UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Física Aplicada

DIGESTIÓN GASTROINTESTINAL DE PROTEÍNAS ALIMENTARIAS Y MECANISMOS DE ACCIÓN DE PÉPTIDOS CON EFECTO SOBRE LA SALUD DIGESTIVA

GASTROINTESTINAL DIGESTION OF FOOD PROTEINS AND MECHANISMS OF ACTION OF PEPTIDES ON DIGESTIVE HEALTH

SAMUEL FERNÁNDEZ TOMÉ

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

Madrid 2016

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Física Aplicada

DIGESTIÓN GASTROINTESTINAL DE PROTEÍNAS ALIMENTARIAS Y MECANISMOS DE ACCIÓN DE PÉPTIDOS CON EFECTO SOBRE LA SALUD DIGESTIVA

Memoria presentada por:

Samuel Fernández Tomé

Para optar al grado de

DOCTOR EN BIOLOGÍA Y CIENCIAS DE LA ALIMENTACIÓN

CON MENCIÓN DE "DOCTORADO INTERNACIONAL"

Instituto de Investigación en Ciencias de la Alimentación

Trabajo realizado bajo la dirección de:

Dra. Isidra Recio Sánchez

Dra. Blanca Hernández Ledesma

ISIDRA RECIO SÁNCHEZ, PROFESORA DE INVESTIGACIÓN DEL CSIC, Y BLANCA HERNÁNDEZ LEDESMA, CONTRATADA RAMÓN Y CAJAL DEL CSIC, DEL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

INFORMAN:

Que el presente trabajo titulado "DIGESTIÓN GASTROINTESTINAL DE PROTEÍNAS ALIMENTARIAS Y MECANISMOS DE ACCIÓN DE PÉPTIDOS CON EFECTO SOBRE LA SALUD DIGESTIVA [GASTROINTESTINAL DIGESTION OF FOOD PROTEINS AND MECHANISMS OF ACTION OF PEPTIDES ON DIGESTIVE HEALTH]", que constituye la Memoria que presenta el Licenciado en Veterinaria SAMUEL FERNÁNDEZ TOMÉ para optar al grado de Doctor con Mención de Doctorado Internacional, se ha realizado bajo su dirección en el Departamento de Bioactividad y Análisis de los Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM).

Y para que conste, firmamos el presente informe en Madrid a 5 de Octubre de 2016.

Fdo. Isidra Recio Sánchez **Fdo. Blanca Hernández Ledesma**

AGRADECIMIENTOS

Al echar la vista atrás y recordar todo lo vivido durante esta etapa, resulta bastante difícil poder plasmar en tan solo unas líneas el agradecimiento debido a todas las personas e instituciones que han facilitado la realización de esta Tesis. En primer lugar, me gustaría mostrar un agradecimiento muy especial a mis directoras, las Dras. Isidra Recio Sánchez y Blanca Hernández Ledesma, por confiar en mí y darme la oportunidad de realizar este trabajo, por todo el tiempo, asesoramiento científico, paciencia y consejos dedicados que han hecho posible el buen fin de cada uno de los resultados recogidos en la siguiente memoria. Gracias por haber dejado huella tanto a nivel profesional como personal, por transmitirme orientación ante cada duda y dejarme aprender de vuestra capacidad de trabajo y experiencia.

Gracias al Ministerio de Economía y Competitividad por la concesión de una beca pre-doctoral asociada al proyecto AGL2011-24643. Al proyecto COST Action INFOGEST FA1005 por financiar mi asistencia a "2nd PhD Training School". Un agradecimiento personal dirigido a mi grupo de investigación, Bioactividad y Alergenicidad de Proteínas y Péptidos Alimentarios (BIOPEP), por todos los momentos compartidos con ellos. Mi reconocimiento a la Dra. Rosina López-Fandiño por su excelente trabajo como jefa de grupo y amabilidad. Un agradecimiento especial a la Dra. Lourdes Amigo, por su magnífica ayuda y consejo, aportando una actitud tan necesaria llena de entusiasmo, que me ha acompañado desde el inicio. También agradecer a las Dras. Beatriz Miralles, por todo el tiempo y atención dedicada, Elena Molina, por su carisma y labor organizativa, Marta Miguel y Josefina Belloque, por su simpatía. Me gustaría agradecer al Instituto de Investigación en Ciencias de la Alimentación (CIAL), bajo la dirección de la Dra. Mª Victoria Moreno Arribas, por la acogida y todos los recursos humanos y técnicos facilitados. Gracias a todo el personal del centro por su gran labor de asistencia diaria. A las Dras. Nieves Corzo, jefa del Departamento de Bioactividad y Análisis de los Alimentos, y Susana Santoyo, por aceptar la tutoría de este trabajo.

La realización de esta Tesis me ha permitido colaborar con varias instituciones a las que me gustaría mostrar mi más sincero agradecimiento por la cálida acogida, disponibilidad y ayuda brindada. Quiero agradecer a los Dres. Luis Goya y Sonia Ramos por sus enseñanzas transmitidas, cercanía y simpatía constantes durante mi estancia en el Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), también a los Dres. María Ángeles Martín, Isabel Cordero y José Manuel Silván y a todos los integrantes del grupo Metabolismo y Bioactividad de Fitoquímicos por la agradable etapa vivida allí. Gracias a los Dres. Rocío Girón y Carlos Goicoechea y a todo su grupo de investigación, con mención especial a Guadalupe Pablo, por toda su ayuda, gentileza y disposición durante mi paso por la Universidad Rey Juan Carlos. Igualmente agradecer los momentos transcurridos en el Instituto de Investigación Sanitaria del Hospital Universitario La Paz (IdiPAZ) junto a las Dras. María Tabernero y Carlota Largo, y en la Estación Experimental del Zaidín (EEZ) con los Dres. Alfonso Clemente y Cristina Delgado. I would like to truly thank to Dr. Hang Xiao for allowing me to perform a PhD internship at the

University of Massachusetts in Amherst (USA) and to his entire lab, especially Fei (Jason) Xu, Xiaokun Cai, and Minqi Wang, as well as to the "big international family" I met there.

Una mención especial va dirigida a todos mis compañeros del CIAL. Quiero empezar con un reconocimiento muy personal hacia el Dr. Daniel Martínez Maqueda, de quien aprendí tanto, por su ejemplar guía, disponibilidad y simpatía constantes. Un agradecimiento destacado y, tan extenso como todas las vivencias, risas y anécdotas compartidas desde que comenzamos juntos este camino, a Laura Perezábad, Alba Pablos y Daniel Lozano, por haber hecho único cada día. A Tomás Herrero, por ser uno de esos compañeros de trabajo y amigos que uno tiene la suerte de encontrarse en la vida. No me olvido de todos aquellos que me acogieron tan bien, Sara Benedé, Marta Garcés, Almudena García, Sara Junco, Mónica Ullate, Beatriz Fernández, Nuria Martínez, Isabel Herranz, Alberto Fernández, Iván López, Constanza Talavera, Alberto Valdés… y especialmente Elvia Cruz y Laura Sánchez, por su cercanía y consejos. Gracias a los que han ido llegando aportando tanto, Silvia Moreno, Diego Morales, Rubén Vilcacundo, Mónica Martínez, Leticia Pérez, Sara Lara, Juancar Ramos, Nuria Lara, Helena Rodríguez, Marta Santos, y con una mención especial a Javier Sanchón e Irene Pastor, por su ayuda y apoyo en esta etapa final. A todos los que han hecho su paso por el laboratorio, Fernanda, Aurora, Blanca, Sebnem, David, Xiaojuan, Susana, Miriam, Roberto, Estefanía, Irati, Ángela, Isabel, Amandine, Pablo, Fátima, Cindy, Diego, Sonia, Rubén, Leire, Javier… así como a mis compañeros de la sala postdoctoral, que me han alentado durante estos largos últimos pasos. Gracias a todos por vuestro apoyo y ayuda, pero, sobre todo, por cada uno de los momentos vividos que han hecho de estos años una aventura inolvidable.

Muchas gracias a mis amigos de siempre, Andy, Bea, Carmen, David, Isa, Joaquín, Laura, Lore, María, Martas, Patri, Paula, Ra, Samuel, Sergio, Sesa, Varo, Vila y "agregados" por sacar siempre lo mejor de mí y seguir acumulando tantísimas vivencias juntos, sin las que hubiera sido mucho más complicado llegar a este punto.

Un profundo agradecimiento a toda mi familia, a mis padres por los esfuerzos hechos que me han dado el soporte por el que hoy puedo estar aquí. A mi madre y hermana por toda su comprensión, apoyo y ánimo. Con un afecto muy personal, a mis abuelos Cecilia Romo y Nicomedes Fernández por haberme transmitido muchísimo más de lo que ellos se pueden imaginar. Un agradecimiento "diferente" para "Ron", gracias por todo lo que me acompañaste con cada gesto y paseo y, aunque no sea igual, seguir conmigo. Por último, "no quiero irme sin decirte una cosa", el agradecimiento más especial es para ti, Patricia, por haberte cruzado en mi camino, por iluminarlo, por solo sonreír y hacerme tan feliz... gracias por todo.

Vale la pena luchar por lo que vale la pena tener.

Samuel

“Las que conducen y arrastran al mundo
no son las máquinas, si no las ideas”

Victor Hugo

ÍNDICE

ABSTRACT

In this Thesis several aspects related to the physiological effect of bioactive peptides on the digestive tract have been studied by a double approach. It has been investigated not only the modifications that food proteins and peptides undergo during the gastrointestinal digestion, but also the mechanisms of action involved in the biological function that bioactive peptides might exert due to their contact with digestive cells and receptors. Peptide lunasin, casein and whey proteins, and milk peptides were analyzed.

Initially, the behavior of peptide lunasin under digestive conditions simulating the transit through the gastrointestinal tract in the absence or presence of soybean Bowman-Birk isoinhibitor 1 (IBB1), in both active and inactive states, was evaluated. IBB1 isoinhibitor exerted a protective effect on lunasin degradation during digestion. Protection against the action of pepsin was due to the presence of IBB1 and its higher size in comparison with that of peptide lunasin, independently of activity. However, an IBB1 dose-dependent protective effect was found at intestinal level, related to its inhibitory activity of pancreatic enzymes trypsin and chymotrypsin. The peptide profiles of gastric and gastrointestinal digests were characterized. It was demonstrated the notable resistance during the digestive process of some domains of peptide lunasin, especially regions ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{19}LTPCEKHIME^{28} and ^{29}KIQGRGDDDDDDDDD^{43}. The transepithelial transport of these four fragments and the precursor lunasin was evaluated using Caco-2 cell monolayers. While some regions of these peptides were susceptible to epithelial brush-border peptidases, a marked resistance was found for others, particularly for fragments ^{1}SKWQHQQDSC^{10} and ^{29}KIQGRGDDDDDDDDD^{43}. The transepithelial transport of lunasin and fragment ^{11}RKQLQGVN^{18} was mediated through diffusion via the paracellular pathway. Additionally, lunasin, as well as its gastrointestinal digests in presence/absence of IBB1, and some lunasin-derived fragments identified in these digests were evaluated

for their effect against the viability of gastric cancer AGS and colorectal cancer HT-29 and Caco-2 cells. It is highlighted that the fragment ^{1}SKWQHQQDSC^{10} was the main responsible for the demonstrated inhibitory effect of lunasin on cellular viability, particularly in HT-29 cells. The chemopreventive mechanisms of action of lunasin against the proliferation of bulk colorectal cancer HCT-116 cells and the expansion of their tumorsphere-derived cancer stem-like subpopulation were evaluated. Peptide lunasin inhibited the viability of bulk tumor cells, as well as the tumorsphere-forming capacity of HCT-116 cells. The inhibitory activity was mediated by inducing apoptosis and arresting cell cycle at G1 phase. These effects were associated to a stimulatory effect on molecular marker caspase-3 linked to a cleavage on PARP signal, and a modest activation of p21 protein expression. Upon chemical-induced oxidative stress, lunasin-treated liver HepG2 cells showed an increased cellular viability. This protective effect was mediated through raising intracellular glutathione levels, and decreasing oxygen reactive species production and glutathione peroxidase and catalase activities. In addition, lunasin protected proteins from oxidative damage and inhibited caspase-3 mediated apoptosis.

In the context of gastrointestinal mucus strengthening by food protein compounds, novel peptides derived from α_{s1}-casein ^{144}YFYPEL^{149} and ^{144}YFYPEL^{148} and, to a lower extent, ^{144}YFY^{146} and ^{143}AYFYPEL^{149} were found, for the first time, to exert an opioid agonist activity. By molecular dynamics simulations of peptides binding with the μ-opioid receptor, it was proved that the carboxi-terminal proline residue from peptide ^{144}YFYP^{147} affected to its interaction with opioid receptor and activity. On intestinal goblet cells, it was demonstrated that peptide ^{144}YFYPEL^{149} was the minimum fragment able to stimulate expression of MUC5AC, the main secreted mucin gene in HT29-MTX cells. Later on, the protective effect of a peptic casein hydrolyzate containing these opioid peptides was studied in the rat intestinal mucus layer. This hydrolyzate stimulated the gene expression of mucins Muc2 and Muc3 in ileum and

colon, and the fecal mucin secretion after its oral administration during two and eight weeks, enhancing *in vivo* the intestinal mucus barrier.

Finally, protein degradation and peptide formation in digests obtained after *in vivo/in vitro* gastrointestinal digestion of casein and whey protein were analyzed. With this purpose, the peptide profile of jejunal digests of 5 human volunteers was characterized and compared with peptides generated during *in vitro* gastrointestinal digestion of milk proteins following a standardized and internationally harmonized static digestion protocol. Whereas protein degradation through the gastrointestinal tract was observed, some protein domains showed resistance against the action of digestive enzymes. *In vivo* and *in vitro* protein degradation and digests peptidome were similar. Spearman correlation coefficients between *in vivo* and *in vitro* digests were within the range of that obtained between the different human volunteers of the study. Therefore, this *in vitro* method represented a suitable model to physiologically simulate the gastrointestinal digestion, at least in the case of milk proteins.

Altogether, these results demonstrated the key role that digestive tract can play on the bioactivity of ingested compounds. Moreover, the results have allowed increasing the knowledge on the mechanisms of action involved in the beneficial effects of peptide lunasin, milk proteins, and derived peptides on digestive health.

RESUMEN

En la presente Tesis Doctoral se ha llevado a cabo el estudio de varios aspectos relacionados con el efecto fisiológico de los péptidos bioactivos sobre el tracto digestivo con un doble enfoque. Por un lado, se han estudiado las modificaciones que sufren las proteínas alimentarias durante la digestión gastrointestinal y, por otro, se han evaluado los mecanismos de acción implicados en la funcionalidad biológica que los péptidos bioactivos pueden ejercer en contacto con las células y los receptores del tracto digestivo. Como sustratos se estudiaron el péptido lunasina, las caseínas y proteínas de suero y distintos péptidos lácteos.

Inicialmente, se estudió el comportamiento del péptido lunasina en condiciones de digestión que simulan el tránsito a través del tracto gastrointestinal, en ausencia o presencia de la isoforma 1 del inhibidor de proteasas Bowman-Birk (IBB1) derivado de la soja, tanto en su estado activo como inactivo. El isoinhibidor ejerció un efecto protector frente a la degradación del péptido lunasina durante la digestión. La presencia del IBB1 y su mayor tamaño con respecto al péptido lunasina, independientemente de su actividad, fueron responsables del efecto protector ejercido a nivel gástrico. Sin embargo, a nivel intestinal, la protección fue dependiente de la dosis de IBB1, relacionado con su actividad inhibitoria de las enzimas pancreáticas tripsina y quimiotripsina. Se caracterizó el perfil peptídico de los digeridos gástricos y gastrointestinales y se identificaron ciertos dominios del péptido lunasina con marcada resistencia durante el proceso digestivo, concretamente las regiones ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{19}LTPCEKHIME^{28} y ^{29}KIQGRGDDDDDDDDD^{43}. Asimismo, se evaluó el transporte transepitelial del péptido lunasina y de los cuatro fragmentos mencionados, empleando el modelo de células Caco-2 en monocapa. Aunque algunas regiones de estos péptidos fueron susceptibles a la acción de las enzimas del epitelio intestinal, se encontró una notable resistencia en otras zonas, especialmente los fragmentos ^{1}SKWQHQQDSC^{10} y ^{29}KIQGRGDDDDDDDDD^{43}. El

transporte del péptido lunasina y del fragmento ^{11}RKQLQGVN^{18} a través de la monocapa celular estuvo mediado por un mecanismo de difusión por la vía paracelular. Paralelamente, se ensayó el efecto del péptido lunasina, de los digeridos gastrointestinales obtenidos a partir de las mezclas con/sin IBB1 y de algunos de los fragmentos identificados en dichos digeridos sobre la viabilidad de células de cáncer gástrico AGS y colorrectal HT-29 y Caco-2. Se pudo concluir que el fragmento ^{1}SKWQHQQDSC^{10} es el principal responsable de la actividad inhibitoria del péptido lunasina sobre la viabilidad celular, especialmente en células HT-29. Se profundizó en los mecanismos de actividad quimiopreventiva del péptido lunasina frente a la proliferación de células nativas de cáncer colorrectal HCT-116 y la expansión de su subpoblación de células madre tumorales. El péptido lunasina inhibió la viabilidad de las células nativas tumorales así como la capacidad de formación de esferas tumorales de las células madre. La actividad inhibitoria estuvo mediada por un efecto inductor de la apoptosis celular y un bloqueo del progreso del ciclo celular en la fase G1. Estos eventos se asociaron a un aumento en la expresión proteica del biomarcador caspasa-3, con la consiguiente disminución de la señal PARP, y un moderado incremento de la expresión de la proteína p21. Frente al estrés oxidativo inducido químicamente en células hepáticas HepG2, las células tratadas con el péptido lunasina mostraron un incremento en la viabilidad. Este efecto estuvo mediado por un aumento del contenido intracelular de glutatión, una disminución en la generación de especies reactivas de oxígeno y de la actividad de las enzimas glutatión peroxidasa y catalasa, así como una recuperación frente al daño oxidativo a proteínas provocado por el agente químico y un bloqueo de la activación pro-apoptótica por caspasa-3.

En el contexto del fortalecimiento del mucus gastrointestinal mediante compuestos alimentarios de naturaleza proteica, se describió por primera vez la actividad agonista opioide de los péptidos lácteos derivados de la α_{s1}-caseína

^{144}YFYPEL^{149} e ^{144}YFYPE^{148} y, aunque con menor potencia, de los péptidos ^{144}YFY^{146} y ^{143}AYFYPEL^{149}. Mediante una modelización de la interacción molecular entre los péptidos y el receptor opioide tipo μ, se observó que la presencia de prolina en el extremo carboxi-terminal del péptido ^{144}YFYP^{147} influía negativamente en su interacción con el receptor y actividad opioide. Adicionalmente, se demostró que el péptido ^{144}YFYPEL^{149} constituye la mínima secuencia con capacidad estimulante de la expresión del gen MUC5AC, que codifica para la principal mucina en las células caliciformes intestinales HT29-MTX. Posteriormente, se estudió el efecto protector de un hidrolizado de caseínas con pepsina que contiene estos péptidos lácteos opioides, sobre la barrera mucosa del intestino de ratas. El hidrolizado aumentó la expresión génica de las mucinas Muc2 y Muc3 en íleon y colon, así como la secreción fecal de mucinas tras dos y ocho semanas de tratamiento, fortaleciendo *in vivo* la capa mucosa intestinal.

Finalmente, se comparó la degradación proteica y el proteoma obtenido tras la digestión gastrointestinal *in vivo/in vitro* de caseínas y proteínas de suero lácteo. Para ello, se caracterizó el perfil peptídico de los digeridos obtenidos a partir del yeyuno humano en 5 voluntarios y se comparó con los péptidos liberados a partir de las proteínas lácteas tras ser sometidas a un protocolo estático de simulación de la digestión gastrointestinal recientemente consensuado a nivel internacional. Se puso de manifiesto la degradación que sufren las proteínas a su paso por el tracto gastrointestinal, mientras que algunos dominios mostraron resistencia al proceso digestivo. La degradación proteica y el peptidoma de los digeridos obtenidos *in vivo* e *in vitro* fueron similares entre ambas digestiones. Tras la comparación, los coeficientes de correlación de Spearman entre los digeridos *in vivo* e *in vitro* fueron del mismo orden a los encontrados al evaluar la variabilidad inter-individual entre los voluntarios del estudio. Por ello, el método empleado de simulación de la digestión gastrointestinal

in vitro, supuso una buena aproximación a la situación fisiológica, al menos en el caso de la digestión de las proteínas lácteas.

En conclusión, se ha demostrado el papel determinante que desempeña el tracto digestivo sobre los compuestos bioactivos ingeridos y se ha avanzado en el conocimiento de los mecanismos involucrados en los efectos beneficiosos del péptido lunasina, de proteínas lácteas y de péptidos derivados sobre la salud digestiva.

LISTA DE ABREVIATURAS

α-La: α-lactoalbúmina

β-Lg: β-lactoglobulina

BBI: inhibidor de proteasas Bowman-Birk

BBIC: concentrado de inhibidor de proteasas Bowman-Birk

CAT: catalasa

CM: casomorfina

CMP: caseinomacropéptido

CN: caseína

CPPs: caseinofosfopéptidos

ECA: enzima convertidora de angiotensina

EFSA: Agencia Europea de Seguridad Alimentaria

FDA: Agencia Estadounidense de Alimentos y Medicamentos

GPx: glutatión peroxidasa

GSH: glutatión reducido

H_2O_2: peróxido de hidrógeno

HPLC-MS/MS: cromatografía de líquidos de alta eficacia acoplada a espectrometría de masas en tándem

IBB1: isoinhibidor-1 de Bowman-Birk

KTI: inhibidor de tripsina Kunitz

LF: lactoferrina

LPS: lipopolisacárido

Mucn: gen correspondiente a la mucina de rata número “n”

MUCn: gen correspondiente a la mucina humana número “n”

qRT-PCR: reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa

ROS: especies reactivas de oxígeno

SDS-PAGE: electroforesis en gel de poliacrilamida

SOD: superóxido dismutasa

t-BOOH: *tert*-butil hidroperóxido

OBJETIVOS Y PLAN DE TRABAJO

El tracto digestivo constituye la mayor superficie de intercambio entre el medio externo y el organismo. Se encuentra continuamente en contacto con los digeridos de los alimentos y con numerosos compuestos que pueden provocar desequilibrios oxidativos y alteraciones proliferativas en sus poblaciones celulares. Además, expresa una gran cantidad de receptores, p.e. receptores opioides, y desempeña un papel decisivo en las modificaciones de los componentes ingeridos. Por tanto, el tracto digestivo es considerado como una de las principales dianas de los compuestos con actividad biológica y es determinante en su posible relevancia fisiológica.

Durante la digestión gastrointestinal se producen una gran cantidad de péptidos por hidrólisis de las proteínas alimentarias, los cuáles podrían desempeñar distintos efectos regulatorios sobre las funciones digestivas. Así, en el estudio de los efectos biológicos de los péptidos alimentarios, resulta esencial conocer cómo se digieren las proteínas alimentarias, identificar la forma activa en el organismo y evaluar los mecanismos de acción implicados en dichos efectos. Esta información es fundamental para establecer la relación entre el compuesto y la funcionalidad biológica. De este modo, estos estudios se presentan como una interesante estrategia para la mejora de la salud digestiva y la prevención de las enfermedades relacionadas.

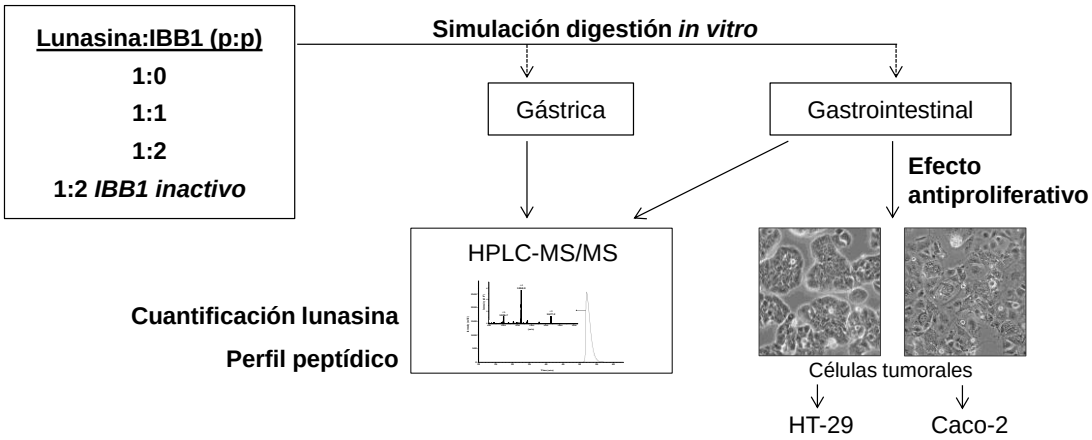
Objetivo general

El **principal objetivo** de esta Tesis Doctoral es la “evaluación de los procesos relacionados con la digestión y la biodisponibilidad de diferentes péptidos de origen alimentario implicados en la salud digestiva, así como el estudio de sus propiedades beneficiosas, a través de su efecto opioide y modulador de la expresión de mucinas intestinales, y de su capacidad protectora frente a procesos de estrés oxidativo y de proliferación celular anormal”. Para cumplir este objetivo se plantearon los siguientes **objetivos parciales** y se diseñó el siguiente **plan de trabajo:**

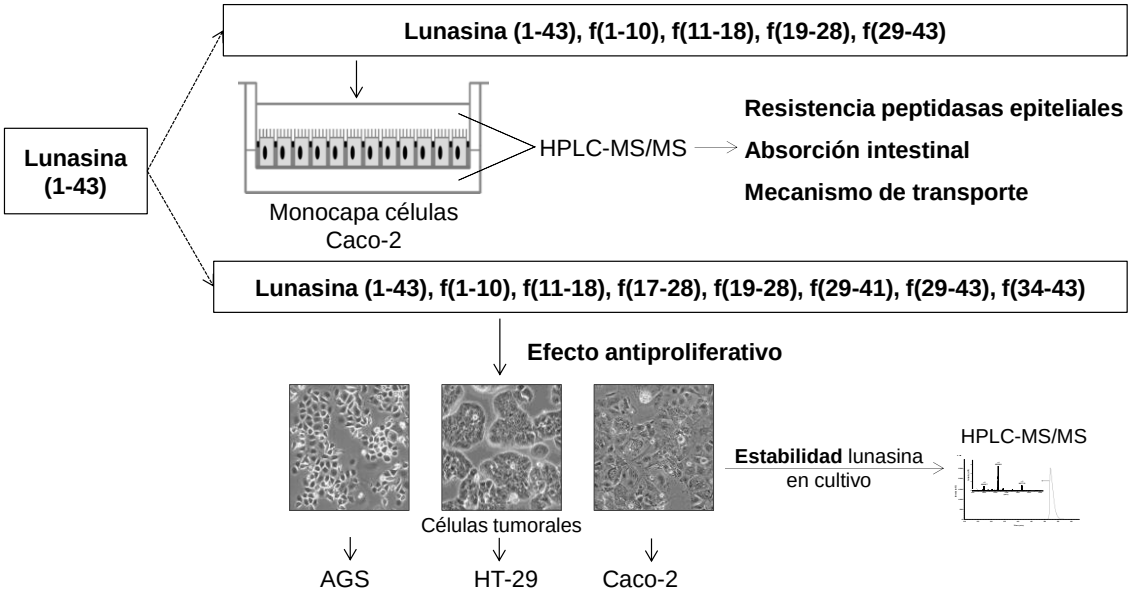
Objetivo 1

Estudio del comportamiento del péptido lunasina frente a la simulación *in vitro* de la digestión y la absorción gastrointestinal. Efectos sobre la viabilidad de células tumorales del tracto digestivo.

1.1. Evaluación del efecto protector del isoinhibidor-1 de Bowman-Birk (IBB1) en la digestión *in vitro* del péptido lunasina. Estudio de la actividad de los digeridos gastrointestinales sobre la viabilidad de células de cáncer colorrectal.



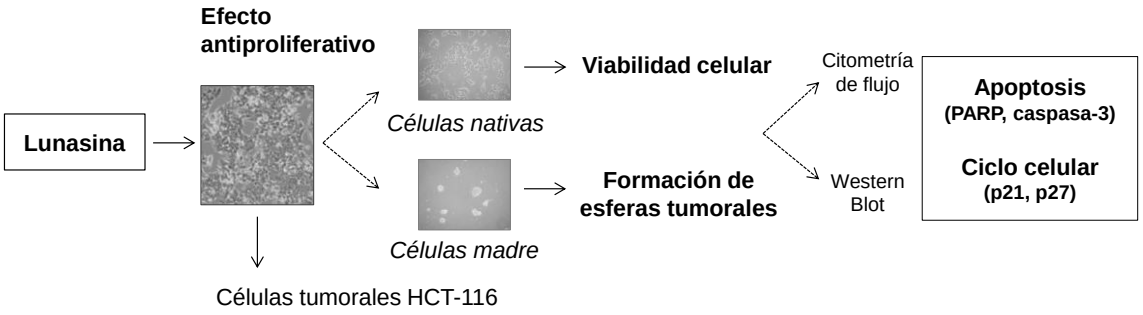
1.2. Evaluación del transporte transepitelial del péptido lunasina y de péptidos liberados a partir de su digestión gastrointestinal. Estudio de la actividad de los péptidos sobre la viabilidad de células de cáncer gástrico y colorrectal.



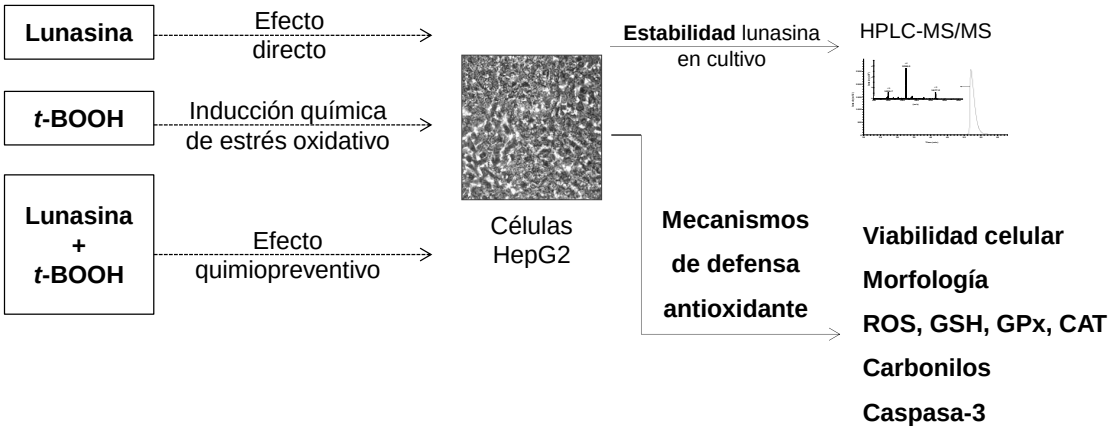
Objetivo 2

Estudio de los mecanismos celulares protectores del péptido lunasina responsables de su actividad quimiopreventiva.

2.1. Efecto del péptido lunasina sobre la proliferación de células de cáncer colorrectal. Evaluación de los mecanismos preventivos en células nativas y células madre tumorales.



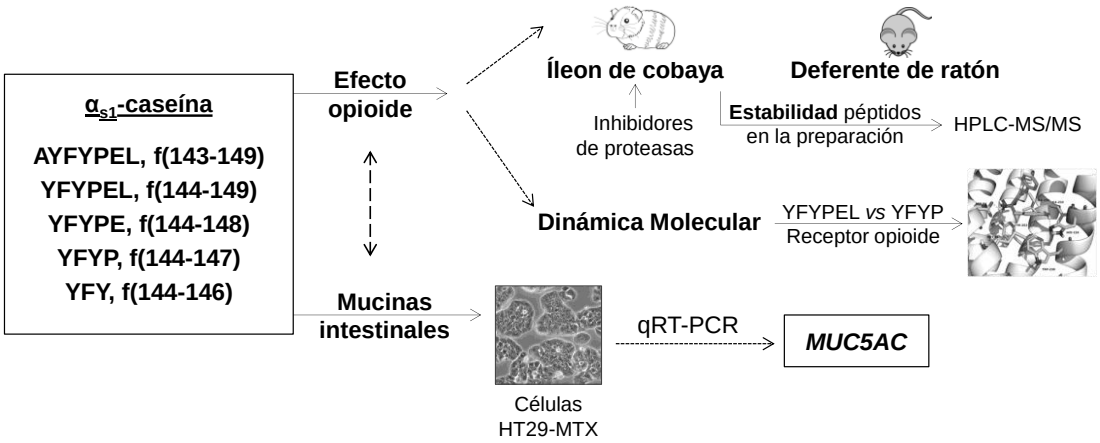
2.2. Evaluación de los mecanismos de defensa antioxidante ejercidos por el péptido lunasina en células hepáticas.



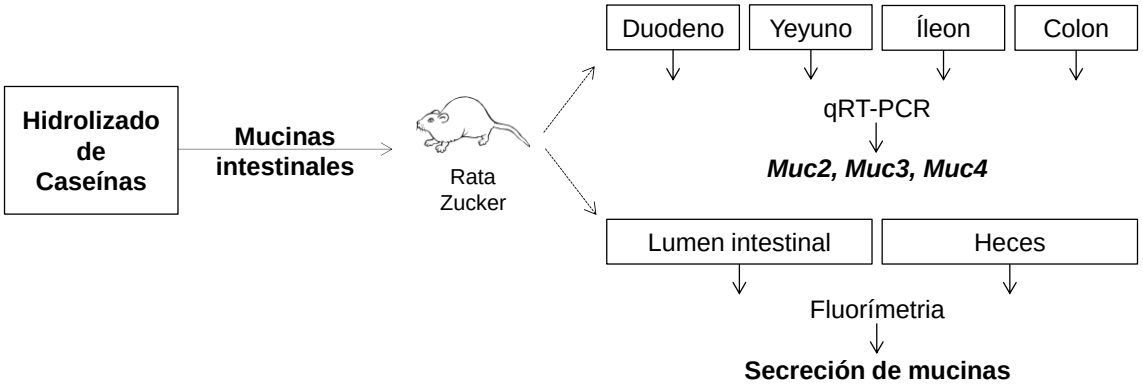
Objetivo 3

Estudio de los mecanismos implicados en la actividad protectora de péptidos lácteos sobre la capa mucosa del tracto intestinal.

3.1. Evaluación de la actividad opioide de péptidos lácteos con efecto en la expresión de mucinas en células caliciformes intestinales.

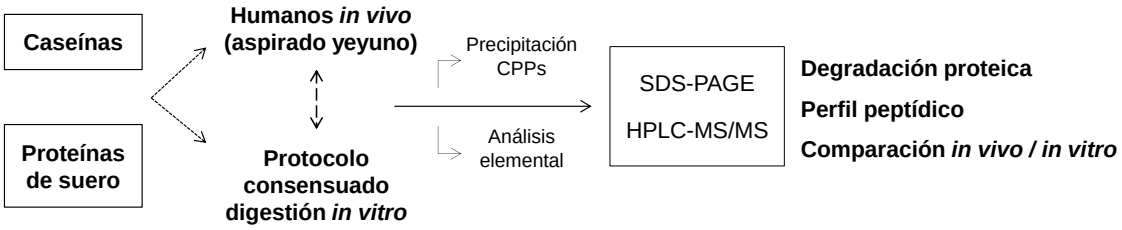


3.2. Efecto modulador *in vivo* de un hidrolizado de caseínas lácteas sobre la producción de mucinas en el intestino de rata.



Objetivo 4

Estudio comparativo de procesos de simulación *in vitro* de la digestión gastrointestinal de proteínas lácteas (caseínas y proteínas de suero) con digeridos intestinales obtenidos en individuos sanos.



1. INTRODUCCIÓN

Un gran número de las funciones del organismo se encuentran reguladas por proteínas y péptidos endógenos que pueden actuar como hormonas, neurotransmisores o reguladores enzimáticos. Durante la digestión gastrointestinal de las proteínas alimentarias se produce una amplia variedad de péptidos. Estos péptidos exógenos pueden ser estructuralmente similares a sus homólogos endógenos, lo que les permite interaccionar con los mismos receptores y, de esta forma, ejercer una determinada actividad biológica. El tracto digestivo se encuentra expuesto de forma continua y directa a los compuestos derivados de la digestión de los alimentos. Además, expresa en su superficie una gran diversidad de receptores, por lo que es en sí mismo una de las principales dianas de los compuestos bioactivos ingeridos. De este modo, la modulación ejercida por la dieta en las funciones fisiológicas del sistema digestivo ha sido reconocida como un elemento esencial para el mantenimiento general y la mejora de la salud (Shimizu, 2010).

En las últimas dos décadas se han descrito varios estudios acerca de las múltiples actividades de los péptidos bioactivos. Sin embargo, los datos sobre la influencia que tiene el tracto digestivo en la relevancia fisiológica de los péptidos, así como los efectos biológicos ejercidos a este nivel son más reducidos. Por un lado, resulta necesario conocer los cambios ocurridos durante la digestión de las proteínas, a través del análisis, tanto de su degradación, como de la consecuente liberación de secuencias peptídicas. Asimismo, se debe considerar que los péptidos bioactivos pueden ejercer su efecto bien a través de interacciones locales con los receptores de la superficie gastrointestinal o bien a nivel sistémico, para lo cual deben resistir el proceso digestivo, atravesar la barrera intestinal y permanecer en estado activo durante su metabolismo y distribución hasta el órgano diana (Foltz y col., 2010). Por otro lado, para establecer una relación causa-efecto entre los péptidos bioactivos y la salud, es necesario evaluar los mecanismos de acción implicados e identificar la forma activa potencialmente responsable de los efectos biológicos observados.

Por todo ello, y en el ámbito de la funcionalidad de las proteínas alimentarias, se ha despertado el interés científico sobre las interacciones entre los componentes del sistema digestivo y los péptidos derivados de los alimentos. Estos estudios pueden proporcionar información muy valiosa y de relevancia fisiológica para la promoción de la salud digestiva y la prevención de las enfermedades crónicas asociadas. Además, pueden ser de utilidad para el avance en el diseño de nuevos ingredientes alimentarios.

1.1. Composición proteica de la soja y la leche

La **soja** presenta un contenido en proteína variable entre el 35 y el 45% (peso/peso). Las proteínas de almacenamiento globulinas son el componente proteico mayoritario (70-83%), principalmente formado por la β-conglicinina y la glicinina, también conocidas como globulina 7S y globulina 11S, respectivamente. Se presentan como proteínas poliméricas unidas mediante puentes disulfuro y uniones no covalentes (Hill y Breidencach, 1974). La fracción proteica de la soja, al igual que la presente en otras especies de leguminosas, está compuesta por una compleja mezcla de proteínas minoritarias y polipéptidos como lectinas, lipoxigenasas, α-amilasas, inhibidores de proteasas de las familias Bowman-Birk (BBI) y Kunitz (KTI), y péptidos bioactivos como la lunasina. Se ha descrito que la composición nutricional y proteica de la soja es variable en función de factores medioambientales, genéticos y de las condiciones de cultivo o del procesado (Zarkadas y col., 2007; Gomes y col., 2014).

La **leche** tiene un contenido medio en proteína entre el 3,0 y el 3,5% (peso/volumen), variable según algunos factores como la especie, la alimentación o el estado de lactancia y/o salud del animal, entre otros (Dalgleish, 1993). Las proteínas lácteas se clasifican en dos grupos: caseínas y proteínas de suero. Las caseínas se caracterizan por precipitar en condiciones ácidas (pH 4,6 y 20 ºC), por su carácter anfipático y por poder estar fosforiladas en los residuos de serina. Las caseínas

representan un 80% del contenido proteico total y comprenden cuatro tipos de cadenas polipeptídicas: α_{s1}-caseína (CN), α_{s2}-CN, β-CN y κ-CN, en proporción aproximada 4:1:4:1, respectivamente. Por ejemplo, en la leche de vaca, el contenido en caseínas es de 29 g/L y estas proteínas se presentan en un 37,6-39,5% (α_{s1}-CN), 7,8-12,1% (α_{s2}-CN), 33,4-44,6% (β-CN) y 9,5-19,7% (κ-CN) (Farrell y col., 2004). En la leche, las caseínas se encuentran estructuralmente en forma de micelas, formando complejos esféricos con sales inorgánicas, principalmente el fosfato cálcico. Además, pueden sufrir modificaciones por glicosilación y proteolisis, como las producidas sobre la β-CN por las propias enzimas presentes en la leche y que dan lugar a las denominadas γ-CN (Swaisgood, 1992). Las proteínas de suero lácteo permanecen solubles en condiciones ácidas (pH 4,6 y 20 ºC). Estas proteínas incluyen la β-lactoglobulina (β-Lg), la α-lactoalbúmina (α-La), la seroalbúmina bovina, las inmunoglobulinas, y otros componentes minoritarios como lactoferrina (LF), lactoperoxidasa, y algunos péptidos de bajo peso molecular que provienen de la proteolisis de las caseínas. La β-Lg es predominante en la leche bovina, mientras que está ausente en la leche humana, en la que la α-La es mayoritaria. Las proteínas de suero tienen una estructura globular en la que predominan los motivos α-hélice. Son proteínas susceptibles a la desnaturalización por calentamiento, aunque disponen de puentes disulfuro intramoleculares que aportan estabilidad a su estructura (Madureira y col., 2007).

La **funcionalidad** de las proteínas está influenciada por sus características estructurales y físico-químicas. Se ha demostrado que dichas características pueden ejercer un papel determinante en la digestión, biodisponibilidad y formación de secuencias peptídicas de relevancia fisiológica, tanto a partir de proteínas de soja (Carbonaro y col., 2015) como de proteínas lácteas (Dziuba y col., 2010).

1.2. Digestión y biodisponibilidad de proteínas de soja y lácteas

Las acciones ejercidas por los procesos fisiológicos del sistema digestivo han sido objeto de estudio en numerosas investigaciones científicas relativas a la nutrición, farmacología, toxicología y microbiología (Sambruy y col., 2001). Los alimentos, tras ser ingeridos, van a sufrir una serie de complejas modificaciones físicas y químicas propias de la digestión que finalmente darán lugar a la liberación y absorción de los nutrientes. Las proteínas alimentarias pueden comportarse de distintas maneras durante el proceso digestivo debido a las diferencias en sus características estructurales, de conformación y tamaño, o a la presencia de inhibidores de proteasas. Además, durante la digestión de las proteínas, se liberan una gran cantidad de péptidos que podrían tener implicaciones fisiológicas, actuando como señales regulatorias de las funciones del organismo (Sánchez-Rivera y col., 2014a), y toxicológicas, como la formación de epítopos con capacidad para inducir reacciones relacionadas con las alergias alimentarias (Jiménez-Saiz y col., 2015). Por ello, resulta fundamental conocer los cambios ocurridos en las proteínas alimentarias en relación a su degradación y a la identificación de regiones proteicas resistentes durante el proceso digestivo. Aunque los métodos de evaluación de la digestión *in vivo*, tanto en animales como en humanos, son los que aportan los resultados fisiológicamente más relevantes, por razones prácticas y éticas, ha sido necesario el desarrollo de modelos *in vitro* de simulación gastrointestinal. En los últimos años, se ha descrito una amplia diversidad de metodologías *in vitro* que difieren en cuanto a variables, como el número de fases de la digestión, fluidos y enzimas empleados, pH y tiempos de incubación, relación enzima:sustrato, adición y retirada de compuestos, mezcla del contenido y uso de co-factores de digestión, entre otros (Hur y col., 2011; Guerra y col., 2012). En este contexto, y en el ámbito de la Acción COST FA 1005 Infogest, recientemente se ha publicado un protocolo en el que se establecen las condiciones estándar para la simulación de la digestión *in vitro* (Minekus y col., 2014). Este método ha sido

aceptado mediante el consenso por parte de diferentes grupos de investigación internacionales, y su reproducibilidad se ha evaluado recientemente en ensayos inter-laboratorio (Egger y col., 2016). Sin embargo, es necesario correlacionar y validar los resultados de estos métodos *in vitro* con aquellos obtenidos *in vivo*, para los que todavía existen muy pocos datos.

Se ha descrito que el tracto gastrointestinal juega un papel decisivo en la relevancia fisiológica de los compuestos bioactivos derivados de los alimentos. En el estudio de los péptidos bioactivos, uno de los primeros aspectos a evaluar es su resistencia a las enzimas digestivas. Asimismo, debe considerarse su estabilidad durante el tránsito digestivo, así como su capacidad de absorción, distribución, metabolismo y excreción (Segura-Campos y col., 2011; Sánchez-Rivera y col., 2014a). La presencia de péptidos bioactivos en el contenido intestinal no es suficiente si no se asegura su bioaccesibilidad y biodisponibilidad (**Figura 1**), es decir, es necesario que

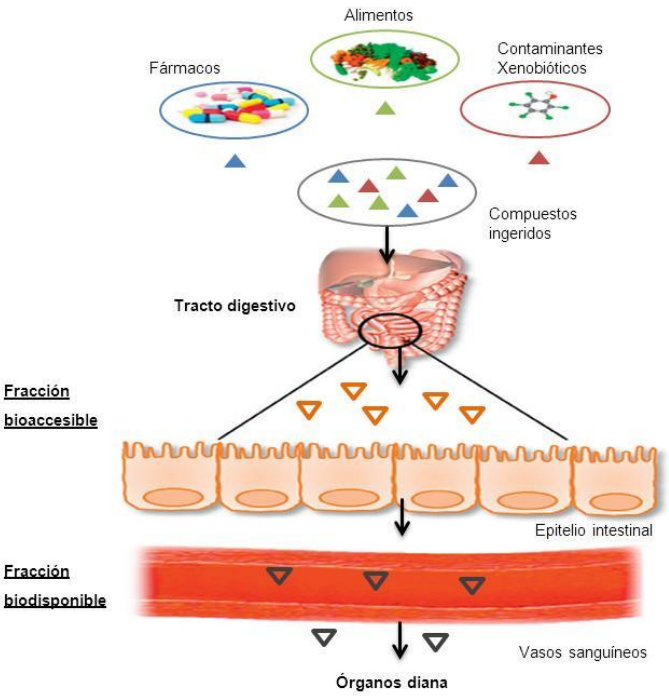


Figura 1. Bioaccesibilidad y biodisponibilidad de los compuestos ingeridos (Adaptada de Guerra y col., 2012).

el péptido interaccione con receptores de la superficie gastrointestinal o, si su sitio de acción implica su absorción, que atraviese la barrera intestinal y alcance el órgano diana en su forma activa (Foltz y col., 2010; Nongonierma y FitzGerald, 2016). Por ello, en los últimos años, se han empleado modelos combinados de simulación *in vitro* de la digestión y absorción intestinal en monocapas de células Caco-2 (Picariello y col., 2013a; 2015). Las células Caco-2 provienen de una línea de adenocarcinoma colorrectal humano. Al cultivarse en soportes adecuados y llegar a confluencia, son capaces de diferenciarse espontáneamente en una monocapa polarizada con microvellosidades, espacios intercelulares, enzimas y sistemas de transporte, presentando las características propias de las células epiteliales intestinales. Este modelo celular ha sido ampliamente utilizado para evaluar la degradación y absorción en el intestino de fármacos y otros compuestos administrados por vía oral (Hubatsch y col., 2007; Antunes y col., 2013). Además, este modelo ha permitido identificar las principales rutas implicadas en el transporte intestinal de proteínas y péptidos derivados (Segura-Campos y col. 2011), tal como se describe en la **Figura 2**.

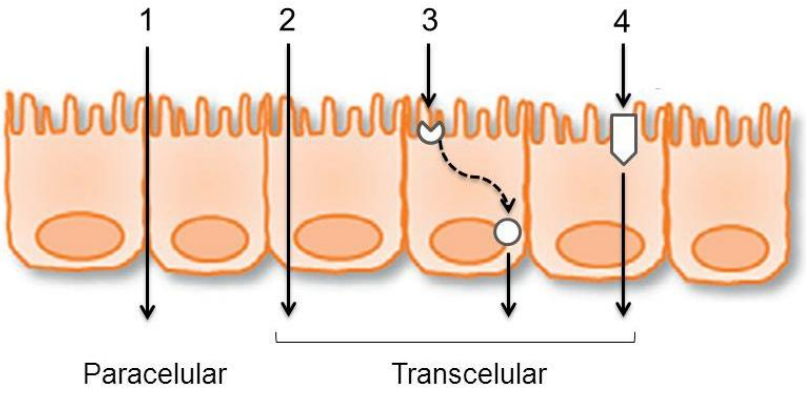


Figura 2. Representación esquemática de los principales mecanismos de transporte de péptidos a través de las células del epitelio intestinal: (1) Transporte paracelular a través de las uniones intercelulares, (2) Transporte transcelular mediante difusión pasiva, (3) Transcitosis, transporte transcelular mediante vesículas, (4) Transporte transcelular mediado por transportadores específicos.

1.2.1. Proteínas de soja. Péptido lunasina

Los trabajos publicados sobre la digestión y/o biodisponibilidad de las proteínas de la soja en relación a sus efectos fisiológicos son escasos. Se ha descrito que la digestión *in vitro* de un aislado de proteínas de soja da lugar a la formación de péptidos antihipertensivos con capacidad inhibidora de la enzima convertidora de angiotensina (ECA) (Lo y Li-Chan, 2005). Siguiendo una estrategia similar, se ha observado como el producto de la digestión enzimática de la proteína β-conglicinina provoca una activación del receptor de colecistoquinina tipo-1, relacionado con la sensación de saciedad (Staljanssens y col., 2012). También se ha visto que la digestión de esta proteína, y de su forma deglicosada, genera péptidos con potencial efecto protector frente a radicales libres (Amigo-Benavent y col., 2014). En este trabajo se identificaron los péptidos presentes en los digeridos gastrointestinales, algunos de los cuáles fueron además biodisponibles en la monocapa intestinal de células Caco-2. En un trabajo reciente, Capriotti y col. (2015) han caracterizado el conjunto de péptidos presentes en digeridos *in vitro* de las proteínas extraídas de semillas y bebidas de soja. Estos autores observaron una intensa degradación de las proteínas durante el proceso digestivo, formándose un total de 2430 secuencias de tamaño variable entre 5 y 49 aminoácidos. Además, se propuso que la longitud de algunos de los péptidos, generalmente más largos que aquellos encontrados en estudios similares con proteínas animales, se justificaba por la presencia de inhibidores de proteasas en las propias proteínas de origen vegetal (Capriotti y col., 2015).

Se ha demostrado que el péptido de 43 aminoácidos **lunasina**, tanto de origen sintético como aislado de la soja, se digiere rápidamente en presencia de pepsina y pancreatina (Park y col., 2007). Sin embargo, en este trabajo se observó que cuando la lunasina se encuentra dentro del extracto proteico de la soja es capaz de resistir parcialmente la acción de las enzimas gastrointestinales. Estos resultados también se han visto en otros estudios con lunasina procedente de otras fuentes proteicas

vegetales, como trigo (Jeong y col., 2007a) y *Solanum nigrum* L. (Jeong y col., 2007b). Por ello, se ha sugerido que los inhibidores de proteasas presentes de forma natural en estos productos, como BBI y KTI, pueden ejercer un efecto protector sobre la digestión enzimática del péptido lunasina. En este sentido, se ha determinado la presencia de los péptidos lunasina y BBI en varios productos comerciales de soja (Hernández-Ledesma y col., 2009a). En este estudio se estableció que la relación de lunasina:BBI (peso:peso) presente en los productos se encontraba en el rango de 1:0,7 – 1:3,9, y se sugirió la influencia de esta relación sobre la resistencia del péptido lunasina frente a la digestión gastrointestinal *in vitro* mediante pepsina y pancreatina. Además, se han publicado algunos estudios *in vivo* que confirman los hallazgos *in vitro* anteriormente mencionados. Hsieh y col. (2010) demostraron la biodisponibilidad de la lunasina en ratones y ratas, determinando su presencia en varios tejidos como hígado, riñón, pulmón, glándula mamaria y próstata. De forma similar, se ha determinado la presencia de este péptido en una forma intacta y bioactiva en el plasma e hígado de ratas alimentadas con dietas de soja (Jeong y col., 2007c), trigo (Jeong y col., 2007a), centeno (Jeong y col., 2009) y cebada (Jeong y col., 2010) enriquecidas en lunasina. Asimismo, Dia y col. (2009) determinaron la presencia de este péptido en el plasma de individuos sanos tras la ingesta de soja, estimando un porcentaje de absorción (intestino-plasma) del 4,5% del total de lunasina ingerida en 50 g de proteínas de soja. Sin embargo, y en el contexto de las modificaciones propias ejercidas durante el proceso digestivo de los péptidos bioactivos, no hay estudios que hayan evaluado en profundidad la influencia de la digestión sobre la secuencia de 43 aminoácidos del péptido lunasina ni la formación e identificación de fragmentos derivados de dicho péptido con posible actividad biológica.

1.2.2. Proteínas lácteas

La digestión de las proteínas lácteas ha sido evaluada en numerosos trabajos mediante métodos ***in vitro*** de simulación gastrointestinal, en los que mayoritariamente

se emplea como herramienta analítica técnicas de separación e identificación por cromatografía acopladas a espectrometría de masas (Picariello y col., 2013b; Capriotti y col., 2016). Estos estudios se han desarrollado con una amplia variedad de objetivos dirigidos, bien a la identificación de secuencias específicas con relevancia fisiológica, o al análisis general de las modificaciones ocurridas en las proteínas y sus péptidos liberados durante la digestión. Por ejemplo, se ha estudiado la formación de péptidos antioxidantes e inhibidores de la ECA en digeridos de leche humana y fórmulas infantiles (Hernández-Ledesma y col., 2007), de los péptidos opioides β-casomorfina (CM)-5 y β-CM-7 tras la digestión de varios productos lácteos comerciales (De Noni y Cattaneo, 2010) y de epítopos relacionados con la alergia a las proteínas lácteas β-Lg (Benedé y col., 2014a) y β-CN (Benedé y col., 2014b). Oshawa y col. (2008) observaron la resistencia de los péptidos antihipertensivos IPP y VPP, fragmentos f(74-76) y f(84-86) de la β-CN, respectivamente, tras una simulación de la digestión con enzimas gástricas, pancreáticas y del epitelio intestinal. Asimismo, se ha demostrado la biodisponibilidad de estos tripéptidos y se han estudiado sus parámetros farmacocinéticos en cerdos (van der Pijl y col., 2008) y humanos (Foltz y col., 2007). De un modo similar, se ha evaluado la estabilidad del péptido antihipertensivo LHLPLP, correspondiente al fragmento f(133-138) de la β-CN, durante la digestión (Quirós y col., 2009) y la biodisponibilidad del péptido derivado HLPLP, β-CN f(134-138), en células Caco-2 (Quirós y col., 2008) y en ratas (Sánchez-Rivera y col., 2014b). Con un enfoque más amplio, recientemente se ha evaluado el conjunto de péptidos generados durante la fermentación y posterior digestión gastrointestinal de un yogur (Jin y col., 2016). También se ha comparado el perfil de péptidos formados en los digeridos de dos matrices alimentarias lácteas distintas, queso Valdeón y leche en polvo evaporada (Sánchez-Rivera y col., 2014c). Dupont y col. (2010) estudiaron el comportamiento de las proteínas β-CN y β-Lg sometidas a dos protocolos de digestión *in vitro* distintos acordes a las condiciones propias de la digestión de un organismo adulto o infantil. En el trabajo de Picariello y col. (2010), se demostró la influencia

ejercida por las peptidasas del borde del epitelio intestinal en la digestión mediante pepsina y pancreatina de las caseínas y las proteínas de suero. Estos autores trataron así de aproximar los protocolos *in vitro* de digestión simulada a las condiciones propias *in vivo* del entorno fisiológico del tracto digestivo.

Hay pocos estudios que hayan evaluado la digestión gastrointestinal de las proteínas lácteas en humanos. Se ha descrito que las distintas proteínas lácteas presentan un comportamiento diferente durante la digestión gastrointestinal. Por ejemplo, se ha encontrado que un 64 y 44% de las proteínas β-Lg y α-La, respectivamente, llegan intactas a la parte superior del yeyuno (Mahé y col., 1991). En el caso de la proteína LF, Troost y col. (2001) observaron que más de un 60% no se degrada durante su digestión en el estómago. Se ha reconocido a las caseínas como proteínas de "digestión lenta" y a las proteínas de suero lácteo de "digestión rápida", en función de la cinética de su hidrólisis y de la absorción de aminoácidos provenientes de su digestión (Boirie y col., 1997). Recientemente, se ha propuesto en un meta-análisis de estudios clínicos que estas diferencias podrían tener influencia sobre la utilización postprandial del nitrógeno proteico, la llegada de péptidos y aminoácidos al plasma sanguíneo, la secreción de hormonas y enzimas digestivas y sobre aspectos metabólicos relacionados con el apetito (Bendtsen y col., 2013). Estos autores vieron que las proteínas de suero tenían un mayor efecto sobre la saciedad a corto plazo mediante el estímulo de la secreción de hormonas incretinas (péptido 1 similar al glucagón y péptido insulinotrópico-dependiente de glucosa), mientras que relacionaron a las caseínas con efectos a largo plazo.

Como se muestra en la **Tabla 1**, se han descrito algunos estudios *in vivo* acerca de la presencia de péptidos en el tracto gastrointestinal tras la digestión de proteínas lácteas en humanos y animales. Sin embargo, la diversidad de estos trabajos es bastante amplia en cuanto a la especie utilizada, el producto consumido

Tabla 1. Estudios in vivo de la digestión de productos lácteos y presencia de péptidos derivados en el tracto gastrointestinal (Adaptada de Boutrou y col., 2015).

Especie	Dieta	Muestra	Objetivo del estudio	Referencia
Humano	Leche	Intestino delgado	β-CMs	Svedberg y col., 1985
	Leche, yogur	Estómago, duodeno	Péptidos de caseínas	Chabance y col., 1998
	Yogur, caseínas, proteínas de suero	Yeyuno, íleon	CMP	Ledoux y col., 1999
	LF	Estómago	LF	Troost y col., 2001
	Caseínas, proteínas de suero	Yeyuno	Péptidos de caseínas y suero	Boutrou y col., 2013
Mini cerdo	Caseínas	Intestino delgado	β-CMs, CPPs	Meisel y Frister, 1989
	Leche desnatada	Duodeno	Péptidos de caseínas y suero	Barbé y col., 2014
Vaca	Leche desnatada, caseínas	Estómago	Péptidos de caseínas	Yvon y Pelissier, 1987
	Leche desnatada	Estómago	Péptidos de caseínas	Scanff y col., 1992
Rata	CPPs	Duodeno, yeyuno, íleon	CPPs	Brommage y col., 1991
	Caseínas, CPPs	Intestino delgado, intestino grueso	CPPs	Kasai y col., 1995
	Proteínas de suero, α-La	Estómago, intestino delgado	α-La, péptidos de suero	Pantako y col., 2001
	Dieta suplementada en Phe-CMP	Estómago, intestino delgado	CMP	Fosset y col., 2002

LF: lactoferrina, CPPs: caseinofosfopéptidos, α-La: α-lactoalbúmina, CMP: caseinomacropéptido, β-CMs: β-casomorfinas

(desde matrices lácteas a proteínas aisladas), el segmento del tracto gastrointestinal evaluado y el análisis de las muestras a estudio.

Con un enfoque particular a los trabajos publicados sobre el análisis de contenidos intestinales **humanos**, tras la ingesta de leche se ha detectado en el intestino delgado la presencia de los péptidos opioides β-CM-7, -6, y -4 mediante ensayos inmunológicos (Svedberg y col., 1985). Posteriormente, Chabance y col. (1998) caracterizaron los digeridos gástricos y duodenales de humanos que tomaron leche y yogur. A nivel gástrico, en el estudio de la leche se identificaron un total de 26 péptidos derivados de la α_{s1}-CN, α_{s2}-CN, β-CN y κ-CN, mientras que del yogur se formaron 8 péptidos derivados de la α_{s1}-CN, β-CN y κ-CN. Adicionalmente, se observó la degradación de las proteínas lácteas a su paso por el intestino delgado, así como la formación de péptidos de menor tamaño, entre los que destacan algunas secuencias para las que posteriormente se ha demostrado bioactividad, como el péptido inductor de mucinas intestinales YFYPEL, α_{s1}-CN f(144-149) (Martínez-Maqueda y col., 2013a), y el péptido opioide YPVEPF, β-CN f(114-119) (Jinsmaa y Yoshikawa, 1999), ambas formadas 20 minutos tras la ingestión de leche. Además, se identificó la presencia y resistencia del caseinomacropéptido (CMP), correspondiente al fragmento f(106-169) de la κ-CN, y de varios fragmentos derivados durante el tránsito gastrointestinal. De la misma forma, Ledoux y col. (1999) confirmaron posteriormente la presencia de CMP a nivel de yeyuno e íleon. Con el avance de las técnicas analíticas de proteómica y peptidómica se ha conseguido mejorar la identificación de secuencias en muestras complejas como son los digeridos gastrointestinales. Así, en un estudio reciente realizado con muestras de aspirados de yeyuno, se han detectado y secuenciado un total de 356 y 146 péptidos tras la ingesta de caseínas y proteínas de suero, respectivamente (Boutrou y col., 2013). En el caso de las caseínas, la mayoría de los péptidos identificados pertenecían a la β-CN (61,2%) seguida de la α_{s1}-CN (24,9%). Ciertas regiones de estas proteínas resistieron durante su tránsito digestivo hasta el

yeyuno dando origen a la mayoría de los péptidos encontrados, como son, las regiones 1-14, 57-70, 67-92, 128-140, 144-163 y el dominio C-terminal de la β-CN; y las regiones 23-33, 104-121 y 165-186 de la α_{s1}-CN. De las proteínas de suero, la β-Lg, y principalmente las regiones comprendidas entre los aminoácidos 40-58 y 122-137, dieron lugar a la mayoría de los péptidos identificados (72,0%). Mientras que en las proteínas de suero no se observó la presencia de ningún fragmento con efecto biológico descrito, numerosos péptidos bioactivos se identificaron derivados de las caseínas, y en concreto de la β-CN, como por ejemplo el péptido con efecto inmunomodulante PGPIPN, f(63-68) (Gill y col., 2000) o los péptidos antihipertensivos EMPFPK, f(108-113) (Pihlanto y col., 1998) y LHLPLP, f(133-138) (Miguel y col., 2006). Además, se identificaron 32 y 27 precursores de los tripéptidos antihipertensivos (Nakamura y col., 1995) VPP e IPP, respectivamente, y 15 precursores que incluyen la conocida secuencia opioide YPFPGPI, f(60-66) β-CM-7. Dada la relevancia fisiológica y la alta frecuencia de estos últimos péptidos en los digeridos, se cuantificó la presencia de β-CM-7 estimándose una concentración de 17 μM después de 2 horas tras la ingestión de caseínas, lo que sería compatible con la cantidad necesaria para ejercer su efecto fisiológico *in vivo* (European Food Safety Authority, EFSA, 2009; Boutrou y col., 2013).

1.3. Péptidos alimentarios con efectos biológicos relacionados con la salud digestiva

En las últimas dos décadas muchos estudios han establecido una relación entre las proteínas alimentarias, y los péptidos incluidos en sus secuencias, y diversos efectos beneficiosos sobre los principales sistemas del organismo, como son el sistema cardiovascular, digestivo, endocrino, inmunológico y nervioso (Hernández-Ledesma y col., 2014).

Se ha considerado que el tracto gastrointestinal es el órgano endocrino más extenso del organismo, ya que presenta un amplio rango de receptores y de péptidos endógenos. Se ha demostrado que además de la regulación ejercida por factores internos sobre las funciones del sistema digestivo, los agentes externos también pueden actuar, existiendo un interés creciente sobre la funcionalidad biológica que proteínas y péptidos de origen alimentario pueden ejercer a este nivel (Shimizu y Hachimura, 2011; Martínez-Augustin y col., 2014). Actualmente, más del 70% de los productos comercializados como alimentos funcionales en Japón "Food for Specified Health Uses (FoSHU)" se encuentran relacionados con las funciones del sistema digestivo. Sin embargo, la mayoría de estos compuestos se refieren a bacterias probióticas y compuestos prebióticos como la fibra dietética y oligosacáridos no digestibles (Shimizu, 2012). Con respecto al grupo de compuestos de origen proteico, la EFSA, hasta el momento, tan solo ha aprobado el efecto de las proteínas alimentarias sobre el mantenimiento normal del sistema óseo, del crecimiento y de la masa muscular (EFSA, 2010a), mientras que no se han aprobado efectos a nivel digestivo. En relación con la salud digestiva, la EFSA ha aprobado la utilización de compuestos de naturaleza glucídica, como la fibra de salvado de trigo para aumentar el peso del bolo fecal y reducir el tiempo de tránsito intestinal (EFSA, 2010b), y de las bacterias vivas del yogur para mejorar la digestión de la lactosa en individuos con maladigestión de este azúcar (EFSA 2010c). Por lo tanto, resulta evidente la necesidad de más estudios que evalúen el efecto de las proteínas alimentarias y sus péptidos derivados sobre la fisiología del tracto digestivo y la prevención de las enfermedades asociadas.

Dada la baja biodisponibilidad descrita para varios péptidos bioactivos, se ha sugerido que ciertos efectos fisiológicos pueden estar mediados a través de interacciones con receptores localizados a nivel de la superficie gastrointestinal (Moughan y col., 2007). Aunque generalmente la afinidad de estos péptidos por los

receptores celulares es relativamente baja, las altas concentraciones alcanzadas en el lumen durante el proceso digestivo podrían desencadenar propiedades remarcables. Algunas de las actividades relacionadas con la modulación gastrointestinal se han referido al efecto sobre la saciedad, la regulación del vaciado gástrico y de la motilidad intestinal, la capacidad secretora y de absorción de las células intestinales, el metabolismo de nutrientes y a propiedades inmunomodulantes (Pang y col., 2012). Muchos de los péptidos que ejercen un efecto modulador directo sobre la función digestiva presentan actividad opioide, agonista o antagonista (Rutherfurd-Markwick, 2012). Además, pueden ejercer capacidad antioxidante, contrarrestando situaciones de estrés oxidativo e inflamación (Chakrabarti y col., 2014), así como efectos preventivos frente a la proliferación de células tumorales (Hernández-Ledesma y Hsieh, 2015), fenómenos íntimamente implicados en el origen, desarrollo y agravamiento de múltiples alteraciones patológicas digestivas.

1.3.1. Actividad opioide. Efectos sobre la producción de mucinas intestinales

Los receptores opioides se encuentran ampliamente distribuidos en el organismo, principalmente formando parte de los sistemas fisiológicos nervioso, endocrino, inmunológico y digestivo (Filizola y Devi, 2012). En 1975 se descubrió la actividad opioide de dos pentapéptidos endógenos, Met-encefalina y Leu-encefalina (Hughes y col., 1975). Posteriormente, se ha descrito actividad opioide para otras dos familias de péptidos endógenos, endorfinas y dinorfinas, y para diversos péptidos exógenos derivados de los alimentos. En relación a su estructura, ambos grupos de péptidos, endógenos y exógenos, muestran una secuencia conservada a nivel del extremo N-terminal con un residuo de tirosina en la primera posición acompañado de otro aminoácido aromático en la tercera o cuarta posición (Meisel, 1997). Se ha descrito que esta disposición estructural es un factor esencial para la interacción del péptido con el sitio de unión del receptor opioide (Teschemacher y col., 1997).

Los primeros péptidos exógenos derivados de proteínas alimentarias que demostraron actividad opioide se denominaron β-CMs, al encontrarse en un hidrolizado lácteo de β-CN (Brantl y col., 1979). Tal como se detalla en la **Tabla 2**, numerosos estudios posteriores han caracterizado péptidos alimentarios opioides entre los que destacan algunos derivados de las proteínas lácteas, y mayoritariamente de las caseínas. Sin embargo, los péptidos opioides también han sido identificados en otras proteínas animales, como las proteínas de suero lácteo y la hemoglobina, y en proteínas vegetales, como el trigo, el arroz, la espinaca, y la soja (Teschemacher, 2003; Garg y col., 2016).

Las primeras investigaciones sobre péptidos opioides alimentarios determinaron la presencia de compuestos con características “similares a la morfina” a partir de muestras de leche humana y bovina en un rango de concentración entre 200-500 ng/L (Hazum y col., 1981), que fueron principalmente atribuidos a las secuencias YPFPGPI, β-CN f(60-66), denominada β-CM-7 (Brantl y col., 1981), RYLGYLE, α_{s1}-CN f(90-96) (Loukas y col., 1983), y sus fragmentos derivados. Dependiendo de la proteína de origen y el efecto opioide, los péptidos lácteos han sido clasificados como agonistas opioides: α-exorfinas (derivados de la α-CN), β-CMs (derivados de la β-CN), α-lactorfinas (derivados de la α-La) y β-lactorfinas (derivados de la β-Lg), y antagonistas opioides: casoxinas (derivados de la κ-CN) y lactoferroxinas (derivados de la LF) (**Tabla 2**). El grupo de las β-CMs ha sido el más investigado dentro de los péptidos exógenos con actividad opioide (EFSA, 2009). La β-CM-7 bovina fue el primer ejemplo de péptido de origen natural en el que la sustitución de los residuos glicina-glicina (GG) de los péptidos endógenos por un residuo de prolina no producía un descenso de la actividad opioide en el ensayo en músculo longitudinal del plexo mientérico del íleon de cobaya (Brantl y col., 1979; 1981). Posteriormente, algunos péptidos derivados de esta secuencia, como la β-CM-5 (YPFPG) y β-CM-4 (YPFP),

Tabla 2. Péptidos opioides derivados de proteínas alimentarias (Adaptada de Teschemacher, 2003; Garg y col., 2016).

Origen	Proteína[1]	Péptido opioide			Receptor opioide		Referencia
		Denominación	Secuencia	Fragmento	A/AN[2]	Selectividad	
Animal	α-CN_B	α-Exorfina (1-7)	RYLGYLE	f(90-96)	A	κ > μ/δ	Loukas y col., 1983
		α-Exorfina (2-7)	YLGYLE	f(91-96)	A	μ/δ	
	α-CN_H	α-Casoxina D	YVPFPPF	f(158-164)	AN	μ/δ	Yoshikawa y col., 1994
		α-Casomorfina (1-5)	YVPFP	f(158-162)	A/AN	κ > μ/δ	Kampa y col., 1996
		α-Casomorfina (1-5)-NH_2	YVPFP-NH_2		A/AN	κ/δ > μ	
	β-CN_B	β-Casomorfina-8	YPFPGPIP	f(60-67)	A	μ > κ/δ	Petrilli y col., 1984
		β-Casomorfina-7	YPFPGPI	f(60-66)	A	μ	Brantl y col., 1979; 1981
		β-Casomorfina-6	YPFPGP	f(60-65)	A	μ	Brantl y col., 1981
		β-Casomorfina-5	YPFPG	f(60-64)	A	μ	
		β-Casomorfina-4	YPFP	f(60-63)	A	μ	
		Morficeptina	YPFP-NH_2		A	μ > δ > κ	Chang y col., 1981
		Neocasomorfina-6	YPVEPF	f(114-119)	A	μ	Jinsmaa y Yoshikawa, 1999
	β-CN_H	β-Casomorfina-8	YPFVEPIP	f(51-58)	A	μ > δ > κ	Koch y col., 1985
		β-Casomorfina-7	YPFVEPI	f(51-57)	A	μ > δ > κ	
		β-Casomorfina-5	YPFVE	f(51-55)	A	μ > δ > κ	
		β-Casomorfina-4	YPFV	f(51-54)	A	μ > δ > κ	
		Valmuceptina	YPFV-NH_2		A	μ > δ	Yoshikawa y col., 1986
		β-Casorfina	YPSF-NH_2	f(41-44)	A	μ	
	κ-CN_B	Casoxina A	YPSYGLN	f(35-41)	AN	μ > κ/δ	Chiba y col., 1989
		Casoxina B	YPYY	f(57-60)	AN	μ	
		Casoxina C	YIPIQYYLSR	f(25-34)	AN	μ	
		Casoxina-6	SRYPSY-OCH_3	f(33-48)	AN	μ > κ	
		Casoxina-5	RYPSY-OCH_3	f(34-48)	AN	μ > κ	
	α-La_{BH}	α-Lactorfina	YGLF	f(50-53)	A	μ	Yoshikawa y col., 1986
		α-Lactorfina-NH_2	YGLF-NH_2		A	μ	
	β-Lg_B	β-Lactorfina	YLLF	f(102-105)	A	μ	
		β-Lactorfina-NH_2	YLLF-NH_2		A	μ	

	LF_H	Lactoferroxina A	YLGSGY-OCH_3	f(318-323)	AN	μ	Tani y col., 1990
		Lactoferroxina B	RYYGY-OCH_3	f(536-540)	AN	μ	
		Lactoferroxina C	KYLGPQY-OCH_3	f(673-679)	AN	μ	
	$Seroalbúmina_B$	Serorfina	YGFQNA	f(399-404)	A	δ > μ	Tani y col., 1994
	$Hemoglobina_B$	Hemorfina (1-4)	YPWT	β-cadena (34-47)	A	μ/δ > κ	Brantl y col., 1986
Vegetal	Glutenina	Exorfina A5	GYYPT	-	A	δ > μ	Fukudome y Yoshikawa,
		Exorfina A4	GYYP	-	A	δ > μ	1992; 1993
		Exorfina B5	YGGWL	-	A	δ	
		Exorfina B4	YGGW	-	A	δ	
		Exorfina C5	YPISL	-	A	δ > μ	
	α-Gliadina	Gliadorfina (1-7)	YPQPQPF	f(43-49)	A	-	Payan y col., 1987
	Alérgeno	Oryzatensina (1-9)	GYPMYPLPR	f(47-55)	A	μ	Takahashi y col., 1994
	RA5B	Oryzatensina (5-9)	YPLPR	f(51-55)	A	μ	
	Rubisco	Rubiscolina-6	YPLDLF	f(103-109)	A	δ	Yang y col., 2001
		Rubiscolina-5	YPLDL	f(103-108)	A	δ	
	β-Conglicinina	Soymorfina-7	YPFVVNA	f(323-329)	A	μ	Ohinata y col., 2007
		Soymorfina-6	YPFVVN	f(323-328)	A	μ	
		Soymorfina-5	YPFVV	f(323-327)	A	μ	

[1] $_B$: bovina, $_H$: humana

[2] A: agonista, AN: antagonista

CN: caseína, α-La: α-lactoalbúmina, β-Lg: β-lactoglobulina, LF: lactoferrina

mostraron un mayor efecto opioide en este ensayo con valores de inhibición (concentración necesaria para disminuir en un 50% la actividad, IC_{50}) de 0,5 y 3,6 μM, respectivamente, comparado con el valor de 5,1 μM, determinado para la β-CM-7 (Koch y col., 1985). Con respecto a las β-CMs de origen humano, caracterizadas por la presencia de los residuos valina-ácido glutámico (VE) en la cuarta y quinta posición desde el extremo N-terminal, en vez de los residuos prolina-glicina (PG) propios de las bovinas, estos autores encontraron un efecto entre 3 y 30 veces menor en el mismo ensayo de íleon de cobaya. Por lo tanto, las β-CMs de la leche humana también mostrarían efecto opioide, aunque significativamente menor al descrito para sus homólogos bovinos (Koch y col., 1985).

El **mecanismo opioide** ha sido determinado comúnmente por distintos métodos: (i) inhibición de la actividad de la enzima adenilato ciclasa, (ii) inhibición de la contracción inducida eléctricamente en preparaciones *ex vivo* de tejidos de íleon de cobaya, deferente de ratón y deferente de conejo, y (iii) estudios de unión con radioligandos. Las preparaciones animales *ex vivo* han sido ampliamente utilizadas debido a su mayor relevancia fisiológica, menor coste y mínimo impacto tóxico (Taylor, 2011). Además, los efectos opioides en tejidos de íleon de cobaya, deferente de ratón y deferente de conejo están principalmente mediados por los subtipos μ-, δ- y κ-, respectivamente, del receptor opioide, lo que ha permitido el uso de este tipo de preparaciones para evaluar la selectividad de los péptidos opioides (Janecka y col., 2004). Gracias a estos estudios se ha evidenciado que los péptidos exógenos de origen animal tienen una mayor selectividad por receptores del subtipo μ-opioide, y los de origen vegetal, con excepción de los descritos en soja y arroz, por el subtipo δ-opioide (**Tabla 2**). Como ejemplos, los valores IC_{50} descritos para los péptidos vegetales exorfina A5, rubiscolina-5 y soymorfina-5 en preparaciones de deferente de ratón fueron 60, 51 y 50 μM, respectivamente, *versus* 1000, 1110 y 6 μM en el ensayo

en íleon de cobaya (Fukudome y Yoshikawa, 1993; Yang y col., 2001; Ohinata y col., 2007).

Dependiendo de la localización de los sistemas opioides implicados, los **efectos fisiológicos** atribuidos a los péptidos opioides son diversos y pueden presentar actividad relacionada con numerosas funciones regulatorias y neuroendocrinas (Yoshikawa, 2015; Garg y col., 2016). Se han descrito efectos moduladores analgésicos, locomotores y sobre la conducta social de roedores tras la administración intraperitoneal o intracerebral de péptidos opioides (Teschemacher, 2003). En diversos estudios *in vivo*, las β-CMs administradas por vía oral prolongaron el tiempo del tránsito gastrointestinal (Daniel y col., 1990; Defilippi y col., 1994) e inhibieron la motilidad y el vaciado gástrico (Froetschel, 1996), aumentaron la secreción de polipéptido pancreático, insulina y somatostatina (Schusdziarra y col., 1983a; b; c), estimularon la ingesta vía enterostatina (White y col., 2000), e incrementaron la insulina plasmática y la actividad de las enzimas antioxidantes superóxido dismutasa (SOD) y catalasa (CAT) en ratas diabéticas (Yin y col., 2010). Además, se ha conseguido identificar la presencia de β-CMs en el contenido de digeridos intestinales de mini cerdos (Barbé y col., 2014) y humanos (Boutrou y col., 2013) tras la ingesta de productos lácteos. Sin embargo, en individuos adultos, no ha sido posible demostrar la presencia de estos péptidos en el plasma tras la administración oral de sus precursores. Este hecho, junto a la baja permeabilidad intestinal mostrada en modelos *in vitro* del epitelio con células Caco-2 (Iwan y col., 2008; Jarmolowska y col., 2013), sugiere que los efectos de estos péptidos pueden estar mediados por interacciones con receptores a nivel digestivo (Teschemacher, 2003).

Considerando los efectos locales producidos en el tracto gastrointestinal, varios estudios han evaluado una posible relación entre la activación de receptores opioides intestinales y la protección ejercida por la **barrera mucosa digestiva**. Usando el

modelo *ex vivo* de yeyuno de rata, se ha descrito que la β-CM-7 tiene un potente efecto inductor sobre la secreción de mucinas intestinales a través de la activación del sistema nervioso entérico y los receptores opioides (Claustre y col., 2002; Trompette y col., 2003). Posteriormente, se demostró el efecto directo de este péptido sobre células caliciformes intestinales de rata (DHE) y humanas (HT29-MTX), con capacidad secretora de mucinas y presencia de receptores opioides (Zoghbi y col., 2006). Estos autores encontraron un aumento significativo en la secreción de mucinas y la sobreexpresión de los principales genes implicados en ambos modelos celulares (Muc2 en DHE, y MUC5AC en HT29-MTX). Igualmente, la aplicación de un agonista μ-opioide, encefalina D-Ala(2),N-Me-Phe(4),glicinol(5), reprodujo estos efectos, mientras que el pretratamiento con un antagonista μ-opioide, cipromida, los anuló. Los péptidos lácteos μ-opioides α-lactorfina y β-lactorfina han demostrado efectos equiparables en células HT29-MTX (Martínez-Maqueda y col., 2012; 2013a). Por el contrario, otros péptidos a los que no se les atribuye actividad opioide y cuyas secuencias no presentan los requerimientos propios de los ligandos opiodes, como el fragmento de la β-CN f(94-123) (Plaisancié y col., 2013) y los péptidos derivados f(94-108) y f(117-123) (Plaisancié y col., 2015), también han manifestado efectos regulatorios en la producción de mucinas intestinales en células HT29-MTX y en el intestino delgado de rata. Además, Plaisancié y col. (2015) recientemente demostraron que el péptido opioide neocasomorfina-6, fragmento f(114-119) de la β-CN, inducía la sobreexpresión del gen que codifica para la mucina asociada a membrana MUC4 en células HT29-MTX, mientras que no tenía ningún efecto sobre las mucinas secretadas MUC2 y MUC5AC, ni en la secreción de mucinas. En estos trabajos se ha sugerido que la activación de los mecanismos opioides podría tener influencia sobre la producción de mucinas intestinales. Sin embargo, los efectos demostrados son variables dependiendo del tipo de mucina analizada y se han evaluado escasamente en modelos animales. Además, no se ha conseguido establecer una relación directa entre ambas actividades, ni se ha descartado la implicación de otros posibles mecanismos.

1.3.2. Actividad antioxidante

En condiciones fisiológicas, en las células del organismo existe un equilibrio entre la formación de especies reactivas de oxígeno (comúnmente denominadas ROS por sus siglas en inglés “reactive oxygen species”) y su neutralización por parte de los mecanismos de defensa antioxidantes. Las ROS se originan en la célula fundamentalmente durante el metabolismo endógeno del oxígeno para la obtención de energía. También pueden tener origen externo por diversos factores como la radiación, la quimioterapia, los agentes contaminantes, el tabaco, el alcohol y los xenobióticos, entre otros. En situación de homeostasis, las ROS cumplen funciones esenciales como eficientes moléculas de señalización celular y componentes del sistema inmunológico. Las células presentan un eficaz sistema antioxidante, tanto enzimático, en el que destaca la actividad de las enzimas glutatión peroxidasa (GPx), glutatión reductasa, CAT y SOD, como no-enzimático, cuyo principal agente es el tripéptido ECG, denominado glutatión (GSH). Cuando se produce un desequilibrio debido a un aumento en la producción de ROS o a una reducción en los mecanismos de defensa antioxidante propia de situaciones patológicas (Ray y col., 2012), la célula entra en un estado anómalo conocido como **estrés oxidativo**. En este estado, las ROS pueden interaccionar con lípidos, proteínas, ácidos nucleicos, enzimas y otras moléculas celulares, y debido a su alta reactividad y carácter de radicales libres, pueden oxidar y alterar sus estructuras químicas (**Figura 3**).

Se ha reconocido el papel que juegan los desequilibrios oxidativos celulares como agente causal subyacente en el desarrollo de numerosos procesos patológicos crónicos, como la inflamación, las enfermedades cardiovasculares, digestivas y neurodegenerativas, la diabetes, el envejecimiento y el cáncer (Reuter y col., 2010; Rains y Jain, 2011; Mittal y col., 2014). Además, las reacciones de oxidación participan en el origen de múltiples procesos relacionados con la pérdida de calidad y deterioro nutricional que se producen en el alimento.

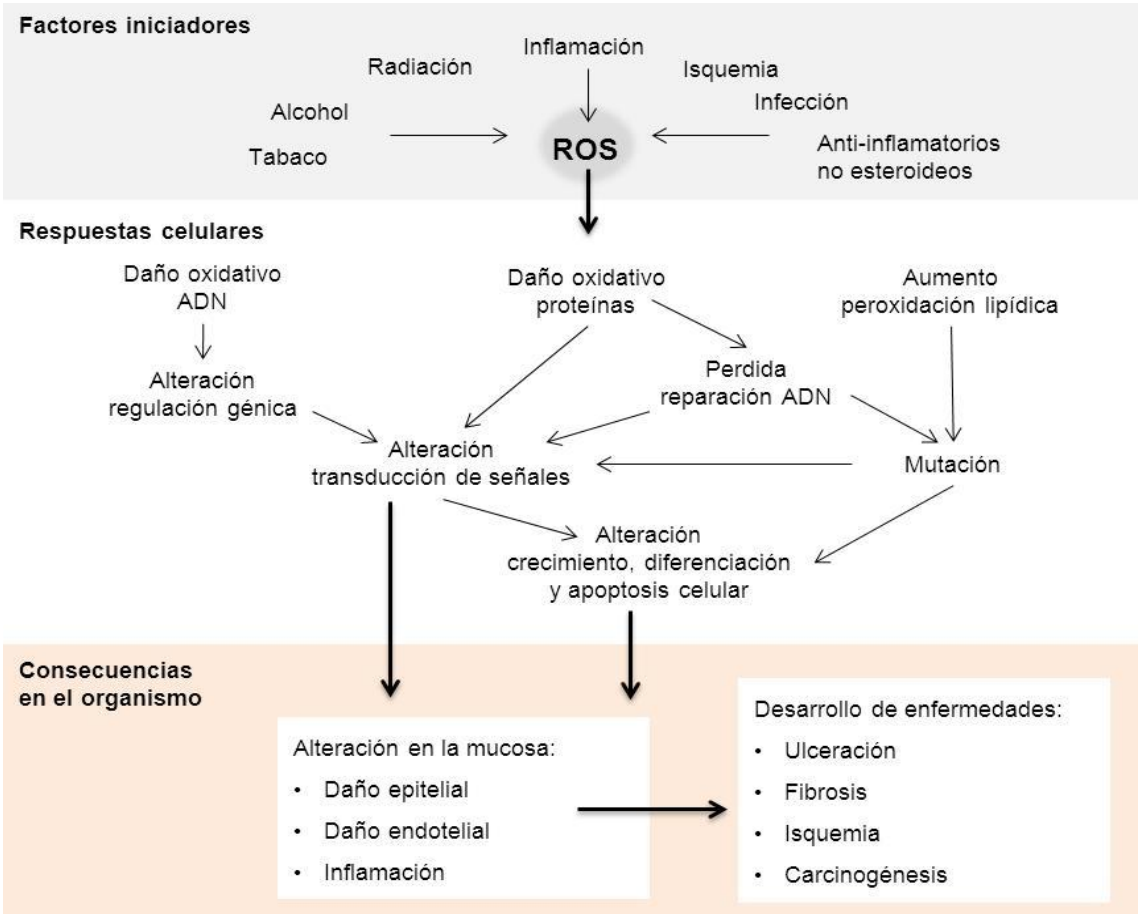


Figura 3. Representación esquemática de la inducción del estado de estrés oxidativo y sus efectos patofisiológicos (Adaptada de Bhattacharyya y col., 2014).

El tracto gastrointestinal se encuentra continuamente sometido al ataque oxidativo de altas concentraciones de ROS en el contenido luminal como consecuencia de ciertos compuestos pro-oxidantes de la dieta, fármacos o tóxinas, así como aquellas especies reactivas generadas de forma endógena en el metabolismo del propio organismo (Zhu y Li, 2012). Además, en íntima relación con el sistema digestivo, se considera que el hígado es particularmente sensible a los agentes tóxicos y oxidativos al estar directamente conectado con los órganos gastrointestinales y el bazo mediante la circulación portal hepática. Por todo ello, en numerosos estudios se han vinculado los desequilibrios del sistema redox con el inicio y la progresión de varias patologías digestivas, como las enfermedades inflamatorias intestinales, las disfunciones hepáticas y el cáncer colorrectal (Alzoghaibi, 2013; Bhattacharyya y col., 2014).

Las estrategias actuales de intervención farmacológica están principalmente basadas en el uso de corticosteroides y otros agentes inmunosupresores. Sin embargo, estos fármacos presentan varios efectos adversos tras su administración prolongada, como alteraciones gastrointestinales, anemia, hepatotoxicidad, nefrotoxicidad y reacciones de hipersensibilidad (Rutgeerts y col., 2009). Además, el uso crónico de estos fármacos se ha asociado a una falta de efectividad de los mismos y/o una menor respuesta farmacológica (Barnes y Adcock, 2009). Por ello, y con el objeto de evitar estos inconvenientes, numerosas investigaciones se han centrado en la búsqueda e identificación de nuevos agentes de origen natural con potencial efecto antioxidante (Moura y col., 2015). Entre ellos, destacan las investigaciones relacionadas con los compuestos fenólicos derivados de frutas y vegetales (Goya y col., 2016; Umeno y col., 2016). Además, estos agentes se presentan como una alternativa más segura frente al uso de antioxidantes artificiales para retardar y evitar las alteraciones del propio alimento relacionadas con las reacciones de oxidación. Debido a la interacción directa y continua entre el tracto digestivo y los componentes alimentarios derivados de la digestión, como son los péptidos bioactivos, estos compuestos pueden representar una interesante estrategia preventiva para la protección del organismo a este nivel.

Se han descrito numerosos **métodos** para evaluar la capacidad antioxidante de un compuesto. Inicialmente, la mayoría de las investigaciones se centraron en distintos métodos químicos *in vitro*, basados en la complejidad de las reacciones de oxidación que ocurren en los sistemas biológicos. Estos métodos químicos pueden clasificarse en ensayos basados en la transferencia de un átomo de hidrógeno, entre los que destacan el ensayo “oxygen radical absorbance capacity (ORAC)” y el ensayo “total radical trapping antioxidant parameter (TRAP)”; y los basados en la transferencia de electrones, como el ensayo “Trolox equivalent antioxidant capacity (TEAC)”, el ensayo “ferric ion reducing antioxidant parameter (FRAP)” y el ensayo “diphenyl-1-

picrylhydrazyl (DPPH) radical scavenging capacity assay", entre otros (Huang y col., 2005). Sin embargo, ninguno de estos métodos proporciona resultados inequívocos, existiendo además una falta de estandarización entre ellos, por lo que se ha recomendado aplicar una combinación de los mismos para evaluar conjuntamente diferentes condiciones de oxidación y mecanismos de acción de las sustancias (Carocho y Ferreira, 2013). Además, estas metodologías presentan varias limitaciones ya que solamente reflejan reactividad química, pero no demuestran evidencias de relevancia fisiológica (Sarmadi e Ismail, 2010). En este sentido, en los últimos años se han empleado los modelos *in vitro* en cultivos celulares ya que permiten evaluar la biodisponibilidad, el metabolismo y la bioactividad de los compuestos antioxidantes, y son una herramienta alternativa y previa a los modelos animales y a los ensayos clínicos que suponen un mayor tiempo, coste e implicaciones éticas (Power y col., 2013; Wan y col., 2015). Por ello, únicamente los resultados obtenidos mediante ensayos en cultivos celulares o estudios *in vivo* se consideran a continuación.

La actividad antioxidante de péptidos **lácteos** ha sido ampliamente evaluada mediante métodos químicos. Sin embargo, hay pocos estudios que hayan demostrado estos efectos en modelos celulares o *in vivo* (Power y col., 2013; Nongonierma y FitzGerald, 2015). Las proteínas de suero lácteo han sido las más estudiadas en cuanto a sus potenciales efectos antioxidantes. En el modelo celular de estrés oxidativo inducido por peróxido de hidrógeno (H_2O_2) en células de feocromocitoma de rata PC12, Jin y col. (2013) encontraron un efecto protector antioxidante para hidrolizados de proteínas de suero mediado por la actividad de las enzimas SOD y CAT y el marcador de daño oxidativo malondialdehído. Esta protección, además, se asoció a un efecto estabilizador de los niveles de ROS y Ca^{2+} y a una inhibición de la apoptosis (Jin y col., 2013). Posteriormente, Zhang y col. (2015) obtuvieron resultados similares, y propusieron que la naturaleza hidrofóbica de los hidrolizados se relacionaba con su potencial antioxidante. La hidrólisis enzimática de un concentrado

de proteínas de suero fue capaz de estimular el GSH intracelular y la actividad de la CAT, así como de regular la expresión de varios genes implicados en los mecanismos de defensa antioxidantes y de detoxificación de células endoteliales humanas HUVECs (O´Keeffe y FitzGerald, 2014). También se ha demostrado *in vivo* el efecto protector antioxidante de las proteínas de suero en modelos de ratas diabéticas (Ebaid y col., 2011), ratas con hepatotoxicidad inducida por tetracloruro de carbono (Gad y col., 2011) y ratas con envejecimiento inducido por suplementación en exceso de D-galactosa (Peng y col., 2014). En un estudio clínico se observó como la ingesta de un aislado de proteínas de suero enriquecido en cisteína mejoraba la funcionalidad hepática, mediante un incremento del GSH plasmático y de la capacidad antioxidante total de pacientes con esteatosis hepática de origen no alcohólico (Chitapanarux y col., 2009). Además, Lollo y col. (2014) demostraron que un hidrolizado de proteínas de suero inducía efectos reductores en los niveles de lactato deshidrogenasa y creatinquinasa, marcadores enzimáticos de daño oxidativo tisular, en individuos sometidos a esfuerzo físico.

En cuanto a la fracción de caseínas de la leche, los caseinofosfopéptidos (CPPs) han mostrado efecto preventivo a nivel del epitelio digestivo usando células Caco-2 bajo estrés oxidativo inducido por H_2O_2 (García-Nebot y col., 2011). Estos autores vieron que las células pretratadas con CPPs estaban protegidas frente al daño oxidativo y mantenían la viabilidad y el progreso del ciclo celular, mediante un aumento del GSH intracelular y de la actividad de la CAT y una disminución de la peroxidación lipídica. Usando este mismo modelo de daño oxidativo en células hepáticas HepG2, se comprobó la actividad antioxidante de caseínas hidrolizadas mediante la acción de la enzima Alcalasa® y enzimas gastrointestinales (Xie y col., 2013). En este estudio, destacó el efecto llevado a cabo por la fracción biodisponible y menor de 1000 Dalton del hidrolizado de caseínas con Alcalasa®, aunque no se identificaron los péptidos potencialmente responsables de la actividad. Recientemente, Bessette y col. (2016)

han demostrado que el péptido β-CN f(94-123) es capaz de proteger *in vivo* frente a lesiones del epitelio intestinal inducidas por el anti-inflamatorio no esteroideo indometacina. Estos autores observaron como la administración oral de este péptido en ratas reducía la severidad del daño oxidativo a la mucosa intestinal, mejorando su capacidad de barrera y disminuyendo la apoptosis celular.

La **soja** también ha sido descrita como una fuente natural de compuestos con capacidad antioxidante. Vernaza y col. (2012) demostraron que las harinas de soja germinadas durante 72 h e hidrolizadas con Alcalasa® tenían un efecto reductor sobre varios marcadores de estrés inflamatorio y oxidativo inducido por lipopolisacárido (LPS) en macrófagos murinos RAW 264.7. En este modelo celular, Dia y col. (2014) encontraron resultados similares para varios hidrolizados de productos comerciales de soja con pepsina y pancreatina. Recientemente, se ha visto en ratas que el consumo de una dieta normocalórica enriquecida en harinas de soja mejora los mecanismos antioxidantes enzimáticos y el contenido de GSH reducido hepáticos, en comparación con dietas basadas en caseínas (Razzeto y col., 2015). Además, Celec y col. (2013) observaron en un estudio clínico con individuos sanos como el consumo de soja (2 g/Kg/día) durante 7 días aumentaba la capacidad antioxidante total en hombres y mujeres, y disminuía la oxidación de proteínas en mujeres. En estos estudios, además de las proteínas de la soja y sus péptidos derivados, otros componentes de naturaleza no proteica como polifenoles, isoflavonas, saponinas, oligosacáridos y fosfolípidos podrían contribuir a la actividad antioxidante observada. Sin embargo, hasta el momento, los agentes fitoquímicos responsables de estos efectos no han sido identificados.

En relación a la fracción proteica de la soja, Hwang y col. (2011) han descrito que una mezcla de péptidos purificados puede ejercer efectos antioxidantes y de disminución de la muerte celular en miocitos C2C12 químicamente inducidos por H_2O_2, mediante la vía de quinasas reguladas por señales extracelulares. Además, esta

actividad se ha asociado con un efecto anti-trombótico en un modelo de rata (Hwang y col., 2011). En el estudio de Amigo-Benavent y col. (2014), se mostró que la deglicosilación de la proteína β-conglicinina mejoraba el estado oxidativo de células Caco-2 estimuladas con H_2O_2, facilitando además su digestión gastrointestinal y la absorción transepitelial de los péptidos generados. En un modelo *in vivo* de esteatosis hepática no alcohólica inducida por el consumo de una dieta grasa, Yang y col. (2011) determinaron que las ratas que habían recibido una suplementación con proteína de soja presentaban un mayor potencial antioxidante gracias a la activación de las enzimas CAT y SOD y a la disminución de la expresión del citocromo P450 2E1. La protección hepática observada en este trabajo se relacionó, además, con una disminución de los niveles de colesterol y triglicéridos y una mejora en la resistencia a la insulina. Young y col. (2012) observaron que un hidrolizado de proteínas de soja enriquecido en di- y tri-péptidos presentaba propiedades preventivas en un modelo de inflamación intestinal porcino. Este efecto se asoció, posteriormente, a la actividad del péptido VPY a través del transportador PepT1 (Kovacs-Nolan y col., 2012). Entre los péptidos bioactivos presentes en la soja con potencial antioxidante destacan los inhibidores de proteasas y el péptido lunasina. El inhibidor de proteasas BBI, en forma de un concentrado de proteína de soja enriquecido en BBI (BBIC), fue capaz de reducir los niveles de ROS en fibras musculares de ratón (Arbogast y col., 2007). Además, Li y col. (2011) demostraron su capacidad protectora frente al incremento de ROS y citoquinas pro-inflamatorias en macrófagos sometidos a estrés oxidativo por LPS. Paralelamente, el péptido lunasina ha mostrado efectos similares en diversos ensayos químicos y cultivos celulares. Se ha demostrado la actividad beneficiosa de este péptido al disminuir el estado oxidativo de macrófagos RAW 264.7 estimulados con LPS (Hernández-Ledesma y col., 2009b). Además, esta protección celular se ha vinculado con un potente efecto anti-inflamatorio mediado por mecanismos celulares del factor de transcripción nuclear-κB (Cam y de Mejia, 2012) y la expresión de la integrina αVβ3 (Cam y col., 2013). De una forma similar, García-Nebot y col. (2014)

observaron resultados equiparables en células intestinales Caco-2 sometidas a estrés oxidativo mediante los agentes químicos H_2O_2 y *tert*-butil hidroperóxido (*t*-BOOH). De todo ello, se concluye que la actividad protectora de estos péptidos frente a estadios de estrés oxidativo e inflamatorio podría contribuir a su potencial efecto quimiopreventivo (Hernández-Ledesma y Hsieh, 2015).

1.3.3. Actividad antiproliferativa

El cáncer es una enfermedad crónica reconocida como una de las principales causas de muerte a nivel mundial. A pesar de los avances médicos que se han alcanzado en los últimos años relativos a los tratamientos de quimioterapia, radioterapia y cirugía, recientemente se ha estimado que para el año 2030, aproximadamente 27 millones de nuevos casos de cáncer serán diagnosticados y 17 millones de pacientes de cáncer morirán a causa de esta enfermedad (Ferlay y col., 2015). Tan sólo un 5-10% de los casos de cáncer se han relacionado con factores genéticos, mientras que entre el 90-95% se han atribuido a agentes medioambientales externos y al estilo de vida (Anand y col., 2008). Entre ellos, los hábitos dietéticos podrían relacionarse con aproximadamente un 35% de los casos de cáncer (Davis y Milner, 2007). Además, numerosos estudios *in vitro*, *in vivo* y evidencias epidemiológicos han propuesto el papel preventivo que podrían tener los componentes encontrados en los alimentos (de Kok y col., 2008; Amin y col., 2009).

La **quimioprevención** se ha definido como “el uso de sustancias químicas, naturales o sintéticas, que bloquean, retrasan o revierten el proceso de la carcinogénesis”. Un gran número de los agentes quimiopreventivos conocidos hasta el momento están presentes en los alimentos, pudiendo tener actividad antiproliferativa reduciendo el riesgo de cáncer e, incluso, sensibilizando a las células tumorales frente a las terapias anti-cancerígenas, además de evitar los efectos adversos propios de este tipo de tratamientos (Pratheeshkumar y col., 2012). Los polifenoles son uno de los

grupos de compuestos alimentarios más estudiados por sus propiedades quimiopreventivas, destacando algunos, como la quercetina, las catequinas, el resveratrol y la curcumina (Shay y col., 2015; Bimonte y col., 2016; Lewandowska y col., 2016), entre otros. En los últimos años, numerosos agentes quimiopreventivos se han descrito también dentro del grupo de las proteínas alimentarias y los péptidos derivados (Blanco-Míguez y col., 2016). Estos compuestos presentan ciertas ventajas con respecto a otros tipos de moléculas quimioterapéuticas, como su gran afinidad y especificidad por ciertas dianas celulares, su facilidad de penetración en los tejidos, así como su bajo coste, toxicidad y efectos adversos demostrados y su alta aceptación por parte de los consumidores (Bhutia y Maiti, 2008).

Los estudios llevados a cabo para elucidar los mecanismos moleculares de la carcinogénesis han propuesto que las células cancerígenas presentan seis alteraciones esenciales que conjuntamente desembocan en el desarrollo del proceso tumoral (Hanahan y Weinberg, 2011). Tal como se representa en la **Figura 4**, las células cancerígenas son capaces de ignorar las señales de inhibición del crecimiento celular, presentan sus propias señales de proliferación, muestran un umbral de apoptosis muy elevado y capacidad de evadir la muerte celular programada, y son potencialmente capaces de replicarse sin límite, e invadir tejidos y capilares dando lugar a los procesos de angiogénesis y metástasis.

Varios estudios han demostrado el efecto quimiopreventivo de los péptidos bioactivos como agentes moduladores del proceso tumoral mediante, la inducción de la apoptosis, el bloqueo de vías de señalización celular, la inhibición de la angiogénesis, así como mediante efectos anti-inflamatorios, antioxidantes e inmunomodulantes (**Figura 5**).

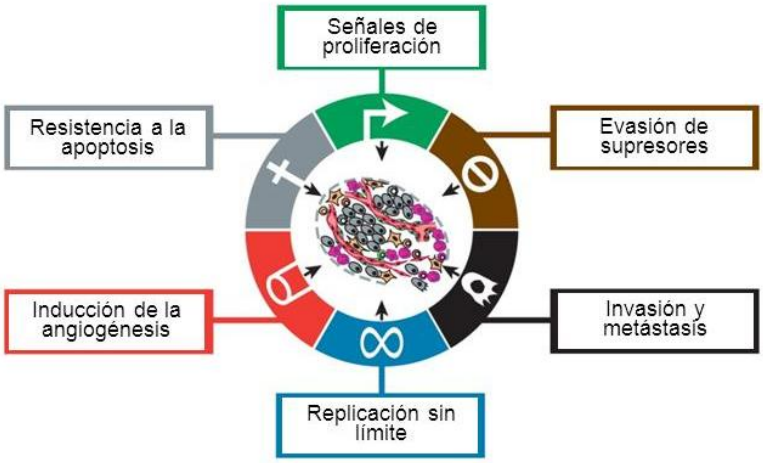


Figura 4. Representación esquemática de las alteraciones distintivas de las células tumorales (Adaptada de Hanahan y Weinberg, 2011).

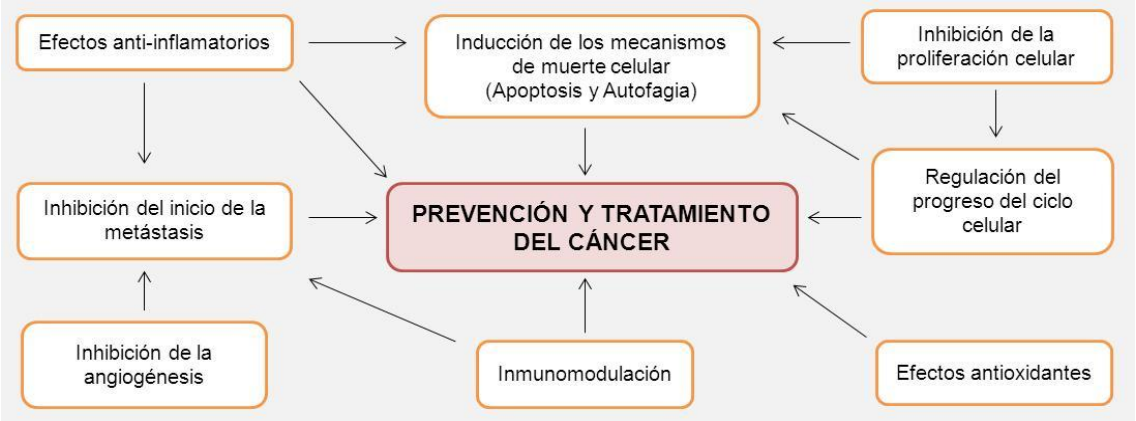


Figura 5. Representación esquemática de los mecanismos celulares implicados en la actividad quimiopreventiva de los péptidos antiproliferativos (Adaptada de de Mejia y Dia, 2010).

En este sentido, se ha descrito que los péptidos bioactivos pueden regular la expresión de proteínas y genes implicados en los distintos estadios del inicio y progreso del desarrollo tumoral (de Mejia y Día, 2010; Blanco-Míguez y col., 2016). Numerosos trabajos han revisado la actividad antiproliferativa ejercida por proteínas y

péptidos lácteos y de soja frente a cultivos celulares de cáncer de mama, próstata, colorrectal, hepático, pulmonar, gástrico, ovárico, fibrosarcoma, leucemia y linfoma, entre otros (de Mejia y Día, 2010; Pepe y col., 2013; Clemente y Arqués, 2014; Hernández-Ledesma y Hsieh, 2015; Sah y col., 2015).

En el contexto de la interacción directa entre los digeridos de compuestos de naturaleza proteica y el tracto digestivo, se ha establecido una evidencia creciente sobre los potenciales efectos quimiopreventivos ejercidos a este nivel (Half y Arber, 2013; Khoogar y col., 2016). En la **Tabla 3** se detallan aquellos efectos relacionados con la protección de la salud digestiva demostrados mediante estudios en **cultivos celulares** para proteínas y péptidos lácteos y de soja, así como los mecanismos de acción implicados en la actividad antiproliferativa. Dentro de las proteínas lácteas, destaca la actividad de la proteína de suero LF y del péptido lactoferricina, derivado de la proteína parental mediante digestión enzimática por pepsina. Se ha sugerido que el posible mecanismo de su acción quimiopreventiva podría relacionarse, por un lado, con su naturaleza catiónica, que le permite interaccionar con la superficie de la célula cancerígena caracterizada por su carga negativa, causando la desestabilización de la membrana tumoral (Hoskin y Ramamoorthy, 2008), y por otro, con su capacidad inhibitoria de la angiogénesis (Mader y col., 2006). Además se ha determinado que la unión de la LF con el ácido oleico da lugar a complejos proteína-ácido graso con efectos citotóxicos en varias líneas tumorales (Pepe y col., 2013; Fang y col., 2014), al igual que se ha demostrado para la proteína α-La. La α-La humana y bovina, en su unión al ácido oleico forma los complejos conocidos como “human alpha-lactalbumin made lethal to tumor cells (HAMLET)” y “bovine alpha-lactalbumin made lethal to tumor cells (BAMLET)”, respectivamente, que también han mostrado eficacia quimiopreventiva dependiente del tipo celular (Brinkmann y col., 2013). Una mezcla comercial de péptidos obtenidos a partir de proteínas de suero mediante digestión enzimática mostró capacidad inductora de la apoptosis y de condensación nuclear y

Tabla 3. Proteínas y péptidos lácteos y de soja con actividad antiproliferativa a nivel digestivo.

Proteína-Péptido[1]	Tipo de cáncer	Línea celular	Mecanismos de acción				Referencia
			Citotoxicidad (IC_{50})	Ciclo celular	Apoptosis	Otros	
LF_L	Colorrectal	HCT-116	~ 5,0 mg/mL	-	-	Efectos antioxidantes	Habib y col., 2013
$LFcin_L$	Carcinoma oral	SAS	~ 1,0 mg/mL	-	Inducción vía caspasa-3 y PARP	Fosforilación vías ERK1/2 y JNK/SAPK	Sakai y col., 2005
	Colorrectal	C26	111,0 µM	-	-	Disrupción de la membrana celular	Eliassen y col., 2002
		HT-29 Colo-35	~ 0,1 mg/mL	-	Inducción de la apoptosis Fragmentación del ADN	-	Mader y col., 2005
		Caco-2	-	Bloqueo en fase S	-	Descenso ciclina E1	Freiburghaus y col., 2009
$LFcin_L$-AO	Colorrectal	HT-29	5,0 µM	-	Inducción de la apoptosis	-	Fang y col., 2014
	Hepato-carcinoma	HepG2	4,9 µM	-	Inducción vía mitocondrial y receptor de muerte celular	-	
α-La_L-AO	Colorrectal	HT-29	45,8 µM	-	Inducción de la apoptosis	-	
	Hepato-carcinoma	HepG2	50,2 µM	-	Inducción de la apoptosis	-	
$CPPs_L$	Colorrectal	HT-29	-	-	Inducción vía caspasa-3/7	Activación de canales de voltaje dependientes de Ca^{2+}	Perego y col., 2012
$Lunasina_S$	Colorrectal	HT-29	61,7 µM	Bloqueo en fase G2/M Incremento p21	Inducción vía mitocondrial Incremento Bax, nCLU y caspasa-3 Descenso Bcl-2	Efecto sinérgico con cisplatino	Dia y de Mejia, 2010

		KM12L4	13,0 µM	Bloqueo en fase G2/M Incremento p21 y p27	Inducción vía mitocondrial Incremento Bax, nCLU, citocromo c, caspasa-3/2/9 Descenso Bcl-2 Condensación nuclear y fragmentación del ADN	Inhibición de las vías FAK/ERK y NF-κB Unión a integrina $\alpha_5\beta_1$ Modulación de angiogénesis y metástasis Efecto sinérgico con cisplatino	Dia y de Mejia, 2011a; b
		HCT-116	26,3 µM	-	-	Inhibición de las vías FAK/ERK y NF-κB	
		RKO	21,6 µM	-	-	Inhibición de las vías FAK/ERK y NF-κB Inhibición de la migración celular	
BBI_S	Hepato-carcinoma	HepG2	140,0 µM	-	-	-	Ho y Ng, 2008
	Colorrectal	HT-29	46,0 µM (BBI) 48,3 µM (IBB1) 39,9 µM (IBBD2)	Bloqueo en fase G0/G1	-	Actividad relacionada con la capacidad inhibitoria de proteasas	Clemente y col., 2010
KTI_S	Hepato-carcinoma	HepG2	-	-	-	Inducción de citoquinas	Fang y col., 2010
			> 25,0 µM	-	-	-	Ye y Ng, 2009
$Lectina_S$	Hepato-carcinoma	HepG2	4,1 µM	-	-	-	Lin y col., 2008

[1] $_L$: lácteo, $_S$: soja

LF: lactoferrina, LFcin: lactoferricina, AO: ácido oleico, α-La: α-lactoalbúmina, CPPs: caseinofosfopéptidos, BBI: inhibidor Bowman-Birk, KTI: inhibidor de tripsina Kunitz, IBB1 y IBBD2: iso-inhibidores Bowman-Birk, PARP: poli-ADP ribosa polimerasa, nCLU: cluster nuclear, ERK: quinasas reguladoras por señales extracelulares, JNK: quinasas c-Jun N-terminal, SAPK: quinasas activadas por estrés, FAK: quinasas de adhesión focal, NF: factor de transcripción nuclear

fragmentación del ADN en células de cáncer colorrectal HT-29 (Kreider y col., 2011). Sin embargo, estos autores no identificaron los péptidos responsables de dichos efectos. Las caseínas también representan una fuente de péptidos con propiedades antitumorales. Perego y col. (2012) determinaron el efecto pro-apoptótico de los CPPs en células HT-29 mediante su habilidad de unión al Ca^{2+}, mientras que se ha propuesto que los péptidos β-CM-7 y β-CM-5 pueden bloquear el progreso del ciclo celular de células tumorales de cáncer de mama y colorrectal mediante su interacción con receptores opioides y de somatostatina (De Simone y col., 2009; Pepe y col., 2013). El péptido lunasina es uno de agentes de la fracción proteica de la soja que más se ha estudiado por sus propiedades quimiopreventivas (**Tabla 3**). Este péptido ha demostrado efectos antiproliferativos en cultivos celulares de cáncer de mama, próstata, pulmón, colorrectal, leucemia y linfoma, mediante diversos mecanismos relacionados con el control del ciclo celular, la apoptosis y la metástasis, atribuyéndosele también una acción regulatoria de naturaleza epigenética (Hernández-Ledesma y Hsieh, 2015). En el caso de los inhibidores de proteasas presentes en la soja, destaca la acción quimiopreventiva de las familias BBI y KTI, que se ha relacionado principalmente con su capacidad para inhibir la acción enzimática de las proteasas tripsina y quimiotripsina en el inicio de la carcinogénesis (Clemente y Arqués, 2014). Además, estos inhibidores son particularmente resistentes a las condiciones del tracto digestivo (Clemente y col., 2011). Adicionalmente, Rayaprolu y col. (2013) demostraron que la hidrólisis y fraccionamiento de aislados de proteína de soja daba lugar a la formación de compuestos resistentes a la digestión gastrointestinal *in vitro* y con actividad antitumoral dosis-dependiente en células de cáncer colorrectal (Caco-2 y HCT-116), hepático (HepG2) y pulmonar (NCL-H1299). Sin embargo, estos autores no identificaron la secuencia de los péptidos generados y potencialmente responsables de los efectos observados.

Los resultados demostrados en cultivos celulares han dado origen a algunos estudios con el objetivo de trasladar dichos efectos a evidencias ***in vivo***. Mediante el uso de modelos animales de metástasis, se mostró que la actividad quimiopreventiva del péptido lactoferricina se relacionaba con su habilidad para suprimir la angiogénesis y la metástasis a los tejidos hepáticos y pulmonares (Eliassen y col., 2002; 2006; Mader y col., 2006). Paralelamente, se ha observado que la administración oral de este péptido induce un efecto quimiopreventivo, anti-metastásico e inmunomodulador en la mucosa intestinal mediante la activación de caspasa-1, interleuquina-18, interferón-γ y células de defensa T y "natural killer" (Kuhara y col., 2000; Iigo y col., 2004). El péptido lunasina ha demostrado efectos *in vivo* en el modelo de metástasis hepática inducido por células de cáncer colorrectal KM12L4 (Dia y de Mejia, 2011b; 2013). En estos estudios se observó una reducción del antígeno de proliferación celular, relacionado con el bloqueo del progreso del ciclo celular, y una inducción de la apoptosis mediante modulación de los marcadores Bcl-2:Bax. Sin embargo, se encontraron diferencias entre la administración por vía oral e intraperitoneal del péptido, que hicieron difícil obtener resultados definitivos sobre el potencial efecto *in vivo* de la lunasina frente a la metástasis del cáncer colorrectal (Dia y de Mejia, 2011b; 2013). Utilizando modelos animales de lesiones intestinales inflamatorias y pre-cancerígenas inducidas por los agentes químicos 1,2-dimetilhidrazina y sulfato de dextrano sódico, se ha evidenciado el papel protector del BBI derivado de la soja. Este péptido ha sido capaz de prevenir la formación de lesiones neoplásicas e inflamatorias y de reducir la incidencia y frecuencia de los tumores colorrectales y la tasa de mortalidad (St Clair y col., 1990; Ware y col., 1999; Kennedy y col., 2002; Carli y col., 2012). En el año 1992, la Agencia Estadounidense de Alimentos y Medicamentos (FDA) aprobó la investigación del BBIC en ensayos clínicos. Desde entonces, se han llevado a cabo seis ensayos clínicos en pacientes de hiperplasia prostática benigna, leucoplasia oral y colitis ulcerativa (Clemente y Arqués, 2014). En concreto, en el estudio clínico de colitis ulcerativa, Lichtenstein y col. (2008) describieron que el

tratamiento durante 12 semanas (800 unidades/día) con BBIC presentaba notables beneficios en relación a la remisión de la sintomatología clínica (50,0%) y recuperación de la enfermedad (36,0%), comparado con el grupo placebo (respuesta sintomatológica parcial 29,0%, y recuperación 7,1%). En estos estudios se ha indicado el potencial efecto quimiopreventivo que pueden tener las proteínas alimentarias y sus péptidos derivados frente a diversos factores de riesgo relacionados con el inicio y progreso de enfermedades crónicas, como el cáncer (Hernández-Ledesma y Hsieh, 2015). Sin embargo, todavía es necesario un mayor número de investigaciones para evidenciar cómo estos péptidos interaccionan con el organismo y poder demostrar científicamente su efectividad en estudios clínicos.

2. RESULTS / RESULTADOS

Estudio del comportamiento del péptido lunasina frente a la simulación *in vitro* de la digestión y la absorción gastrointestinal. Efectos sobre la viabilidad de células tumorales del tracto digestivo.

2.1: Publicación I: **The protective role of the Bowman-Birk protease inhibitor in soybean lunasin digestion: the effect of released peptides on colon cancer growth.**

FOOD & FUNCTION, *6* (2015) 2626-2635

2.2. Publicación II: **Transepithelial transport of lunasin and derived peptides with inhibitory effects on the viability of gastrointestinal cancer cells.**

FOOD & FUNCTION (submitted)

Food & Function

PAPER

Cite this: *Food Funct.*, 2015, **6**, 2626

The protective role of the Bowman-Birk protease inhibitor in soybean lunasin digestion: the effect of released peptides on colon cancer growth†

Elvia Cruz-Huerta,‡[a] Samuel Fernández-Tomé,‡[a] M. Carmen Arques,[b] Lourdes Amigo,[a] Isidra Recio,[a] Alfonso Clemente[b] and Blanca Hernández-Ledesma*[a]

Received 27th April 2015,
Accepted 30th May 2015

DOI: 10.1039/c5fo00454c

www.rsc.org/foodfunction

Lunasin is a naturally-occurring peptide demonstrating chemopreventive, antioxidant and anti-inflammatory properties. To exhibit these activities, orally ingested lunasin needs to survive proteolytic attack of digestive enzymes to reach target tissues in active form/s. Preliminary studies suggested the protective role of protease inhibitors, such as the Bowman-Birk inhibitor and Kunitz-trypsin inhibitor, against lunasin's digestion by both pepsin and pancreatin. This work describes in depth the behaviour of lunasin under conditions simulating the transit through the gastrointestinal tract in the absence or presence of soybean Bowman-Birk isoinhibitor 1 (IBB1) in both active and inactive states. By liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), the remaining lunasin at the end of gastric and gastro-duodenal phases was quantified. Protection against the action of pepsin was independent of the amount of IBB1 present in the analyzed samples, whereas an IBB1 dose-dependent protective effect against trypsin and chymotrypsin was observed. Peptides released from lunasin and inactive IBB1 were identified by MS/MS. The remaining lunasin and IBB1 as well as their derived peptides could be responsible for the anti-proliferative activity against colon cancer cells observed for the digests obtained at the end of simulated gastrointestinal digestion.

1. Introduction

Lunasin is a naturally-occurring peptide corresponding to the small subunit peptide of 2S albumin.[1] Its amino acid sequence (SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDDD, National Center for Biotechnology Information, NCBI, accession number AAP62458) is characterized by the presence of a predicted α-helix structure, a tri-peptide RGD cell adhesion motif and a continuous sequence of nine aspartic acid residues at the C-terminus. A recent molecular dynamics study of this peptide has suggested the important role played by its α-helicity and highly negatively-charged C-terminal tail in the recognition of and binding to the chromatin residue, and thus, in the anti-mitotic activity of lunasin in several mammalian cell lines.[2]

Recent studies have revealed the potential role of lunasin against established breast, colon, and prostate cancer, and leukemia cell lines, through its ability to inhibit cell proliferation by arresting the cell cycle and inducing apoptosis.[3] The chemoprotective effects of lunasin against skin,[4] breast,[5,6] and colon cancer[7,8] have also been evaluated in animal models. Moreover, lunasin has been shown to restrain the oxidative status caused by chemical agents in both intestinal Caco-2 and hepatic HepG2 cells, and to inhibit inflammation in cultured RAW 264.7 macrophages. These antioxidant and anti-inflammatory properties have been suggested to contribute to the anti-cancer activity of lunasin.[3]

Food-derived peptides are expected to exhibit their health beneficial properties after being orally taken. Even though these peptides can elicit hormone-like functions locally in the gastrointestinal tract, they are generally required to flow into the blood to exhibit specific activities at a systemic level. In the last few years, the number of studies evaluating bioavailability aspects of dietary peptides, such as their resistance to digestive enzymes and their absorption/distribution rates has notably increased.[9] In addition, the application of peptidomics tools

[a]*Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain. E-mail: b.hernandez@csic.es; Fax: +34 910017905; Tel: +34 910017970*

[b]*Estación Experimental del Zaidín (EEZ-CSIC), Profesor Albareda, 1, 18008 Granada, Spain*

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c5fo00454c

‡Both authors equally contributed to this work.

in these analyses has markedly emerged.[10] It is increasingly obvious that gastrointestinal digestion has an important influence on the biological activity of food-derived peptides, with the release of new active fragments from their precursors or, on the contrary, giving rise to fragments with less or null activity.[11–15]

Park *et al.*[16] reported that isolated lunasin, either synthetic or soybean-purified, is easily digested after 2 min incubation with simulated gastric or intestinal fluids. However, lunasin survives, at least partially, the attack of digestive enzymes when it is present in crude protein extracts purified from soybean or other plant sources. By western-blot analysis, Jeong *et al.*[17] demonstrated that approximately 85% of the original lunasin contained in *Solanum nigrum* L. crude protein extract remained intact after 120 min digestion with simulated gastrointestinal fluids. In the case of lunasin present in soybean crude protein extract, 60% and 80% of the initial lunasin was detected by immunoblotting after proteolysis by pepsin and pancreatin for 120 min, respectively.[16] The results from these studies suggested that the presence of naturally-occurring protease inhibitors in sufficient amounts, such as the Bowman-Birk inhibitor (BBI) or the Kunitz-trypsin inhibitor, might exert a protective effect against lunasin's proteolysis by digestive enzymes. Hernández-Ledesma *et al.*[18] also observed the protective effect exerted by BBI when lunasin present in soybean-derived foods was subjected to a sequential digestion with pepsin and pancreatin, suggesting the importance of the lunasin : BBI ratio in this protection. However, to our knowledge, the behavior of lunasin during its transit through the gastrointestinal tract has not been previously evaluated. Thus, the present study aims to simulate physiological conditions using an *in vitro* digestion model for lunasin in the absence and/or presence of a major soybean Bowman-Birk isoinhibitor (IBB1). Liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) analysis was carried out to quantify the remaining intact lunasin and to identify the peptides released during the digestive process. The anti-proliferative effect against colon cancer cells (HT-29 and Caco-2) of digests obtained at the end of the digestive process was also evaluated.

2. Materials and methods

2.1. Reagents

Peptide lunasin (>95% of purity) was synthesized by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China). BBI from soybean (T9777), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), porcine pepsin (EC 3.4.23.1), pancreatic bovine trypsin (EC 232-650-8), pancreatic bovine α-chymotrypsin (EC 232-671-2, Type I-S), pancreatic porcine lipase (EC 232-619-9, Type VI-S), pancreatic porcine colipase (EC 259-490-1), phosphatidylcholine (PC), sodium taurocholate, sodium glycodeoxycholate, trifluoroacetic acid (TFA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Madrid, Spain). Other chemicals used were of HPLC grade.

2.2. Purification of IBB1 isoinhibitor from soybean

IBB1 was purified from commercially available soybean BBI following the protocol of Arques *et al.*[19] Briefly, the sample consisted of a mixture of protease inhibitors that was loaded onto a MonoS 5/50 GL cation exchange column (GE Healthcare, Uppsala, Sweden) connected to an AKTA FPLC system (GE Healthcare), using a linear gradient of 0–0.22 M NaCl in 25 mM sodium acetate buffer, pH 4.4, at a flow rate of 1 mL min^{-1}. The elution was monitored at 280 nm and 0.5 mL fractions were collected. Measurements of trypsin inhibitory activity of the eluted samples were carried out in flat-bottom microtitre plates by using BAPNA as the specific substrate, and assay products were measured at $OD_{405\ nm}$, as previously described.[20] The unbound sample, containing both trypsin and chymotrypsin inhibitory activity – the latter measured by using BTEE as the specific substrate[21] – was dialyzed extensively against distilled water and freeze-dried until use. The identification of IBB1 was carried out by peptide mass fingerprinting, as previously reported.[22]

To abolish the inhibitory activity of soybean IBB1, chemical inactivation *via* reduction of disulphide bonds and subsequent alkylation of the cysteinyl sulfhydryl groups was carried out.[22] Ten milligrams of soybean IBB1 were dissolved in 50 mM Tris-HCl (pH 8.2) and reduced with 100 μL 0.5 M dithiothreitol (DTT) for 2 min at 100 °C, and alkylated with 500 μL of 0.25 M iodoacetamide for 15 min at 50 °C under dark conditions. In order to remove residual DTT and iodoacetamide, samples were dialyzed extensively against distilled water and freeze-dried.

2.3. Measurement of protease inhibitory activities

IBB1 and its inactive form were assessed for trypsin and chymotrypsin inhibitory activities. Trypsin inhibitory activity was measured using a modified small-scale quantitative assay with BAPNA as the specific substrate using 50 mM-Tris pH 7.5 as enzyme assay buffer. A trypsin inhibitor unit is defined as that which gives a reduction in absorbance at 410 nm of 0.01, relative to trypsin control reactions, in 10 min in a defined assay volume of 10 mL.[23] Chymotrypsin inhibitory activity was measured by using BTEE as the specific substrate. A chymotrypsin inhibitor unit is defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to chymotrypsin control reactions, in 5 min in a defined assay volume of 10 mL, as described previously.[21] Specific trypsin and chymotrypsin inhibitory activities, expressed as inhibitor units per mg of protein, were calculated and used to assess the chemical inactivation of IBB1.

2.4. *In vitro* simulated gastrointestinal digestion

The *in vitro* digestibility of lunasin in the presence or absence of IBB1, either active or inactive, was evaluated by using an *in vitro* model system mimicking *in vivo* gastric and duodenal digestion according to Moreno *et al.*[24] with some modifi-

cations.[25] The digestions were assessed under the following conditions: lunasin in the absence of IBB1, lunasin in the presence of IBB1 [lunasin : IBB1 ratios of 1 : 1 and 1 : 2 (w : w)] and lunasin in the presence of chemically inactivated IBB1 [lunasin : IBB1 ratio of 1 : 2 (w : w)].

Lunasin and IBB1 were dissolved in simulated gastric fluid (SGF, 35 mM NaCl, pH 2.0) containing phospholipid vesicles at a total protein concentration of 1.5 mg mL^{-1}. The solution was adjusted to pH 2.0, preheated for 15 min at 37 °C, and subjected to gastric digestion for 60 min at 37 °C by adding 182 units of porcine pepsin per mg of protein. Gastric reaction was stopped by raising pH up to 7.0–7.5. For intestinal digestion, the pH of the gastric digest was adjusted to 6.5–6.8 by addition of 1 M $CaCl_2$, 0.25 M Bis–Tris, and a 0.125 M bile salt equimolar mixture of sodium glycodeoxycholate and sodium taurocholate. The pancreatic bovine trypsin and α-chymotrypsin, and pancreatic porcine lipase were added to the mixture at the enzyme : substrate ratios of 34.5, 0.4 and 24.8 units per mg of protein, respectively. Pancreatic porcine colipase was added at an enzyme : substrate ratio of 1 : 895 (w : w). Duodenal digestion was carried out for 60 min at 37 °C and stopped by raising the temperature to 95 °C for 10 min. Aliquots were taken before digestion at the end of both gastric and duodenal phases. The simulated gastrointestinal digestion was carried out in duplicate for each lunasin : IBB1 ratio.

2.5. Analysis of digests by RP-HPLC-MS/MS

Synthetic lunasin (at concentrations ranging from 0.125 to 1.50 mg mL^{-1}) and samples collected during simulated gastrointestinal digestion were subjected to HPLC-MS/MS. The samples were analyzed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik, Bremen, Germany) and equipped with an electrospray ionization (ESI) source. The analyses were carried out using a Mediterranea Sea_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain). The injection volume was 50 μL and the flow rate was set at 0.2 mL min^{-1}. Peptides were eluted with a linear gradient (0–70%) of solvent B [acetonitrile : TFA, 1000 : 0.27 (v : v)] in A [water : TFA, 1000 : 0.37 (v : v)] in 75 min. Spectra were recorded over the mass/charge (m/z) range 200–3000. Each sample was analyzed in duplicate. Data obtained were processed and transformed to spectra representing mass values using the Data Analysis program (version 4.0, Bruker Daltonik). BioTools (version 3.2, Bruker Daltonik) was used to process the MS/MS spectra and to perform peptide sequencing.

2.6. Cell viability assay

Two human colorectal adenocarcinoma cell lines (Caco-2 and HT-29) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HT-29 and Caco-2 cells were grown in McCoy's medium (Lonza Group Ltd, Basel, Switzerland) and Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical), respectively, supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest, Nuaillé, France), and 1% (v/v) penicillin/streptomycin/amphotericin B solution (Biowest). In the case of Caco-2 cells, DMEM was also supplemented with 1% (v/v) non-essential amino acids (Lonza Group Ltd). Cell cultures were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C.

The effect of digests on cell viability was evaluated by using the MTT assay. Caco-2 and HT-29 cells were seeded in 48-well plates (VWR International, Radnor, PA, USA) at a density of 7×10^4 cells per cm^2, and incubated for 24 h. The cells were treated with gastrointestinal digests (at total protein concentration of 0.3, 0.2 and 0.1 mg mL^{-1}) for 24 h, and washed with phosphate buffer saline (PBS, Lonza Group Ltd). MTT solution (0.5 mg mL^{-1} at the final concentration) was added to each well, and cells were incubated for 2 h at 37 °C. The supernatant was aspirated and insoluble formazan crystals formed were solubilized in dimethylsulfoxide : ethanol (1 : 1, v : v), measuring the absorbance at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Experiments were carried out in triplicate and the results were expressed as a percentage of viable cells compared to the control, and as IC_{50} values (protein concentration needed to inhibit 50% of viable cells).

2.7. Statistics

Data were evaluated using one-way ANOVA followed by the Bonferroni *post hoc* test, and expressed as the mean ± SD of the different experiments carried out. GraphPad Prism 5.0 software (San Diego, CA, USA) was used to perform statistical analyses. Differences with a P value <0.05 (*) were considered significant.

3. Results

3.1. Behavior of lunasin under gastric digestion

Commercial BBI consisted of a mixture of two major BBI isoinhibitors, IBB1 and IBBD2, differing in potency and specificity against trypsin and chymotrypsin. While IBB1 shows the ability to inhibit both trypsin and chymotrypsin, IBBD2 only inhibits trypsin.[22] By cation-exchange chromatography, both forms were isolated, IBB1 being unbound to the Mono-S column, whereas IBBD2 was eluted in the NaCl gradient. From the functional point of view, the obtained IBB1 demonstrated a high potency against both trypsin and chymotrypsin showing K_i values at the nanomolar level (30 ± 4 and 3 ± 1 nM, respectively). Its specific inhibitory activity against trypsin and chymotrypsin was 3828 ± 209 trypsin inhibitor unit per mg of protein and 2917 ± 292 chymotrypsin inhibitor unit per mg of protein, respectively. Following the reduction of disulphide bonds and further alkylation of the cysteinyl sulphydryl groups, its inhibitory activities decreased in more than 95%. In agreement with the inhibitory assays, the MS/MS analysis of inactive IBB1 revealed the presence of a major peak corresponding to reduced/alkylated IBB1, and a minor peak corresponding to the active isoinhibitor that represented 2% of total IBB1. This indicated that residual IBB1 remained active after the inactivation process carried out in this study.

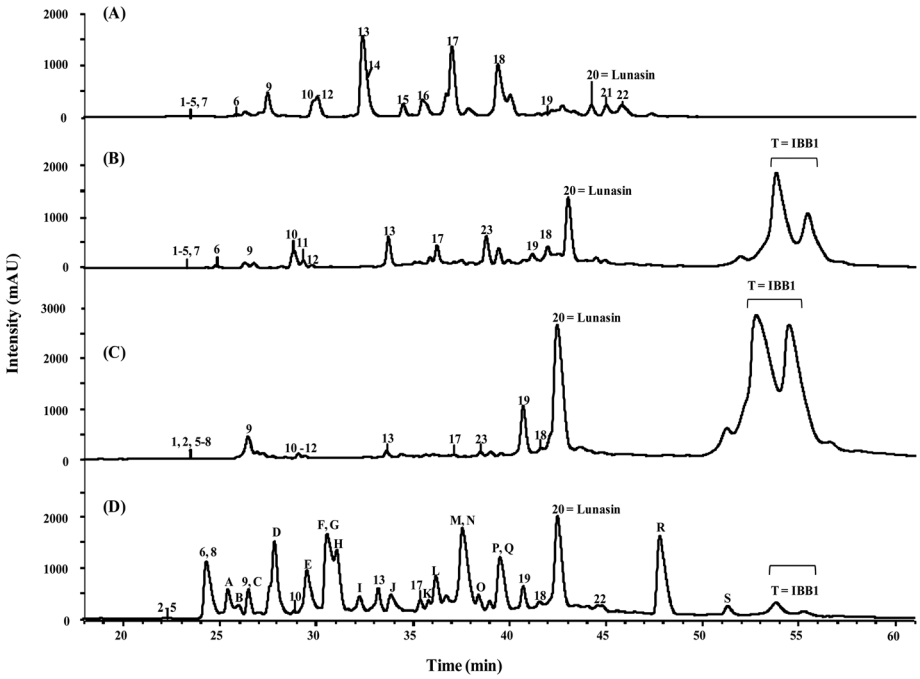


Fig. 1 UV-chromatograms obtained after the gastric phase of the digestive process simulating the gastrointestinal digestion of lunasin : IBB1 mixtures at ratios of (A) 1 : 0 (w : w), (B) 1 : 1 (w : w), (C) 1 : 2 (w : w), and (D) 1 : 2 (w : w) with inactive IBB1. Chromatographic peaks (numbers or letters) corresponding to the lunasin- and IBB1-derived peptides, respectively, were identified by mass spectrometry in tandem, and shown in the ESI, Table S1.†

In order to evaluate the resistance of lunasin to digestive enzymes, mixtures of this peptide without and with IBB1 in active or inactive states were prepared. The mixtures were subjected to a two-stage hydrolysis process simulating gastrointestinal digestion. Fig. 1A–D show the UV-chromatograms of the different digests obtained at the end of the gastric phase. Chromatographic peaks (numbers or letters) corresponded to the lunasin- and IBB1-derived peptides, respectively. Lunasin (peak 20) eluted after 43 minutes, with ions confirming the identity of this peptide (5028.3, experimental mass; 5028.0, theoretical mass). Two peaks were visible for IBB1 (peak T). One of them eluted after 53 minutes and corresponded to the intact polypeptide (7858.6, experimental mass; 7858.8, theoretical mass). The additional peak was detected at a retention time of 55 min, whose ions suggested the presence of IBB1 lacking the C-terminal tetrapeptide ^{68}DKEN^{71} (7380.9, theoretical mass).

The percentage of intact lunasin after pepsin treatment was calculated by using a lunasin's standard curve (0.125–1.5 mg mL^{-1}) analyzed under the same conditions (Table 1). In the absence of IBB1, only 2.6% of lunasin was resistant to the action of pepsin, indicating the high susceptibility of this peptide to the gastric enzyme. However, in the presence of IBB1 at lunasin : IBB1 ratios of 1 : 1 and 1 : 2, more than 34% of lunasin remained intact after pepsin digestion, without observing statistical differences between both ratios. No significant differences were observed when lunasin was mixed with inactive IBB1 in comparison with the samples containing active IBB1, with more than 28% of this peptide remaining intact after the gastric phase. The higher size of IBB1 in comparison with that of lunasin could make this isoinhibitor act as a "chaperone" encasing lunasin and preserving its integrity during pepsin hydrolysis. In addition, it cannot be excluded that the presence of a small percentage of active IBB1 during the inactivation process was capable to protect lunasin from the effects of pepsin (Fig. 1D).

Table 1 Intact lunasin (expressed as % of the initial intact lunasin) measured in the digests obtained after the gastric and the intestinal phases of the digestion process simulating gastrointestinal conditions from the lunasin : IBB1 mixtures at different ratios. Digestion was carried out in duplicate and two HPLC-MS/MS analyses were run for each digest[a]

	Intact lunasin (%)	
Lunasin : IBB1 ratio (w : w)	Gastric digest	Gastric + intestinal digest
1 : 0	2.6 ± 0.4^{a}	0.1 ± 0.1^{a}
1 : 1	34.3 ± 3.7^{b}	1.8 ± 0.4^{b}
1 : 2	35.1 ± 2.8^{b}	5.3 ± 0.4^{c}
1 : 2 (inactive IBB1)	28.4 ± 3.6^{b}	1.5 ± 0.2^{b}

[a] Different superscript letters within the same column denote statistically significant differences ($p < 0.05$).

The peptidic profiles of gastric digests in the presence of active IBB1 (Fig. 1B and C) were similar, but slightly different from that observed for lunasin's digest in the absence of the

isoinhibitor (Fig. 1A). The HPLC-MS/MS analysis allowed identifying the peptides released from lunasin and inactive IBB1 during the simulated gastrointestinal digestive process (Fig. 2A–D, Fig. 3A, and ESI Table S1†). It is remarkable that no peptides derived from active IBB1 were identified in this study. In the gastric digests, up to 22 peptides derived from lunasin and 19 derived from inactive IBB1 were identified, although the presence or absence of each one of these peptides depended on the mixture digested (Fig. 2 and 3). As expected, the higher number of fragments released from lunasin was detected in the digest of sample in the absence of IBB1, with 20 of the 22 total identified peptides. Five peptides, whose sequences were ^{11}RKQLQGVNLTPCEKHIME^{28}, ^{17}VNLTPCEKHIME^{28}, ^{19}LTPCE^{28}, ^{20}TPCEKHIME^{28}, and ^{21}PCEKHIME^{28}, were identified in the digest without IBB1 but not in those digests containing the isoinhibitor in its active state. These fragments were released from the central region of lunasin sited between amino acids 11 and 28, indicating that this area could be predominantly enclosed and protected from the action of pepsin when the protease isoinhibitor is present (Fig. 2A–D).

In the gastric digest of mixtures lunasin : inactive IBB1 (ratio 1 : 2), 19 peptides released from inactive IBB1 were identified (Fig. 3A and ESI Table S1†), confirming the vulnerability of inactive IBB1 to the action of this gastric enzyme, and the consequent release of an important number of IBB1-derived fragments.

3.2. Effects of pancreatic enzymes on lunasin

Once the gastric phase was complete, the mixtures were digested with a combination of pancreatic enzymes, and the resulting digests were analyzed by HPLC-MS/MS (Fig. 4) to quantify the intact lunasin (Table 1). In the absence of IBB1, the residual lunasin was almost completely degraded after the action of pancreatic proteases (Table 1 and Fig. 4A). However, active IBB1 exerted a protective effect on lunasin against pancreatic hydrolysis that could be explained by its ability to inhibit trypsin and chymotrypsin. The enzymatic inhibitory properties of IBB1 together with its molecular structure supported by a network of seven disulphide bridges are responsible for its resistance to duodenal digestion, thus the active isoinhibitor was clearly visible at the end of simulated digestion at lunasin : IBB1 ratios 1 : 1 and 1 : 2 (Fig. 4B and C, respectively). It is remarkable that in contrast to the protection against the action of pepsin, inhibitory effects on duodenal digestion were dose-dependent, the resistance of lunasin being more evident when IBB1 was present at the higher concentration (lunasin : IBB1 ratio 1 : 2). In this case, the residual lunasin at the end of simulated gastrointestinal digestion was 5.3% while the residual value for mixtures lunasin : IBB1 at the ratio 1 : 1 was 1.8%. The latter value was similar to that determined when mixtures lunasin : inactive IBB1 were subjected to intestinal digestion (1.5% of residual lunasin, Table 1). As indicated previously, inactive IBB1 did not show trypsin and chymotrypsin inhibitory activities. However, lunasin was slightly protected from the action of these pancreatic enzymes when inactive IBB1 was present in the mixture. The small percentage of IBB1 remaining active during the inactivation process could be responsible for this slight protection, although it cannot be excluded that the presence of the isoinhibitor (in both the active and inactive state) could be enough to encase lunasin and protect it from the proteolytic action of trypsin and chymotrypsin. In addition, some of the IBB1-derived peptides might contribute to the protective action against lunasin's digestion.

Of 20 lunasin-derived peptides identified in the gastric digest of IBB1 free-sample, only three, corresponding to ^{24}KHIME^{28}, ^{28}EKIQGR^{33}, and ^{29}KIQGRGDDDDDDDDD^{43}, appeared at the end of the whole digestive process, suggesting their resistance to the action of trypsin and chymotrypsin (Fig. 2E). The rest of peptides were not detected in the duodenal digest, indicating that without the protective role of IBB1, pancreatic enzymes acted on those sequences released during the gastric phase resulting in the liberation of shorter peptides and/or free amino acids. Up to 15 new peptides released from lunasin were identified in the hydrolyzate obtained at the end of simulated gastrointestinal digestion when IBB1 was not present (Fig. 2E and ESI Table S1†). The release of eight of these peptides, corresponding to fragments ^{4}QHQQDSCR^{11}, ^{15}QGVNLTPCEK^{24}, ^{16}GVNLTPCEK^{24}, ^{17}VNLTPCEK^{24}, ^{19}LTPCEK^{24}, ^{25}HIME^{28}, ^{29}KIQGR^{33}, and ^{34}GDDDDDDDDD^{43}, could be explained by the action of trypsin on susceptible peptide bonds. The rest of them could be released by the combined action of trypsin and chymotrypsin used during the simulated intestinal phase. None of the peptides identified in the IBB1-free digest was identified in digests containing active IBB1, suggesting the ability of this isoinhibitor to protect both lunasin and derived peptides against the action of trypsin and chymotrypsin. It was also interesting to note the absence of those new peptides when the digestion was carried out on a mixture containing inactive IBB1. These results confirm that the presence of IBB1 in both active and inactive forms was enough to protect lunasin and derived fragments against digestion by duodenal enzymes.

Only four of the nineteen peptides released from inactive IBB1 by the action of pepsin were degraded by trypsin and chymotrypsin, which were not detected at the end of simulated gastrointestinal digestion. The sequences of these peptides were ^{24}CSDMRLNSCHSA^{35}, ^{43}LSYPAQC^{49}, ^{58}CYEPCKPSEDDKEN^{71}, and ^{61}PCKPSEDDKEN^{71}. The remaining peptides were identified at the end of digestion, indicating their resistance to the action of pancreatic enzymes (Fig. 3). Among them, the presence of the trypsin inhibitory domain of IBB1 localized between amino acids ^{14}C and ^{22}C was remarkable, which could protect lunasin and its derived fragments from further digestion by trypsin.[22]

3.3. Anti-proliferative action of gastrointestinal digests against colon cancer cells

The digests at the end of the simulated digestive process were assayed for their effect against the cell viability of two colon adenocarcinoma cell lines, HT-29 and Caco2, using the MTT protocol. In order to evaluate the dose-response, the digests

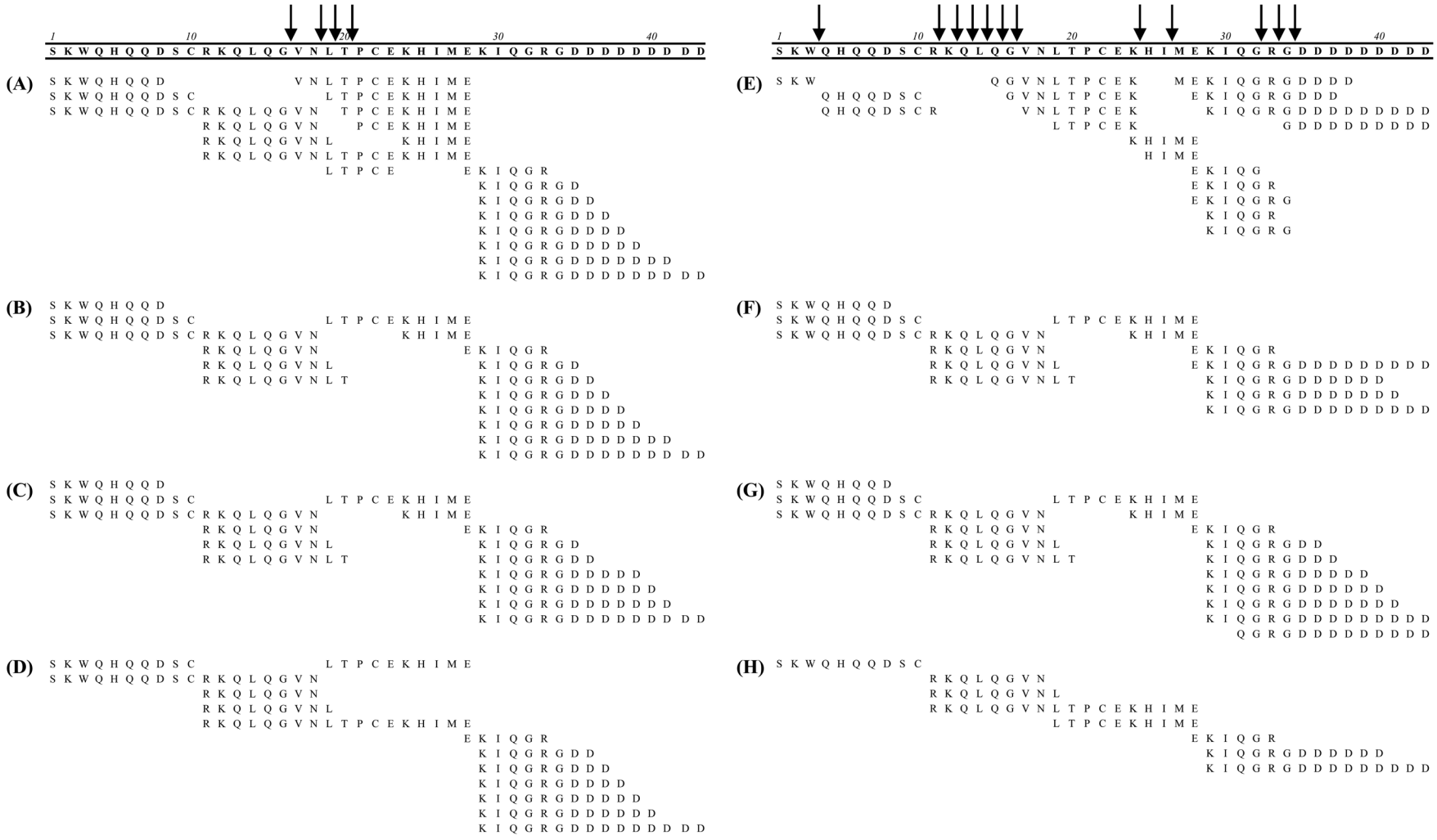


Fig. 2 Lunasin-derived peptides identified in the digests obtained after the (A–D) gastric and (E–H) intestinal phase of the simulated gastrointestinal digestion from lunasin : IBB1 mixtures at ratios of (A and E) 1 : 0 (w : w), (B and F) 1 : 1 (w : w), (C and G) 1 : 2 (w : w), and (D and H) 1 : 2 (w : w) with inactive IBB1. Susceptible peptide bonds in the absence of IBB1 but resistant in the presence of this isoinhibitor are indicated with an arrow.

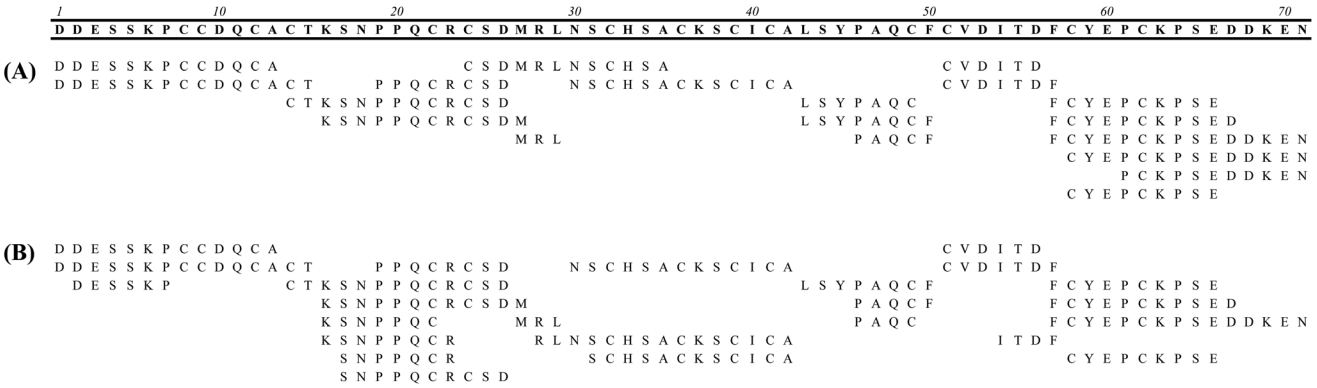


Fig. 3 IBB1-derived peptides identified in the digests obtained after the (A) gastric and (B) intestinal phase of the simulated gastrointestinal digestion from the lunasin : IBB1 inactive mixture (1 : 2, w : w).

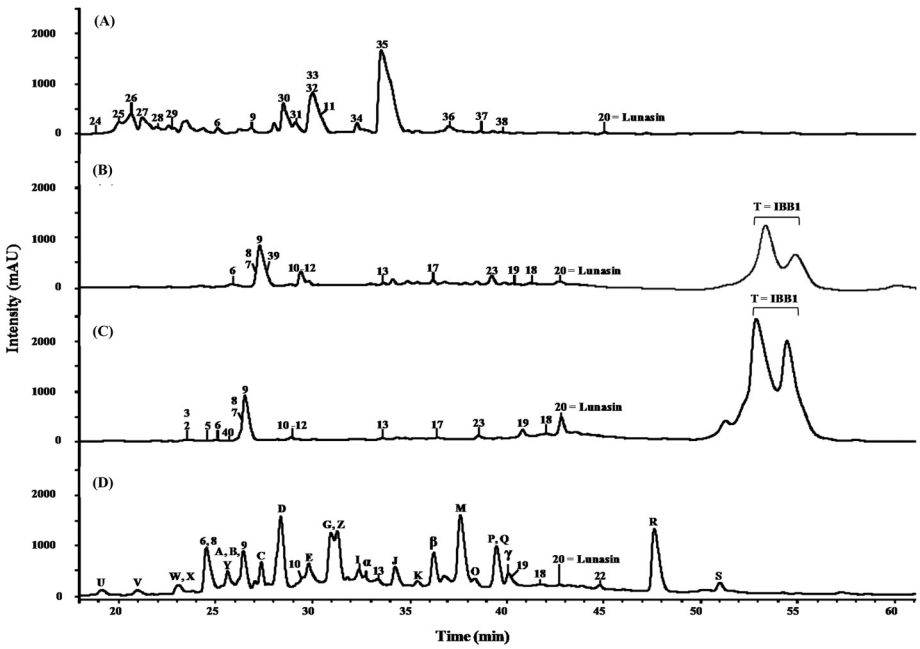


Fig. 4 UV-chromatograms obtained at the end of simulated gastrointestinal digestion of lunasin : IBB1 mixtures at ratios of (A) 1 : 0 (w : w), (B) 1 : 1 (w : w), (C) 1 : 2 (w : w), and (D) 1 : 2 (w : w) with inactive IBB1. Chromatographic peaks (numbers or letters) corresponding to the lunasin- and IBB1-derived peptides, respectively, were identified by mass spectrometry in tandem, and shown in the ESI, Table S1.†

were analyzed at the initial protein concentration (0.3 mg mL^{-1}) and after dilution in growth media 1.5 and 3-times. Digestion media in the absence of lunasin and IBB1 were assayed to evaluate whether any substances used to simulate digestion were capable of decreasing the cell viability (Fig. 5). An anti-proliferative effect was observed when the non-diluted digest was added to HT-29 and Caco2 cells, with values of viable cells of 70.5 ± 8.0% and 86.6 ± 4.0%, respectively. The anti-proliferative effect was significantly higher when digests from lunasin : IBB1 mixtures were assayed. As shown in Fig. 5A, all the tested gastro-duodenal digests affected the HT-29 cell viability in a dose-dependent manner. The digests obtained in the absence of IBB1 provoked a decrease of viable cells of 52.4% at the highest protein concentration assayed. The IC_{50} value calculated for this digest was 0.29 mg mL^{-1}. Since the amount of lunasin in this hydrolyzate was practically undetectable (0.1%), the anti-proliferative activity seems to be mainly due to the peptides released during lunasin digestion. However, these peptides did not exert any significant protective effect against Caco2 cells (Fig. 5B), indicating that their activity might be dependent on the cell line studied. Higher anti-proliferative effects in both the colon cancer cell lines were observed when hydrolyzates obtained from the mixtures containing lunasin and IBB1 were assayed, indicating that both intact polypeptides lunasin and IBB1 and the peptides released from them could cooperate to decrease the viability of

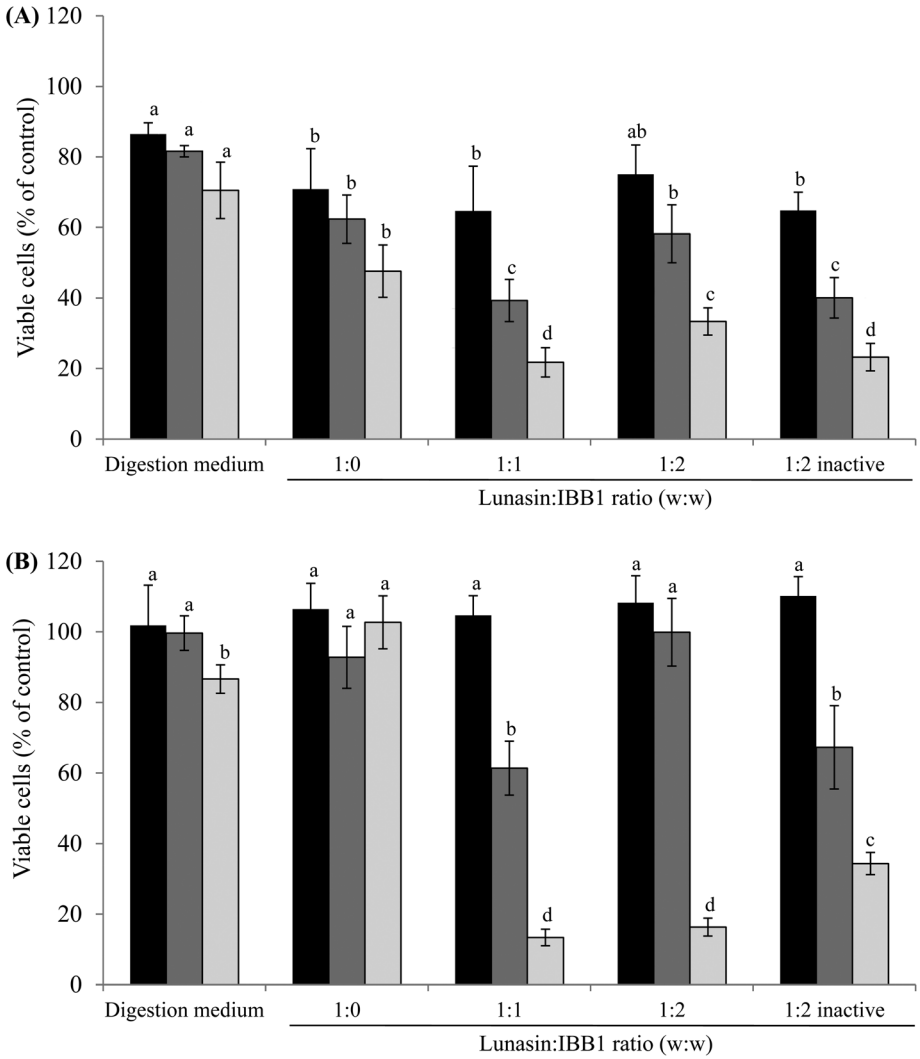


Fig. 5 Effect on proliferation of human colon adenocarcinoma (A) HT-29 and (B) Caco-2 cells shown by the digests obtained at the end of simulated gastrointestinal digestion from digestion medium without lunasin and IBB1, and lunasin : IBB1 mixtures at different ratios and protein concentrations, (■) 0.1 mg mL^{-1}, (■) 0.2 mg mL^{-1}, and (□) 0.3 mg mL^{-1}. Cell viability was evaluated by the MTT assay. Experiments were carried out in triplicate, and the results were expressed as a percentage of viable cells compared to control non-treated cells, considered as 100%. Different letters denote statistically significant differences ($p < 0.05$) between digests at the same protein concentration and the corresponding dilution of digestion medium without peptides.

colon cancer cells. The calculated IC_{50} values for these hydrolyzates were lower (0.16–0.23 mg mL^{-1}) when HT-29 cells were treated with digests, suggesting the higher vulnerability of this cell line to the action of lunasin, IBB1 and their derived-peptides.

4. Discussion

Our findings indicate that, in the absence of IBB1, during the simulated gastric phase, pepsin acts on lunasin hydrolyzing more than 97% of the initial peptide and releasing a great number of peptides. Previous studies have reported the ability of pepsin to degrade lunasin, although the levels of the residual peptide measured after incubation with this gastric enzyme were different in each study. Two immunological studies revealed the complete disappearance of both synthetic and soybean-isolated lunasin after 2-min incubation with the SGF containing pepsin in the absence of BBI.[16,17] However, in BBI-free soymilk, a significant resistance (up to 60%) of lunasin to pepsin treatment was reported.[18] The discrepancies among the studies could be due to multiple factors, such as the occurrence of other protease inhibitors, such as Kunitz-trypsin and the isoinhibitor IBBD2, differences in the lunasin : BBI ratio present in products subjected to simulated pepsin digestion as well as the methodology used to evaluate the percentage of residual lunasin after pepsin proteolysis. To quantify lunasin, the MS analysis used shows some advantages in

comparison with western-blotting and ELISA methods. On one hand, this analysis allowed quantification of lunasin that remained intact after gastric and gastro-duodenal digestion.[26,27] On the other hand, the application of MS to assess digests allowed, for the first time, to identify the peptides released from lunasin by the action of digestive enzymes.

The qualitative and quantitative data obtained confirm the protective role of IBB1 against lunasin hydrolysis by both gastric and pancreatic enzymes. In the presence of active IBB1 at both lunasin : IBB1 ratios, lunasin is partially resistant to the action of pepsin, with more than 34% of the original peptide being observed in the gastric digest. Lunasin protection is not likely associated with pepsin inhibition by IBB1 given its null or extremely low inhibitory activity against this gastric enzyme.[28,29] The absence of peptides released from IBB1 during the gastric phase indicated the extraordinary resistance of this isoinhibitor to pepsin. It is well known that this resistance to digestive proteases and thermal treatment is due to its correct folding and functional structure maintained by the disulphide bridge network.[20,30,31] Conformational changes resulting from the reduction of disulphide bridges and alkylation of the cysteinyl sulfhydryl groups almost completely abolish both trypsin and chymotrypsin inhibitory activities of BBI, decreasing its resistance to enzymes and to high temperatures.[32] Our study confirms that after inactivation of IBB1 by reduction/alkylation, no inactive IBB1 was visible after gastric digestion, although the minimum quantity of IBB1 remaining active during the inactivation process was enough to partly protect lunasin from pepsin action. IBB1-derived peptides released by the action of pepsin might also contribute to protect lunasin against this gastric enzyme.

In the case of the simulated duodenal phase, the trypsin and chymotrypsin inhibitory activities of IBB1 were responsible for the protective role on lunasin from digestion by these enzymes. The absence of IBB1 in digested mixtures provoked the almost complete disappearance of lunasin, as previously reported by Hernández-Ledesma *et al.*[18] when BBI-free soymilk samples were digested with pancreatin over 60 min. However, the presence of BBI has been suggested to provide protection from lunasin's digestion.[33] These authors, by using immunological assays, reported that 3% of lunasin present in lunasin-enriched soybean was detected at the end of a simulated pepsin–pancreatin digestion. In our study, when lunasin : IBB1 mixtures at 1 : 2 ratio were digested, more than 5% of lunasin remained intact. The consumption of 25 g of soybean protein per day of a diet low in saturated fat and cholesterol, recommended by the US Food and Drug Administration to reduce the risk of heart disease, leads to a total intake of 0.94 g of lunasin.[33] Of this oral intake, 5% will survive gastrointestinal digestion resulting in 47 mg of biologically active lunasin, with the ability to act at both local and systemic levels.

Intact soybean lunasin and IBB1 have demonstrated to exert anti-proliferative effects against colon cancer using different cell culture models. Dia and de Mejia[34,35] reported the cytotoxic activity of soybean lunasin in colon cancer HT-29, KM12L4, RKO, and HCT-116 cells. Treatment of HT-29 cells with IBB1 has been described to reduce the cell proliferation in a concentration and time-dependent manner, with an IC_{50} value of 39.9 μM.[22] However, to our knowledge, no information on the potential anti-proliferative effects of digests obtained from these two bioactive peptides during their transit through the gastrointestinal tract was available. In our study, a notable colon cancer HT-29 and Caco2 cell proliferation inhibitory effect was provoked after incubation of cells for 24 hours with hydrolyzates obtained at the end of simulated gastrointestinal digestion. The remaining lunasin and IBB1 as well as shorter peptides released after the action of pepsin and duodenal enzymes could be responsible for the observed effects. To date, only one fragment corresponding to lunasin f(23–43), whose sequence is ^{23}EKHIMEKIQGRGDDDDDDDDD^{43}, has demonstrated higher anti-proliferative activity in human breast cancer MDA-MB-231 cells than complete lunasin.[36]

A petition has been filed with the Food and Drug Administration for a health claim that soy protein consumption as part of a low fat diet may reduce the risk of certain cancers, including colon cancer.[37] Among the polypeptides contained in soy protein, lunasin and naturally occurring protease inhibitors, such as BBI and Kunitz-trypsin inhibitors, have become the most extensively studied for their colon cancer preventive properties.[38] Recently, a protein-enriched soybean meal hydrolyzate showing high resistance to simulated gastrointestinal digestion was fractionated and evaluated for its effects on cell proliferation of colon cancer HCT-116 and Caco2 cells.[39] The highest potency was demonstrated for both the low molecular weight peptide fraction (<5 kDa) and the fraction containing polypeptides (10–50 kDa), although the sequences of the responsible peptides were not elucidated. In our study, at the end of the simulated gastrointestinal digestive process, 29 lunasin- and 24 IBB1-derived fragments have been identified, although the presence of each one depended on the mixture digested. Since the final digests have been found to exhibit potent anti-proliferative properties against colon cancer HT-29 and Caco2 cells, the peptides liberated from lunasin and IBB1 might be the main factor responsible for the observed effects. So far, this is the first study reporting that peptides directly released from lunasin and IBB1, during their transit through the gastrointestinal tract, possess anticancer activities. Further studies are currently ongoing to synthesize these peptides and confirm their potential anti-proliferative effects.

Acknowledgements

This work has received financial support from project AGL2011-24643. A.C. acknowledges support by an ERDF co-financed grant from the Spanish CICYT (AGL2011-26353). The authors are participants in the FA1005COST Action INFOGEST on food digestion. S. F.-T. and B. H.-L. acknowledge the Ministry of Economy and Competitiveness (MINECO) for their FPI fellowship and "Ramón y Cajal" post-doctoral contract,

respectively. E. C.-H. thanks the Universidad Veracruzana for awarding the PROMEP/103.5/13/6408 scholarship for supporting PhD studies abroad. The authors thank T. Herrero for the technical assistance.

References

1 A. F. Galvez, M. J. R. Revilleza and B. O. de Lumen, *Plant Physiol.*, 1997, **114**, 1567–1569.
2 P. Singh and K. Bisetty, *S. Afr. J. Chem.*, 2012, **65**, 115–124.
3 S. Fernández-Tomé and B. Hernández-Ledesma, in *Recent Progress in Medicinal Plants*, Studium Press LLC, India, 2014, in press.
4 A. F. Galvez, N. Chen, J. Macasieb and B. O. de Lumen, *Cancer Res.*, 2001, **61**, 7473–7478.
5 C.-C. Hsieh, B. Hernández-Ledesma and B. O. de Lumen, *J. Food Sci.*, 2010, **75**, H311–H316.
6 C.-C. Hsieh, B. Hernández-Ledesma, H. J. Jeong, J. H. Park and B. O. de Lumen, *PLoS One*, 2010, **5**, e8890.
7 V. P. Dia and E. G. de Mejia, *Cancer Lett.*, 2011, **313**, 167–180.
8 V. P. Dia and E. G. de Mejia, *J. Cancer Ther.*, 2013, **4**, 34–43.
9 G. Picariello, G. Mamone, C. Nitride, F. Addeo and P. Ferranti, *Trends Anal. Chem.*, 2013, **52**, 120–134.
10 L. Sánchez-Rivera, D. Martínez-Maqueda, E. Cruz-Huerta, B. Miralles and I. Recio, *Food Res. Int.*, 2014, **63**, 170–181.
11 M. Amigo-Benavent, A. Clemente, S. Caira and P. Stiuso, *Electrophoresis*, 2014, **35**, 1582–1589.
12 J. Ao and B. Li, *Food Res. Int.*, 2013, **52**, 334–341.
13 M. M. Contreras, D. Sánchez, M. A. Sevilla, I. Recio and L. Amigo, *Int. Dairy J.*, 2013, **32**, 71–78.
14 A. B. Nongonierma and R. J. Fitzgerald, *Int. Dairy J.*, 2013, **32**, 33–39.
15 L. Sánchez-Rivera, I. Diezhandino, J. A. Gómez-Ruiz, J. M. Fresno, B. Miralles and I. Recio, *Electrophoresis*, 2014, **35**, 1627–1636.
16 J. H. Park, H. J. Jeong and B. O. de Lumen, *J. Agric. Food Chem.*, 2007, **55**, 10703–10706.
17 J. B. Jeong, J. J. Jeong, J. H. Park, S. H. Lee, J. R. Lee, H. K. Lee, G. Y. Chung, J. D. Choi and B. O. de Lumen, *J. Agric. Food Chem.*, 2007, **55**, 10707–10713.
18 B. Hernández-Ledesma, C.-C. Hsieh and B. O. de Lumen, *Food Chem.*, 2009, **115**, 574–580.
19 M. C. Arques, M. C. Marín-Manzano, L. Clarissa-Brito, B. Hernandez-Ledesma, I. Recio and A. Clemente, *Food Chem.*, 2014, **155**, 24–30.
20 A. Clemente, E. Jiménez, M. C. Marín-Manzano and L. A. Rubio, *J. Sci. Food Agric.*, 2008, **88**, 523–531.
21 A. Clemente, D. A. MacKenzie, D. J. Jeenes and C. Domoney, *Protein Expression Purif.*, 2004, **36**, 106–114.
22 A. Clemente, J. Moreno, M. C. Marín-Manzano, E. Jiménez and C. Domoney, *Mol. Nutr. Food Res.*, 2010, **54**, 396–405.
23 C. Domoney and T. Welham, *Seed Sci. Res.*, 1992, **2**, 147–154.
24 F. J. Moreno, F. A. Mellón, M. S. J. Wickham, A. R. Bottrill and E. N. C. Mills, *FEBS J.*, 2005, **272**, 341–352.
25 R. Jiménez-Saiz, I. López-Exposito, E. Molina and R. López-Fandiño, *Food Hydrocolloids*, 2013, **30**, 597–605.
26 M. Guijarro-Díez, M. C. García, A. L. Crego and M. L. Marina, *J. Chromatogr. A*, 2014, **1371**, 117–124.
27 I. Nakurte, K. Klavins, I. Kirhnere, J. Namniece, L. Adlere, J. Matvejevs, A. Kronberga, A. Kokare, V. Strazdina, L. Legzdina and R. Muceniece, *J. Cereal Sci.*, 2012, **56**, 510–514.
28 N. Hajela, A. H. Pande, S. Sharma, D. N. Rao and K. Hajela, *J. Plant Biochem. Biotechnol.*, 1999, **8**, 57–60.
29 S. Maggo, S. P. Malhotra, K. Dhawan and R. Singh, *J. Plant Biochem. Biotechnol.*, 1999, **8**, 61–65.
30 M. C. Marín-Manzano, R. Ruiz, E. Jiménez, L. A. Rubio and A. Clemente, *Br. J. Nutr.*, 2009, **101**, 967–971.
31 M. V. Trivedi, J. S. Laurence and T. J. Siahaan, *Curr. Protein Pept. Sci.*, 2009, **10**, 614–625.
32 A. Clemente and M. C. Arques, *World J. Gastroenterol.*, 2014, **20**, 10305–10315.
33 E. G. de Mejia, W. Wang and V. P. Dia, *Mol. Nutr. Food Res.*, 2010, **54**, 406–414.
34 V. P. Dia and E. G. de Mejia, *Cancer Lett.*, 2010, **295**, 44–53.
35 V. P. Dia and E. G. de Mejia, *Cancer Lett.*, 2011, **313**, 167–180.
36 B. Hernández-Ledesma, C.-C. Hsieh and B. O. de Lumen, *Mol. Nutr. Food Res.*, 2010, **55**, 989–998.
37 B. P. Singh, S. Vij and S. Hati, *Peptides*, 2014, **54**, 171–179.
38 B. Hernández-Ledesma, C.-C. Hsieh, V. Dia, E. González de Mejia and B. O. de Lumen, in *Soybean and Health*, Intech, Croatia, 2011, pp. 145–166.
39 S. J. Rayaprolu, N. S. Hettiarachchy, P. Chen, A. Kannan and A. Mauromostakos, *Food Res. Int.*, 2013, **50**, 282–288.

Supplementary Table S1. Peptides identified in the digests obtained from the lunasin:IBB1 mixtures at different ratios after the gastric and intestinal phases of digestive process simulating gastrointestinal conditions. The peak (number or letter) corresponds to the peptide peaks and sequences shown in Figures 1, 2, and 3 (number for lunasin-derived peptides and letter for IBB1-derived peptides).

Peak	Protein fragment	Amino acid sequences	Ion *m/z* (charge)	Simulated gastric digests				Simulated gastrointestinal digests			
				1:0	1:1	1:2	1:2i	1:0	1:1	1:2	1:2i
1	f(29-35)	KIQGRGD	773.5(+1)	+	+	+	-	-	-	-	-
2	f(29-36)	KIQGRGDD	888.5(+1)	+	+	+	+	-	-	+	-
3	f(29-37)	KIQGRGDDD	1003.6(+1)	+	+	-	+	-	-	+	-
4	f(29-38)	KIQGRGDDDD	1118.6(+1)	+	+	-	+	-	-	-	-
5	f(29-39)	KIQGRGDDDDD	1233.7(+1)	+	+	+	+	-	-	+	-
6	f(28-33)	EKIQGR	729.7(+1)	+	+	+	+	+	+	+	+
7	f(29-41)	KIQGRGDDDDDDD	1463.7(+1)	+	+	+	-	-	+	+	-
8	f(29-40)	KIQGRGDDDDDD	1348.6(+1)	-	-	+	+	-	+	+	+
9	f(29-43)	KIQGRGDDDDDDDDD	1694.0(+1)	+	+	+	+	+	+	+	+
10	f(11-18)	RKQLQGVN	942.7(+1)	+	+	+	+	-	+	+	+
11	f(24-28)	KHIME	657.5(+1)	+	+	+	-	+	+	+	-
12	f(1-8)	SKWQHQQD	1056.6(+1)	+	+	+	-	-	+	+	-
13	f(1-10)	SKWQHQQDSC	1246.7(+1)	+	+	+	+	-	+	+	+
14	f(19-23)	LTPCE	562.3(+1)	+	-	-	-	-	-	-	-
15	f(21-28)	PCEKHIME	986.6(+1)	+	-	-	-	-	-	-	-
16	f(20-28)	TPCEKHIME	1087.6(+1)	+	-	-	-	-	-	-	-
17	f(1-18)	SKWQHQQDSCRKQLQGVN	1085.8(+2)	+	+	+	+	-	+	+	-
18	f(19-28)	LTPCEKHIME	1200.8(+1)	+	+	+	+	-	+	+	+
19	f(11-19)	RKQLQGVNL	1055.8(+1)	+	+	+	+	-	+	+	+
20	LUNASIN: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGD DDDDDDDD		1677.2(+3) 1258.0(+4)	+	+	+	+	+	+	+	+
21	f(17-28)	VNLTPCEKHIME	1413.9(+1)	+	-	-	-	-	-	-	-
22	f(11-28)	RKQLQGVNLTPCEKHIME	1062.8(+2)	+	-	-	+	-	-	-	+
23	f(11-20)	RKQLQGVNLT	1156.7(+1)	-	+	+	-	-	+	+	-
24	f(28-37)	EKIQGRGDDD	1133.4(+1)	-	-	-	-	+	-	-	-
25	f(4-10)	QHQQDSC	845.5(+1)	-	-	-	-	+	-	-	-
26	f(34-43)	GDDDDDDDDD	1111.4(+1)	-	-	-	-	+	-	-	-
27	f(29-33)	KIQGR	601.5(+1)	-	-	-	-	+	-	-	-
28	f(4-11)	QHQQDSCR	1001.6(+1)	-	-	-	-	+	-	-	-
29	f(28-32)	EKIQG	573.4(+1)	-	-	-	-	+	-	-	-
30	f(28-34)	EKIQGRG	786.6(+1)	-	-	-	-	+	-	-	-
31	f(19-24)	LTPCEK	690.5(+1)	-	-	-	-	+	-	-	-
32	f(25-28)	HIME	529.4(+1)	-	-	-	-	+	-	-	-
33	f(29-34)	KIQGRG	658.5(+1)	-	-	-	-	+	-	-	-
34	f(27-38)	MEKIQGRGDDDD	1378.7(+1)	-	-	-	-	+	-	-	-
35	f(1-3)	SKW	420.4(+1)	-	-	-	-	+	-	-	-
36	f(17-24)	VNLTPCEK	903.7(+1)	-	-	-	-	+	-	-	-
37	f(16-24)	GVNLTPCEK	960.6(+1)	-	-	-	-	+	-	-	-
38	f(15-24)	QGVNLTPCEK	1088.7(+1)	-	-	-	-	+	-	-	-
39	f(28-43)	EKIQGRGDDDDDDDDD	911.9(+2)	-	-	-	-	-	+	-	-
40	f(31-43)	QGRGDDDDDDDDD	1451.5(+1)	-	-	-	-	-	-	+	-
A	f(19-26)	PPQCRCSD	1019.5(+1)	-	-	-	+	-	-	-	+
B	f(14-26)	CTKSNPPQCRCSD	1609.8(+1)	-	-	-	+	-	-	-	+
C	f(1-13)	DDESSKPCCDQCA	1571.7(+1)	-	-	-	+	-	-	-	+
D	f(1-15)	DDESSKPCCDQCACT	1832.8(+1)	-	-	-	+	-	-	-	+
E	f(16-27)	KSNPPQCRCSDM	1479.7(+1)	-	-	-	+	-	-	-	+
F	f(24-35)	CSDMRLNSCHSA	719.3(+2)	-	-	-	+	-	-	-	-

G	f(30-42)	NSCHSACKSCICA	1554.7(+1)	**-**	-	-	+	-	-	-	+
H	f(58-71)	CYEPCKPSEDDKEN	885.9(+2)	**-**	-	-	+	-	-	-	-
I	f(58-66)	CYEPCKPSE	1169.6(+1)	**-**	-	-	+	-	-	-	+
J	f(51-56)	CVDITD	722.4(+1)	**-**	-	-	+	-	-	-	+
K	f(46-50)	PAQCF	622.4(+1)	**-**	-	-	+	-	-	-	+
L	f(43-49)	LSYPAQC	838.4(+1)	**-**	-	-	+	-	-	-	-
M	f(57-71)	FCYEPCKPSEDDKEN	959.5(+2)	**-**	-	-	+	-	-	-	+
N	f(61-71)	PCKPSEDDKEN	1318.7(+1)	**-**	-	-	+	-	-	-	-
O	f(27-29)	MRL	419.3(+1)	**-**	-	-	+	-	-	-	+
P	f(57-67)	FCYEPCKPSED	1431.7(+1)	**-**	-	-	+	-	-	-	+
Q	f(57-66)	FCYEPCKPSE	1316.7(+1)	**-**	-	-	+	-	-	-	+
R	f(43-50)	LSYPAQCF	985.5(+1)	**-**	-	-	+	-	-	-	+
S	f(51-57)	CVDITDF	869.4(+1)	**-**	-	-	+	-	-	-	+
T	IBB1: DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSAC KSCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN		1572.8(+5) 1310.7(+6)	-	+	+	+	-	+	+	-
U	f(2-7)	DESSKP	661.3(+1)	**-**	-	-	-	-	-	-	+
V	f(16-22)	KSNPPQC	830.5(+1)	**-**	-	-	-	-	-	-	+
W	f(17-23)	SNPPQCR	858.4(+1)	**-**	-	-	-	-	-	-	+
X	f(16-23)	KSNPPQCR	986.6(+1)	**-**	-	-	-	-	-	-	+
Y	f(17-26)	SNPPQCRCSD	1220.6(+1)	**-**	-	-	-	-	-	-	+
Z	f(31-42)	SCHSACKSCICA	1440.7(+1)	**-**	-	-	-	-	-	-	+
α	f(28-42)	RLNSCHSACKSCICA	912.5(+2)	**-**	-	-	-	-	-	-	+
β	f(46-49)	PAQC	475.2(+1)	**-**	-	-	-	-	-	-	+
γ	f(54-57)	ITDF	495.2(+1)	**-**	-	-	-	-	-	-	+

Food & Function

ARTICLE

Transepithelial transport of lunasin and derived peptides with inhibitory effects on the viability of gastrointestinal cancer cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

S. Fernández-Tomé,[a] J. Sanchón,[a] I. Recio[a] and B. Hernández-Ledesma[a,†]

Lunasin is a soybean peptide with demonstrated chemopreventive properties. Upon its oral intake, studies dealing with the effect of the digestive process on lunasin's properties are crucial. The present study describes, for the first time, the behavior of lunasin and fragments derived from its digestion in the Caco-2 cell monolayer. The sequences SKWQHQQDSC and KIQGRGDDDDDDDDD showed a notable resistance against the epithelial brush-border peptidases, although some fragments were generated as cellular hydrolysis products. Lunasin and RKQLQGVN were absorbed intact across the intestinal epithelium. The tight junction disruptor cytochalasin D, increased their transport, suggesting that the paracellular passive diffusion was the main mechanism involved. The study on the cancer cells viability showed that lunasin and SKWQHQQDSC exerted the highest effects on colorectal cancer HT-29 cells. The stability of lunasin during the activity assay suggested that the cell line type might be determinant for the different effect of lunasin-derived sequences.

1. Introduction

The gastrointestinal epithelium acts not only as a physical barrier against the external environment, but also is the place where food digestion and absorption of nutrients occur. The gastrointestinal tract is known to play a key role on the physiological relevance of orally administered bioactive components.[1] Firstly, the resistance of bioactive peptides to pepsin and pancreatic enzymes in gastric and intestinal fluids, respectively, must be one of the primary aspects to be addressed before their potential application into functional foods or drugs.[2] Moreover, other factors such as the epithelial brush-border membrane peptidases, the absorption rate through the intestinal barrier, the possibility of active intracellular peptidases in case of transcellular transport, as well as the potential action of serum peptidases can be determinant leading to the formation and/or degradation of bioactive peptides upon oral administration. These peptides might be absorbed and reach the target tissues acting at systemic level or exert their effects locally in the gastrointestinal tract.

In the last years, several studies have employed *in vitro* gastrointestinal digestion and Caco-2 cell absorption models to estimate the bioavailability of bioactive peptides.[3] The human Caco-2 cells are able to spontaneously differentiate into enterocyte-like monolayers with morphological polarity with an apical and basolateral side, and develop feature characteristics such as apical microvillus and brush-border hydrolases, intercellular tight junctions (TJs), and active receptors and transport systems, including those for peptide transport.[4] Among the several routes described for the transport of peptides in the gut, the main mechanisms include the PepT1, a proton-coupled membrane transporter,[5] the paracellular passive pathway through intercellular TJs, the transcellular passive diffusion, and the vesicle-mediated transcytosis.[6] For oligopeptides, susceptibility to brush-border peptidases has been recognized as the primary factor affecting to the apical-to-basolateral transport rate, with both the paracellular and transcytosis transports identified as the principal involved mechanisms.[7]

Lunasin is a 43-amino acid peptide naturally present in soybean and other legumes, with a variety of biological functions demonstrated by *in vitro* cell cultures and animal models.[8] *In vitro* studies simulating the gastrointestinal digestion have proposed that this peptide was able to resist the action of digestive enzymes due to naturally present protease inhibitors in foods, such as Bowman-Birk protease inhibitor (BBI) and Kunitz trypsin inhibitor.[9, 10] Moreover, Hsieh and co-workers[11] showed that lunasin was bioavailable when orally administered to mice and rats, and Dia et al.[12] reported the presence of this peptide in human plasma after consumption of soy protein. These findings suggest that lunasin is absorbed in the intestine and reaches the bloodstream. Although brush-border intestinal peptidases are key factors limiting the half-life and leading to the transformation of dietary peptides in the digestive tract,[13] to our knowledge, the behaviour of the lunasin sequence in contact with this epithelial membrane has not been previously evaluated.

Our group has recently identified the peptide patterns of gastric and gastrointestinal digests of lunasin by *in vitro* digestion and reverse phase-high liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) analysis, confirming the protective role played by the major Bowman-Birk family isoinhibitor (IBB1) on the digestion of this peptide. Moreover, the final digests were found to exhibit anti-proliferative properties against the growth of colorectal cancer HT-29 and Caco-2 cells.[14] Thus, the present study aims to investigate whether lunasin and selected lunasin-derived

[a] *Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain*
† Corresponding author: b.hernandez@csic.es

fragments arising from its digestion are resistant to brush-border peptidases and susceptible to intestinal transepithelial transport in Caco-2 monolayers. Selective inhibitors of different transepithelial routes were evaluated to identify the potential mechanism involved in the intestinal absorption. Additionally, the anti-proliferative effect of these peptides on human adenocarcinoma gastric (AGS) and colorectal (HT-29 and Caco-2) cells was assessed.

2. Experimental

2.1. Reagents and peptides

Hanks´ balanced salt solution (HBSS), trifluoroacetic acid, Lucifer yellow, cytochalasin D, wortmannin, dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Madrid, Spain). The rest of chemicals used were of HPLC grade.

Lunasin and lunasin-derived peptides used in this study (Table 1) were provided by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China) that synthesized them by the conventional Fmoc solid-phase synthesis method.

Table 1. Synthetic lunasin and lunasin-derived peptides released after an *in vitro* simulated digestion model

Peptide	Amino acid sequence	Purity (%, by HPLC)
Lunasin (1-43)	SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD	98.9
f(1-10)[a]	SKWQHQQDSC	87.6
f(11-18)[a]	RKQLQGVN	89.6
f(19-28)[a]	LTPCEKHIME	75.7
f(29-43)[a]	KIQGRGDDDDDDDDD	68.7
f(17-28)[a]	VNLTPCEKHIME	73.0
f(29-41)[a]	KIQGRGDDDDDDD	76.1
f(34-43)[a]	GDDDDDDDDD	86.1

[a] Lunasin-derived fragments were identified by Cruz-Huerta et al.[14]

2.2. Cell cultures

The human gastric adenocarcinoma cell line (AGS) and two human colorectal adenocarcinoma cell lines (HT-29 and Caco-2) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). AGS, HT-29 and Caco-2 cells were grown in RPMI medium (Biowest, Nuaillé, France), McCoy´s medium (Lonza Group Ltd, Basel, Switzerland), and Dulbecco´s Modified Eagle Medium (DMEM, Biowest), respectively, supplemented with 10% (v:v) fetal bovine serum (FBS, Biowest) and 1% (v:v) penicillin/streptomycin/amphotericin B solution (Biowest). A non-essential amino acid solution (Lonza Group Ltd) was also added to DMEM medium (1%, v:v) for the culture of Caco-2 cells. Cells were maintained in plastic 75-cm^2 culture flasks at 37 ºC in a humidified incubator containing 5% CO_2 and 95% air. The culture medium was changed every two days, and cells were kept sub-confluent by using trypsin/EDTA (Lonza Group Ltd) weekly.

2.3. Transport studies

2.3.1. Culture of Caco-2 monolayers

Caco-2 cells were used to evaluate the transepithelial transport of peptides following the recommendations described by Hubatsch et al.[15] Cells were seeded onto 12-well Transwell polycarbonate permeable membrane supports (12 mm diameter, 0.4 µm pore size, 1.12 cm^2 growth surface area, Costar, Corning, NY, USA) at a density of 1.5×10^5 cells/filter, with 0.5 mL of suspended cells in the apical chamber and 1.5 mL of DMEM in the basolateral side. Filters were pre-wet with 0.1 mL of DMEM before seeding. Medium was replaced at days four and seven after seeding, and 24 h before the experiment. To allow transport studies, cells were used nine days after seeding. Previous to the assay, the integrity of the monolayer of differentiated cells was evaluated by measuring the transepithelial electrical resistance (TEER) value with an EVOM epithelial volt/ohm meter (World Precision Instruments, Sarasota, FL, USA). Cells monolayers with values higher than 400 $\Omega \times cm^2$ were considered confluent and used in the assays.[16]

2.3.2. Transepithelial transport of peptides

Transport experiments were performed as described by Quirós and co-workers,[17] with some modifications. Filters were rinsed by transferring cell monolayers in DMEM into new 12-well plates (Costar) containing HBSS (1.5 mL) in the basolateral side, and by carefully adding HBSS (0.5 mL) to the apical side. Filters were equilibrated at 37 ºC for 20 min. Then, peptides were dissolved in HBSS [lunasin at 10 µM, and fragments f(1-10), f(11-18), f(19-28), and f(29-43) at 1000 µM], and added to the apical chamber. Transwell cultures were incubated at 37 ºC for 60 min, and apical and basolateral samples were withdrawn, freeze-dried, and kept at – 20 ºC until analysis by HPLC-MS/MS. At the end of transport experiments, TEER values were measured again and the flux of Lucifer Yellow (a marker for paracellular permeability) was monitored. Chambers were rinsed with HBSS and incubated at 37 ºC for 30 min. Afterwards, Lucifer Yelow in HBSS (50 µM) was added to the apical compartment and incubated at 37 ºC for 60 min. Aliquots from both the apical and the basolateral sides were taken, and fluorescence levels were determined (excitation 485 nm and emission 520 nm) in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). The post-assay TEER values and the Lucifer Yellow transport (<1% added to apical chamber) were used as parameters to confirm the Caco-2 monolayer integrity during experiments.[18] At least, three independent replicates per peptide were evaluated, and the apparent permeability coefficient (P_{app}, cm/s) was calculated according to Contreras et al.[19] as $P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A} \times \frac{1}{C_0}$; where $\frac{\Delta Q}{\Delta t}$ is the transport rate (µmol/s), A is the surface area of the membrane (1.12 cm^2), and C_0 is the initial peptide concentration in the apical chamber (µmol/mL).

To study the mechanism involved in the transepithelial transport of peptides, prior to the experiments described above, cell monolayers were incubated with cytochalasin D (a TJs disruptor, 0.5 µg/mL) and wortmannin (a transcytosis inhibitor, 500 nM) for 30 min before addition of peptides.[17] Cytochalasin D and wortmannin were dissolved in DMSO (final concentration in HBSS 0.044%). As control, DMSO supplementation was used. Experiments were carried out in duplicate, and peptide quantification in the basolateral side was expressed as relative percentage of control.

2.4. Analysis by HPLC-MS/MS

Analysis of synthetic peptides and samples from apical and basolateral solutions was carried out as previously described,[14] with minor modifications. Peptides were eluted with a linear gradient of solvent B (acetonitrile:trifluoroacetic acid, 1000:0.27, v/v) in solvent A (water:trifluoroacetic acid, 1000:0.37, v/v) going from 0% to 45% in 60 min. Spectra were recorded over the mass/charge (*m/z*) 200-2000, selecting the molecular ion of peptides as the target mass, *m/z* 1258 (lunasin), *m/z* 1247 [f(1-10)], *m/z* 943 [f(11-18)], *m/z* 1201 [f(19-28)], and *m/z* 1694 [f(29-43)]. Peptides were identified by their retention times and fragmentation profiles. Data obtained were processed and transformed to spectra representing mass values using the Data Analysis program (version 4.0, Bruker

Daltonik). To process the MS/MS spectra and to perform peptide sequencing BioTools (version 3.2, Bruker Daltonik) was used.

Previous to the analysis, for lunasin transport experiments (10 μM), freeze-dried samples from apical and basolateral solutions were reconstituted in the initial volume, or concentrated twenty times, respectively, in Milli-Q water. In the case of the four lunasin-derived peptides (1000 μM), apical samples were reconstituted in the initial volume and then diluted 1:24 (v:v), and basolateral samples were concentrated ten times. Five-point external calibration curves were prepared with synthetic lunasin (from 0.5 to 10.0 μM), and lunasin-derived fragments (from 0.9 to 46.4 μM). Duplicate injections were performed for each point of the calibration curve. The area under the curve of the extracted molecular ions of peptides and their sodium and potassium adducts, when formed, was measured and linear or polynomial regression curves were estimated depending on the fit: lunasin, $y = -4 \times 10^8 + 3 \times 10^9 x$ (R^2=0.998); f(1-10), $y = 1 \times 10^8 + 7 \times 10^7 x$ (R^2=0.994); f(11-18), $y = 1 \times 10^8 + 1 \times 10^8 x$ (R^2=0.976); f(19-28), $y = 2 \times 10^8 + 1 \times 10^8 x$ (R^2=0.992); and f(29-43), $y = -8 \times 10^6 + 1 \times 10^8 x - 2 \times 10^6 x^2$ (R^2=0.996).

2.5. Cell viability experiments

2.5.1. MTT assay

The MTT assay was performed to evaluate the effect of peptides on the viability of AGS, Caco-2 and HT-29 cells. Cells were seeded in 96-well plates (Costar) at a density of 5 × 10^3 cells/well (AGS),[20] and 5 × 10^4 cells/well (Caco-2 and HT-29).[21] After 24 h incubation, cells were treated with lunasin and all lunasin-derived fragments (Table 1) at different concentrations (10, 50, 100, and 200 μM) for 24, 48, and 72 h. After the different treatment times, cells were washed with phosphate buffer saline (PBS, Lonza Group Ltd), and incubated for 2 h with a MTT solution (0.5 mg/mL final concentration). The supernatants were discarded and insoluble formazan crystals formed were dissolved in DMSO:ethanol (1:1, v:v). After gently mixing, the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech). Experiments were carried out in triplicate and results were expressed as percentage of the control, non-treated cells.

2.5.2. Morphological analysis

AGS, Caco-2 and HT-29 cells were plated in 6-well plates (Costar) at a density of 5.0 × 10^5, 3.5 × 10^4 and 6.7 × 10^5 cells/well, respectively. After 24 h incubation, cells contained in four of six wells were treated with lunasin (100 and 200 μM) for 48 h. Two wells were used as control (untreated) cells. Afterwards, cell images were taken by using an optical microscope Leica DM2500 (Leica Lasertechnik GmbH, Mannheim, Germany) at 10X magnification, coupled to a camera Leica DFC420 C (Leica Lasertechnik GmbH). This assay was carried out in duplicate.

2.5.3. Stability of lunasin

AGS, Caco-2 and HT-29 cells were plated as described above for morphological analysis, and treated with 10 μM lunasin for 72 h, taking aliquots of the medium at 0, 2, 24, 48, and 72 h of incubation. Aliquots of cultures with non-treated medium were also taken at these times. Samples were evaluated in duplicate and analysed by HPLC-MS/MS as indicated above for transepithelial transport experiments.

2.6. Statistics

Data were analyzed using GraphPad Prism 5.03 software (San Diego, CA, USA) by a one-way ANOVA, followed by the Bonferroni *post hoc* test. Differences with a P value < 0.05 were considered significant.

3. Results and Discussion

3.1. Effect of epithelial peptidases on lunasin and lunasin-peptides

In a previous study, our group had demonstrated that more than 5% of synthetic lunasin resisted at the end of an *in vitro* simulated pepsin-pancreatin digestion when IBB1 was present (lunasin:IBB1 ratio of 1:2, w:w). Moreover, the peptides released in the gastric and gastrointestinal digests of lunasin were identified by HPLC-MS/MS.[14] Therefore, in this transepithelial study, the lunasin-derived peptides f(1-10), f(11-18), f(19-28), and f(29-43) arising from its digestion were selected according to their proved resistance to the gastrointestinal enzymes. In order to evaluate a possible structure/activity relationship, peptide lunasin and these four digestion fragments integrating the complete 43-amino acid sequence were assessed in Caco-2 monolayers for their resistance to brush-border peptidases and intestinal transport.

The integrity of the Caco-2 monolayers was confirmed by measuring TEER values and the flux of Lucifer Yellow before and after the experiments. Cell monolayers maintained intact along the incubation with lunasin-derived peptides at 1000 μM. However, lunasin at 50-1000 μM affected the integrity of the monolayers showing a reduction effect on the TJs strength. Lunasin at 10 μM did not exert a drop on TEER values. Previous studies carried out in our group had demonstrated that lunasin at concentrations ranged from 0.5 to 25 μM did not damage cell integrity during the period of incubation of this peptide with differentiated human Caco-2,[16] and liver HepG2 cells.[22] Thus, 10 μM was the concentration selected to carry out the following transport studies. As illustrated in Figure 1A, lunasin was shown to reduce its concentration in a 42.8% ± 1.3, while lunasin-peptides f(11-18) and f(19-28) remained intact in a 79.7 ± 2.1 and 78.7% ± 8.4 of the initial peptide added, respectively. However, peptides f(1-10) and f(29-43) maintained nearly intact after 60 min incubation with Caco-2 peptidases. This confers further resistance for these peptides in the intestine epithelium, beyond that was previously shown against pepsin and pancreatic proteases.[14] Similarly, some food-derived antihypertensive peptides from milk caseins which sequences were VPP and IPP,[23] and HLPLP,[17] and from egg white QIGLF,[24] and the immunomodulatory peptide β-casein f(193-209)[25] have shown remarkable resistance against intestinal brush-border peptidases. As shown in Figure 1B, when lunasin was added to the apical side of Caco-2 monolayers, brush-border intestinal peptidases were found to cleave at the central region at the peptide bonds ^{15}Q-G^{16}, ^{18}N-L^{19}, ^{19}L-T^{20}, ^{22}C-E^{23}, and ^{23}E-K^{24}. These findings suggest a higher susceptibility to epithelial peptidases for the central region of lunasin sequence than the N- and C-terminal domains. Interestingly, this central region sited between amino acids 11 and 28 was also described to be predominantly cleaved by pepsin and pancreatin when lunasin was subjected to a two-stages hydrolysis process simulating gastrointestinal digestion in absence of IBB1, but enclosed and protected from the enzymatic action when the protease inhibitor was present.[14]

In the case of the lunasin-derived peptides, the fragments found were mostly formed as consequence of cleavages at the N-terminus (Figure 1B). Caco-2 cells have been demonstrated to express up to eight membrane peptidases on the apical side. Among them, the serine protease dipeptidyl peptidase IV (DPPIV) that predominantly cleaves at the N-terminal position has shown to have the highest

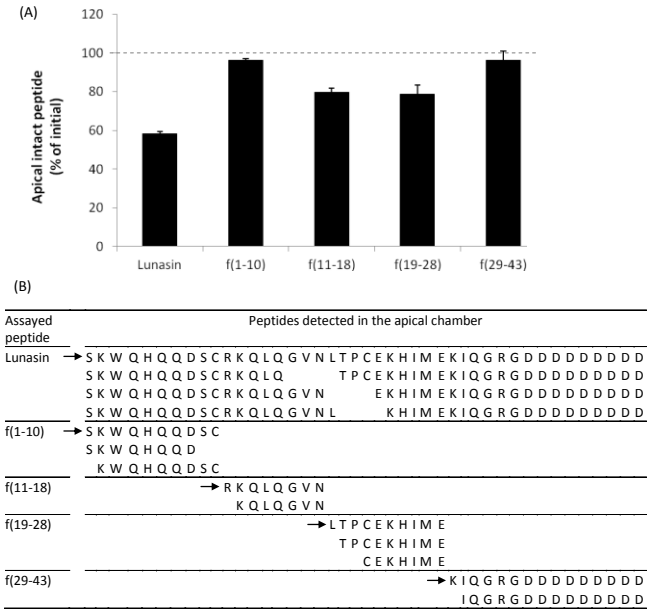


Figure 1. Behaviour of lunasin and lunasin-fragments added to Caco-2 monolayers. Analysis by HPLC-MS/MS of peptides in apical solutions after incubation of lunasin 10 μM and lunasin-fragments 1000 μM with Caco-2 monolayers for 60 min. (A) Remaining intact peptide in apical solutions was evaluated by the extracted molecular ions of lunasin mass/charge (*m/z*) 1258.0 (charge +4) and *m/z* 1677.2 (charge +3), f(1-10) *m/z* 1246.3 (charge +1), f(11-18) *m/z* 942.3 (charge +1), f(19-28) *m/z* 1200.7 (charge +1), and f(29-43) *m/z* 1694.0 (charge +1). Sodium and potassium adducts were considered, when formed. Data are represented as relative percentage of initial peptide added to the chamber, and expressed as mean ± standard error of the mean (SEM) of at least three independent replicates. (B) Intact peptides presented in the apical chamber and derived fragments released by brush-border intestinal peptidases are shown. Sequence of peptide added to the apical solution is marked with an arrow: Lunasin, SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD; f(1-10), SKWQHQQDSC; f(11-18), RKQLQGVN; f(19-28), LTPCEKHIME; and f(29-43), KIQGRGDDDDDDDDD.

activity, especially when Caco-2 cells are completely differentiated.[26] Besides, N-terminal hydrophobic or basic amino acid residues represented a preferred substrate for the enzymatic action of DPPIV, while proline residue was fairly resistant.[27] Degradation by DPPIV presumably occurred at the peptide bonds ^{1}S-K^{2} in f(1-10), ^{11}R-K^{12} in f(11-18), ^{19}L-T^{20} in f(19-28), and ^{29}K-I^{30} in f(29-43), while the peptide bond ^{20}T-P^{21} maintained its integrity. The effect of DPPIV enzyme had been also suggested to be responsible for the N-terminal degradation of bioactive peptides LHLPLP,[17] FRADHPFL,[28] RYLGY and AYFYPEL,[19] and RVPSL,[29] in Caco-2 monolayers.

These results imply the relevance of the resistance to the epithelial brush-border membrane to evaluate the bioavailability of bioactive peptides. Despite the enzymatic attack shown on lunasin and derived fragments prior to their possible transport, these peptides have shown a notable resistance against Caco-2 peptidases, especially f(1-10) and f(29-43), when compared to other bioactives peptides such as the μ-opioid receptor agonists β-casomorphin-5 and -7 from both bovine,[30] and human[31] origins that nearly disappeared after incubation in the apical side of Caco-2 monolayers.

3.2. Transepithelial transport of lunasin and lunasin-fragment f(11-18)

Analysis by HPLC-MS/MS of standard curves prepared with five-point dilutions of synthetic peptides allowed the quantification of peptides in the basolateral solutions. Only peptides lunasin and f(11-18) were absorbed through the cell monolayer, while the rest of lunasin-fragments were not detected at the basolateral side. Any of the released fragments by the action of brush-border peptidases were able to flux across the Caco-2 monolayer. After 60 min incubation in the apical compartment, the P_{app} values calculated for the apical-to-basolateral transport of lunasin and f(11-18) were 3.32 × 10^{-7} cm/s and 2.50 × 10^{-7} cm/s, respectively (Figure 2A). These values were similar to those found for other bioactive peptides on this transepithelial model such as AYFYPEL (2.60 × 10^{-7} cm/s) and RYLGY (2.20 × 10^{-7} cm/s),[19] and VLPVP (2.78 × 10^{-7} cm/s).[32] Several studies have been focused on the *in vitro* transport of bioactive peptides through Caco-2 monolayers, with different permeability values reported for peptides VPP and IPP (0.50 and 1.00 × 10^{-8} cm/s, respectively),[33] RVPSL (6.97 × 10^{-6} cm/s),[29] and SRYPSY (casoxin-6, 9.21 × 10^{-6} cm/s).[30]

Further experiments were conducted in order to evaluate the mechanism involved in the transepithelial transport of intact peptides lunasin and f(11-18). Three main mechanisms have been described for the flux of peptides across intestinal epithelium, the PepT1-mediated transport, the TJs-mediated paracellular pathway, and the vesicle-mediated transcytosis route.[34] Peptides longer than three amino acids residues have been described as not substrates for the PepT1 transporter.[35] Therefore, in this study, Caco-2 monolayers were pre-incubated with wortmannin (a transcytosis inhibitor), and cytochalasin D (a TJs disruptor) for 30 min before the transepithelial experiments.[17] As illustrated in Figure 2B, the presence of wortmannin hardly modified the intensity of neither peptides lunasin nor f(11-18) in the basolateral chamber, suggesting that the vesicle-mediated mechanism was not involved on the transport of these peptides, in disagreement with other peptides such as the β-casein f(193-209).[25] However, cytochalasin D was shown to induce an approximately 1.5-fold increase in the presence of both lunasin and f(11-18) compared to controls. The enhanced transport was accompanied by a TEER value decrease (21.2% average), which also pointed toward the involvement of an expanded intercellular effect on the peptides flux.[25] The paracellular route is characterized by the passive diffusion of molecules between adjacent cells, and is regulated by the intercellular TJs forming a biological barrier with selective permeation function.[2] The aqueous nature of this pathway makes it favourable for the absorption of water-soluble substances including oligopeptides,[36] and allows the flux to the basolateral side avoiding the enzymatic role of intracellular peptidases. Together these findings suggested that the paracellular pathway via TJs was the mainly route for the transepithelial transport of lunasin and f(11-18), consistent with the transport of many peptides, such as VPP,[37] GGYR,[7] HLPLP,[17] QIGLF,[24] RVPSL,[29] TNGIIR,[38] and VLPLP.[32] In addition, the permeability of paracellular-transported compounds has been considered to be underestimated in Caco-2 monolayers because of the colonic origin of these cells that present a relatively higher TJs tightness in comparison with human or animal small intestinal epithelial cells,[39] which ultimately might imply a higher *in vivo* transepithelial transport for the peptides lunasin and derived fragment f(11-18), than that found in this study.

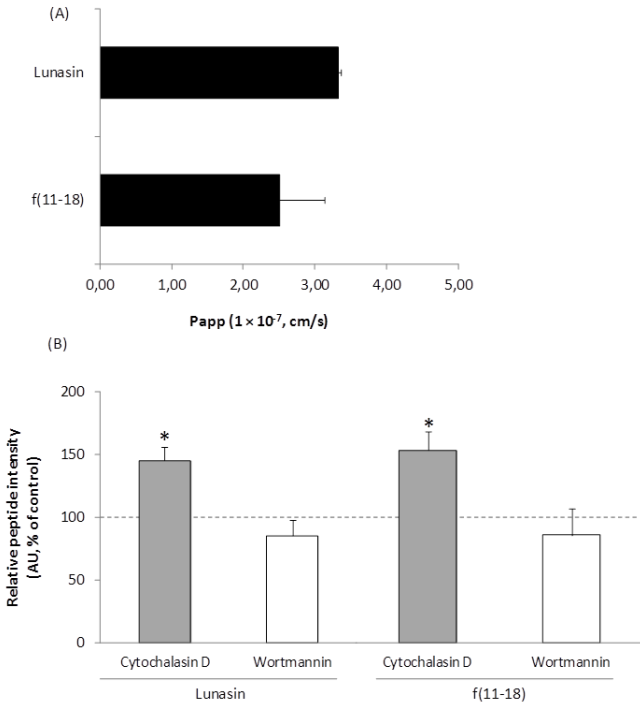


Figure 2. Transepithelial transport of peptides lunasin and f(11-18), RKQLQGVN. (A) Peptides were analyzed by HPLC-MS/MS in the basolateral solutions after incubation of 10 µM lunasin and 1000 µM lunasin-fragment f(11-18). The apparent permeability coefficient (P_{app}) was calculated as described in Materials and methods section, and represented as mean ± standard error of the mean (SEM) of at least three independent replicates per assayed peptide. (B) Effects of cytochalasin D and wortmannin on the transepithelial transport of peptides. Cells were pre-incubated with cytochalasin D (0.5 µg/mL) and wortmannin (500 nM) for 30 min, or Hanks' Balanced Salt Solution (HBSS) with 0.044% dimethylsulfoxide (DMSO) (control), before addition of 10 µM lunasin and 1000 µM lunasin-fragment f(11-18). Experiments were carried out in duplicate, and results were represented as relative peptide intensity in the basolateral chamber (% of control, mean ± SEM). (*, $P < 0.05$) denotes statistically significant differences versus control by a one-way ANOVA followed by the Bonferroni *post hoc* test.

This is the first study that evaluates the behaviour of lunasin and some digestion-fragments on the Caco-2 monolayer. Artursson and Karlsson[40] established an association between the P_{app} coefficient in Caco-2 cells and the oral drug *in vivo* absorption. Later on, numerous studies on this cellular model have also found a high correlation for several compounds compared to the human intestinal transport.[41, 42] In this sense, Dia et al.[12] estimated in humans an average of 4.5% lunasin absorption from the total lunasin ingested in 50 g of soy protein, which is in agreement with the P_{app} coefficient found in this study (3.32×10^{-7} cm/s). Regarding the absorption of lunasin, a relative long 43-amino acid peptide, it is worthy to mention that other polypeptides in this size range such as the therapeutics 32-amino acid calcitonin, and 51-amino acid insulin have been also reported to be transportable in the Caco-2 model.[39] This is similar to the flux found for some dietary whey protein-derived peptides in the β-lactoglobulin sequence f(114-146).[43] The lunasin f(11-18) was the only fragment in this study resembling the transepithelial behaviour of the parent peptide. The RGD-motif found in lunasin sequence at amino acids 33-35 had been demonstrated to allow the attachment of this peptide to the extracellular matrix, suggesting its role in the internalization into mammalian cells.[44] Besides, RGD-motif was suggested as the recognizing sequence for the $\alpha_5\beta_1$ integrin-mediated antiproliferative activity of lunasin on colorectal cancer cells.[45] Nevertheless, in this study, the RGD-containing peptide f(29-43) was not transported across the Caco-2 monolayer. It may be hypothesised whether peptide f(11-18) was the presumable target sequence for the intact absorption of complete lunasin across the intestinal epithelium. However, more studies by modifying this region of the sequence are thus required to confirm these findings.

3.3. Effects of lunasin and derived peptides on the viability of gastric and colorectal cancer cells

Several food-derived peptides have been described to exert potential chemo-preventive properties against the proliferation of malignant cells.[46] Peptide lunasin has demonstrated anti-proliferative activity in colorectal cancer HCT-116, HT-29, KM12L4, and RKO cells.[21, 45] Cruz-Huerta et al.[14] recently showed an inhibitory effect for the gastrointestinal digests of lunasin:IBB1 mixtures in colorectal cancer HT-29 and Caco-2 cells, suggesting that remaining intact peptides and derived fragments directly released after their enzymatic digestion could be the responsible agents for the observed protective effects. In this study, lunasin and some new derived fragments (Table 1) were thus evaluated for their potential anti-proliferative effect in the gastrointestinal tract by the MTT protocol.

As shown in Figure 3, peptide lunasin affected the viability of the three cell lines, with relatively higher effects on HT-29 cells. After 24 h treatment, lunasin provoked a ca. 10% significative induction of non-viable cells at all concentrations assessed (Figure 3A). However, after 48 and 72 h treatment, a dose-dependent statistical trend was found for lunasin activity with the maximum inhibitory effect found at 200 µM (19.1 and 23.8%, respectively). Moreover, a time-dependent lunasin´s effect was revealed, showing a significant enhanced activity for this peptide at 50-200 µM from 24 to 72 h (12.4% average). Dia and de Mejia[21] had previously found that lunasin induced a cytotoxic effect ranging from 19.3% (10 µM) to 62.8% (100 µM) on HT-29 cells. While these authors assessed the activity of purified lunasin (~90%) from defatted soybean flour, we have evaluated the effect of the synthetic peptide. The lower activity found in this study might be due to differences in the secondary and tertiary structures between plant-purified lunasin and the synthetic peptide. In Caco-2 cells, lunasin 50-200 µM, and 10-200 µM was able to induce an average ca. 10% significant increase on the number of non-viable cells after 24 and 48 h treatment, respectively (Figure 3B). In these cells, a dose-dependent trend was not clearly shown, however a notable time-dependent difference was demonstrated at 72 h. At this time no anti-proliferative outcome was shown, suggesting that lunasin´s effects at 24 and 48 h were insufficient to induce a marked inhibition on treated cells, which were found to recover and growth at the same ratio as control cells after 72 h. As shown in Figure 3C, lunasin showed a significant dose-dependent activity in AGS cells. At 10 and 50 µM, this peptide did not present any statistical difference from control cells. However, lunasin 100 and 200 µM modestly induced an average inhibitory effect ca. 7% and 15%, respectively. Figure 4 illustrates the morphological analysis of non-treated and lunasin-treated cells, which showed a visual change in viable cells as compared with the homogenous growth of control cells over the plate. With these results, it is not possible to know the mechanisms responsible for the effects of lunasin on MTT, thus additional experiments would be required. Together these findings indicated the higher susceptibility of HT-29 cells than both Caco-2

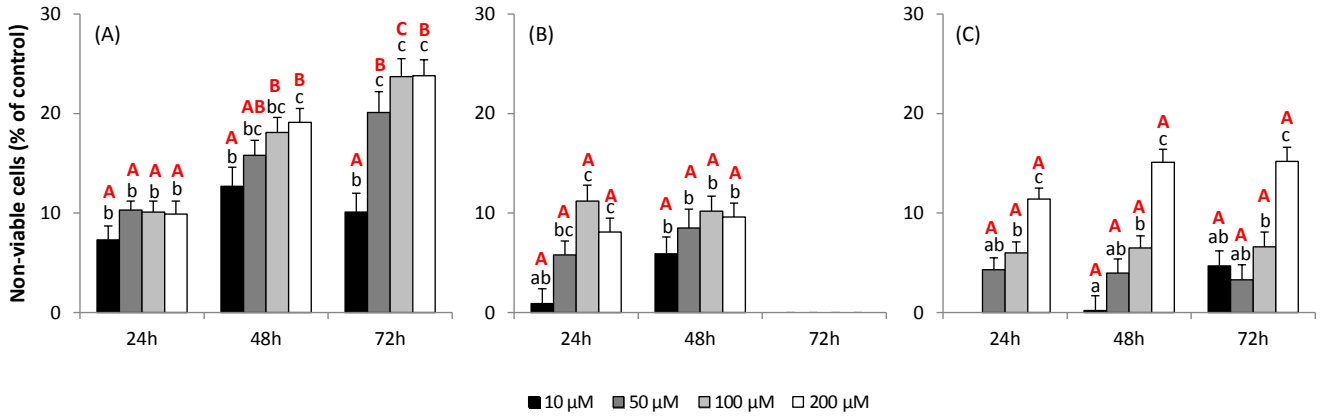


Figure 3. Effects of lunasin on cancer cells viability. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after treatment of human adenocarcinoma colorectal (A) HT-29 and (B) Caco-2 cells and (C) gastric cancer AGS cells with lunasin (10-200 µM, 24-72 h). Results were expressed as percentage of non-viable cells compared to control, considered as 0% (% of control, mean ± standard error of the mean, SEM). Experiments were carried out in triplicate, and analysed by a one-way ANOVA followed by the Bonferroni *post hoc* test. Lower case letters denote statistically significant differences ($P < 0.05$) between doses and control, considered as (a). Capital letters denote statistically significant differences ($P < 0.05$) for the same dose between treatment times.

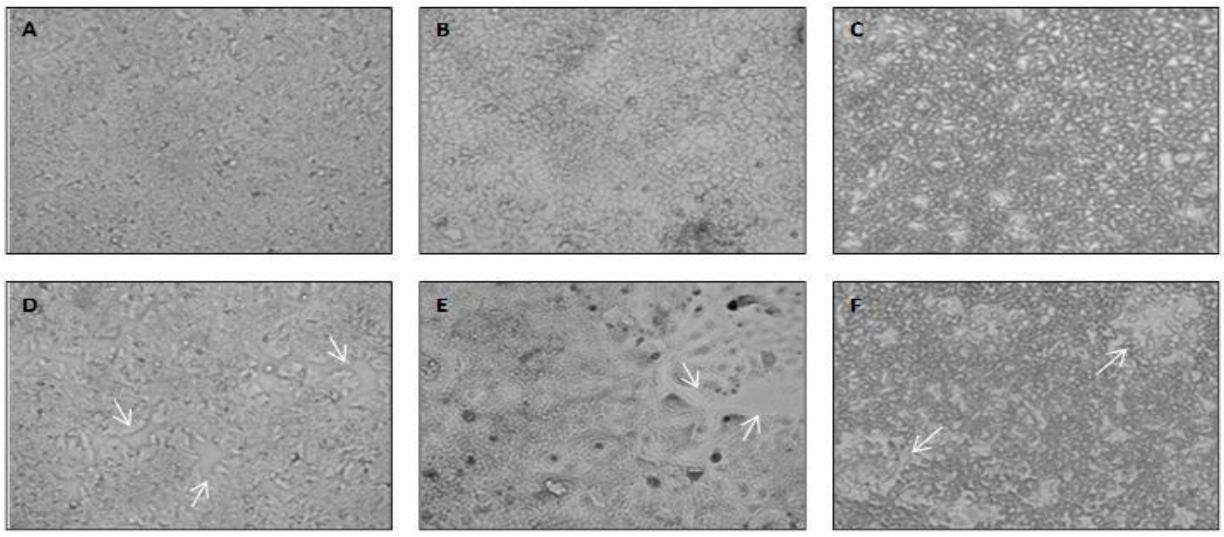


Figure 4. Morphological analysis. Representative images, taken by using an optical microscope at 10X magnification, of HT-29, Caco-2, and AGS (A-C, respectively) non-treated cells, and (D-F, respectively) lunasin-treated cells (200 µM, 48 h). Lunasin-treated cells showed areas with minor cellular density (arrows), compared to non-treated control cells presenting a continuous growth over the plate.

and AGS cells to peptide lunasin, in agreement with previous results that found a more evident anti-proliferative effect for lunasin:IBB1 gastrointestinal digests on the former colorectal cancer cells.[14] Likewise, the cytotoxic activity of this peptide has been proposed to be dependent on the type of cell line treated with inhibitory concentration (IC_{50}) values ranging from 13 µM in colorectal cancer KM12L4 cells[45] to 181 µM in breast cancer MDA-MB-231 cells.[47]

Following the same methodology, gastric and colorectal cancer cells were treated with seven lunasin-derived peptides (10-200 µM, 24-72 h). In HT-29 cells, peptide f(1-10) induced a time-dependent trend on the percentage of non-viable cells compared to control non-treated cells (Figure 5A). This peptide showed an increasing effect with the dose at 24 h from 9.4% to 15.1% inhibition, while no statistical differences between concentrations were demonstrated at 48 and 72 h. The highest effect, 25.8% inhibition, was found at 72 h for the dose of 200 µM. As shown in Figure 5B, peptide f(17-28) reproduced these anti-proliferative properties, but to a lower extent, with a dose-dependent trend (10-100 µM) at 24 and 48 h, and an enhanced activity along the treatment time. The maximum inhibitory effect found for this fragment was observed at 72 h for all the concentrations evaluated (17.3% average). Lunasin-peptides f(19-28), f(29-41), and f(29-43) (10-200 µM) showed a ca. 10% inhibitory activity at 48 and 72 h, while f(11-18) and f(34-43) did not cause significant effect at any condition evaluated in HT-29 cells (data not shown). In Caco-2 cells, f(1-10) and f(17-28) were also the most active peptides among all lunasin-fragments assessed. Lunasin-peptides f(1-10) exhibited the highest effect after 24 h treatment with 200 µM (18.6%) (Figure 5C), and f(17-28) after 48 h treatment with 200 µM (22.8%) with a marked dose-dependent statistically different activity at this time, but not at 24 h (Figure 5D). As it was shown for lunasin treatment on Caco-2 cells, the activity of both fragments was notably decreased after 48 h, with

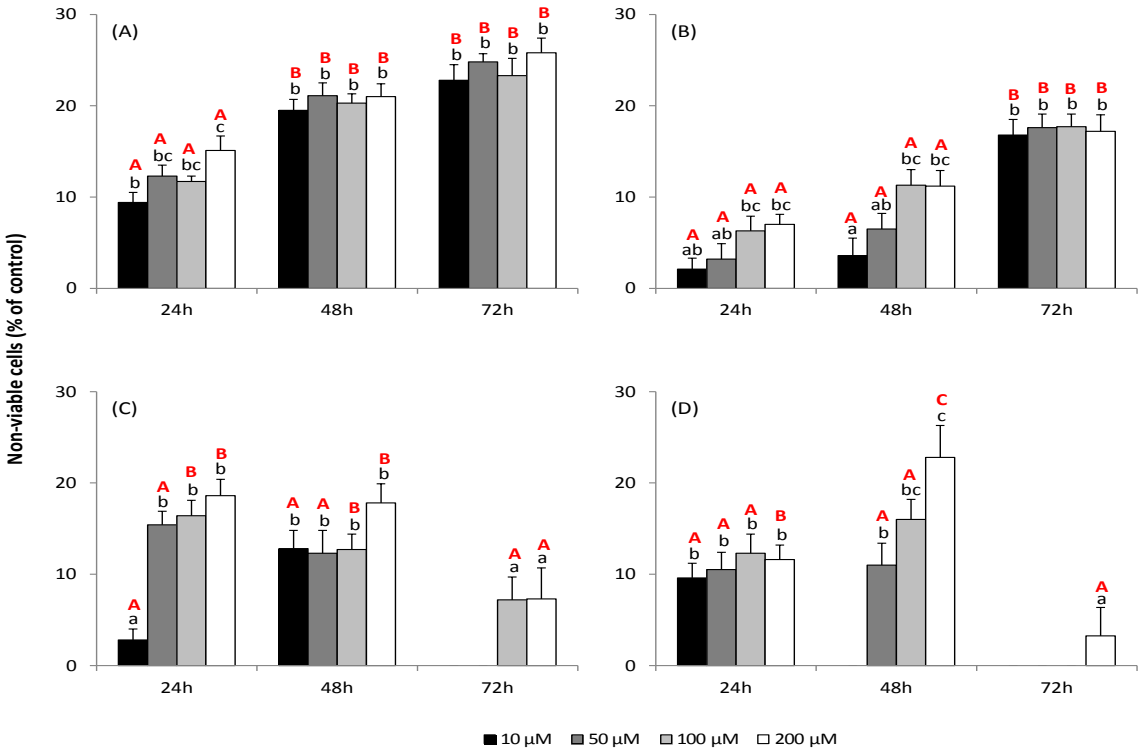


Figure 5. Effects of lunasin-derived fragments on cancer cells viability. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after treatment of human adenocarcinoma colorectal (A and B) HT-29 and (C and D) Caco-2 cells with (A and C) peptide SKWQHQQDSC, f(1-10) and (B and D) peptide VNLTPCEKHIME, f(17-28) at concentrations ranging from 10 to 200 µM for 24-72 h. Results were expressed as percentage of non-viable cells compared to control, considered as 0% (% of control, mean ± standard error of the mean, SEM). Experiments were carried out in triplicate, and analysed by a one-way ANOVA followed by the Bonferroni *post hoc* test. Lower case letters denote statistically significant differences ($P < 0.05$) between doses and control, considered as (a). Capital letters denote statistically significant differences ($P < 0.05$) for the same dose between treatment times.

any statistical difference from control cells at 72 h. While peptide f(29-41) showed a ca. 8% inhibitory activity at 24 and 48 h, none of the rest lunasin-peptides induced cytotoxic effects on these cells (data not shown). Therefore, in colorectal HT-29 cells, peptide f(1-10) might be the main contributor to the anti-proliferative activity proven for the complete lunasin. Despite the inhibitory activity exerted by lunasin and its fragments f(1-10) and f(17-28), the functionality of these peptides on Caco-2 cells was not absolutely established due to the higher resistance of these cells, showing weaker effects that lead to a recovery to control levels after 72 h treatment. Similarly to that was observed for lunasin in gastric AGS cells, all derived fragments showed modest results in the range of 10-15% inhibition, especially at 100 and 200 µM (data not shown). Thus, the effect of lunasin on these cells seems to be unspecific and independent of the assessed region within the complete 43-amino acid peptide. The chemo-preventive properties of lunasin had been attributed to its C-terminal domain that presents: (i) a predicted helix with structural homology to a conserved region of chromatin-binding proteins, (ii) an RGD cell adhesion motif, and (iii) a poly-D tail, with a high negatively charge, known to preferentially bind to positively charged deacetylated histones and hypoacetylated chromatin.[44] To date, to the best of our knowledge, only one study had evaluated a possible structure-activity relationship with different lunasin fragments on breast cancer MDA-MB-231 cells.[47] Whereas peptide lunasin inhibited the malignant growth with an IC_{50} value of 181 µM, these authors proved that the C-terminal region was more active than the complete peptide with IC_{50} values of 175 and 138 µM for lunasin-fragments ^{23}EKHIMEKIQGRG^{34} and ^{23}EKHIMEKIQGRGDDDDDDDDD^{43}, respectively, while the fragment ^{1}SKWQHQQDSCRKQLQGVNLTPC^{22} at the N-terminal domain was less potent (IC_{50}: 323 µM). Therefore, this is the first study suggesting a protective role for the N-terminal region of lunasin, especially for f(1-10) in HT-29 cells. In this line, Chang et al.[48] recently found that a truncated peptide lacking the RGD-motif and the poly-D was equally active than the complete lunasin sequence on the immunomodulatory activation of natural killer cells. However, in the present study, neither lunasin nor lunasin-derived peptides exerted the protective activity previously shown for lunasin:IBB1 digests.[14] Therefore, the inhibitory properties of other compounds in the digests or a possible synergistic effect between the peptides identified could not be discarded in this study. Moreover, it should be highlighted that culture conditions have been recently found to notably modulate the anti-proliferative activity of lunasin on non-small cell lung cancer cells.[49] These authors demonstrated that anchorage-independent growth conditions, more closely mimicking the *in vivo* tumor

environment, increased the sensitivity of cancer cells to lunasin. In our study under anchorage-dependent growth conditions, the moderate biological MTT-activity would be presumably meaningful in a more physiological state.

3.4. Stability of lunasin on gastric and colorectal cancer cells

As shown in Table 2, by HPLC-MS/MS analysis, the residual intact lunasin in the cell cultures was quantified and the derived fragments released by cellular enzymatic action were identified.

Table 2. Stability of peptide lunasin in medium added to AGS and HT-29 cells, and identification of lunasin-derived fragments[a]

Peptides	AGS				HT-29			
	2h	24h	48h	72h	2h	24h	48h	72h
% residual lunasin	100	100	86.8	73.0	97.3	93.1	77.9	68.0
^{23}EKHIMEKIQGRGDDDDDDDDD^{43}	-	-	+	+	-	+	+	+
^{25}HIMEKIQGRGDDDDDDDDD^{43}	-	-	+	+	-	+	+	+
^{29}KIQGRGDDDDDDDDD^{43}	-	-	+	+	-	+	+	+
^{32}GRGDDDDDDDDD^{43}	-	-	-	-	-	-	-	+

[a] Cells were treated with lunasin 10 µM, and samples were withdrawn at 0, 2, 24, 48, and 72 h. Experiments were evaluated in duplicate by RP-HPLC-MS/MS. Results were expressed as % of the initial lunasin, and peptides were identified by the molecular ions and fragmentation patterns of lunasin *m/z* 1258.0 (charge +4) and *m/z* 1677.2 (charge +3), f(23-43) *m/z* 1231.1 (charge +2), f(25-43) *m/z* 1102.5 (charge +2), f(29-43) *m/z* 1694.0 (charge +1), and f(32-43) *m/z* 1324.4 (charge +1).

In AGS cells, lunasin remained intact for up to 24 h incubation, and showed a 13.2% and 27.0% reduction in its content after 48 and 72 h, respectively. Furthermore, at 48 h incubation, the lunasin fragments ^{23}EKHIMEKIQGRGDDDDDDDDD^{43}, ^{25}HIMEKIQGRGDDDDDDDDD^{43}, and ^{29}KIQGRGDDDDDDDDD^{43} were generated and remained in the culture after 72 h. In HT-29 cells, a relatively higher cellular enzymatic activity was evidenced, with residual lunasin percentages being decreased in a time-dependent manner from 2 h (97.3%) to 72 h (68.0%), and the identification of the same three lunasin-derived peptides from 24 h incubation, as well as the new fragment ^{32}GRGDDDDDDDDD^{43} at 72 h. In addition to peptide hydrolysis, degradation by the enzymatic response of cells to incubation with peptide explained the decrease in lunasin concentration. It might be also due to peptide´s internalization into AGS and HT-29 cells, as it has been demonstrated in murine fibroblast C3H10T1/2 cells,[44] and human THP-1 macrophages,[50] colorectal cancer KM12L4 cells,[45] and prostate epithelial RWPE-2 cells.[51] Four lunasin-derived fragments were formed corresponding to the C-terminal region of the sequence from the amino acid residue 23. Among them, fragment ^{29}KIQGRGDDDDDDDDD^{43} coincided with the sequence of one lunasin-peptide that showed a ca. 10% inhibitory activity on AGS and HT-29 cells in this study, and had been identified in *in vitro* gastrointestinal digests of lunasin.[14] In this sense, it is worthy to mention that the lunasin-derived peptide ^{1}SKWQHQQDSC^{10} was not formed during the stability assay, although it was the most active in the MTT assay. The enzymatic action of digestive peptidases would be thus required to release this fragment. Moreover, the fragments ^{25}HIMEKIQGRGDDDDDDDDD^{43}, ^{29}KIQGRGDDDDDDDDD^{43} and ^{32}GRGDDDDDDDDD^{43} had been already identified in a previous study of lunasin´s stability in human liver HepG2 cells,[22] which suggested that these cultures might share enzymatic activities upon incubation with lunasin peptide. However, further studies with these peptides are required to definitively elucidate whether this C-terminal region is, at least partially, involved in the protective properties showed for the intact lunasin. In contrast, neither lunasin decrease nor derived fragments were demonstrated in Caco-2 cells along the incubation time. Lunasin degradation in this culture was thus not the cause of the anti-proliferative activity loss showed after 72 h treatment. García-Nebot et al.[16] had previously conducted the study of lunasin´s stability on differentiated Caco-2 cells, as an *in vitro* model of the human intestine, and also found that this peptide mostly remained intact after 24 h incubation, with no derived fragments identified. Therefore, it might be suggested that the cell line type is determinant in the behavior of lunasin added to the culture medium.

4. Conclusions

The present study has been focused on the analysis of peptides lunasin and lunasin-digestion fragments at the gastrointestinal tract. The results pointed out for the first time a notable resistance against the epithelial brush-border membrane of Caco-2 monolayers, especially for peptides f(1-10) and f(29-43). Lunasin and f(11-18) were demonstrated to cross the intestinal epithelial barrier, with the paracellular route found as the main transport mechanism involved. The f(11-18) might be proposed as preferred target sequence for the transepithelial transport of lunasin peptide. These findings might imply biological relevance, suggesting potential to exert bioactive effects both locally in the gastrointestinal tract as well as at a systemic level. Lunasin and lunasin-released peptides after simulated *in vitro* digestion were shown to play moderate protective properties against the growth of gastric and colorectal cancer cells, with the maximum inhibitory effects shown for lunasin and f(1-10) in HT-29 cells. Therefore, this is the first study that postulates a preventive role for the N-terminal region of lunasin. It has been suggested that the cell line type is determinant for the different behaviour of lunasin-derived sequences.

Acknowledgements

This work has received financial support from project AGL2015-66886-R from the Spanish Ministry of Economy and Competitiveness (MINECO). S. F. -T. and B. H. -L. acknowledge MINECO for their FPI fellowship and “Ramón y Cajal” post-doctoral contract, respectively. J. S. is the recipient of a “CSIC-CM- FSE, Iniciativa de Empleo Juvenil" contract.

References

1 M. Shimizu, S. Hachimura, *Trends Food Sci. Technol.,* 2011, **22**, 646-650.
2 M. Segura-Campos, L. Chel-Guerrero, D. Betancur-Ancona, V. M. Hernandez-Escalante, *Food Rev. Int.* 2011, **27,** 213-226.
3 L. Sánchez-Rivera, D. Martínez-Maqueda, E. Cruz-Huerta, B. Miralles, I. Recio, *Food Res. Int.* 2014, **63**, 170-181.
4 S. Deferme, P. Annaert, P. Augustijns, in *Drug absorption studies – in situ, in vitro, and in silico models*, ed. C. Erhardt, K. –J, Springer, New York, 2008, pp 182-215.
5 B. Brodin, C. –U. Nielsen, B. Steffansen, S. Frøkjær, *Pharmacol. Toxicol.* 2002, **90**, 285-296.
6 E. Ziv, M. Bendayan, *Microscopy Res. Techn.* 2000, **49***,* 346-352.
7 M. Shimizu, M. Tsunogai, S. Arai, *Peptides,* 1997, **18**, 681-687.
8 S. Fernández-Tomé, B. Hernández-Ledesma, in *Recent Progress in Medicinal Plants*, ed. J. N. Govil, Studium Press LLC, 2016. Vol. 43, in press.

9 H. J. Jeong, J. B. Jeong, D. S. Kim, B. O. de Lumen, *J. Agric. Food Chem.* 2007, **55**, 632-637.
10 J. H. Park, H. J. Jeong, B. O. de Lumen, *J. Agric. Food Chem.* 2007, **55**, 10703-10706.
11 C. –C. Hsieh, B. Hernández-Ledesma, H. J. Jeong, J. H. Park, B. O. de Lumen, *PLoS ONE,* 2010, **5**, e8890.
12 V. P. Dia, S. Torres, B. O. de Lumen, J. W. Erdman, E. G. de Mejia, *J. Agric. Food Chem.* 2009, **57**, 1260-1266.
13 G. Picariello, B. Miralles, G. Mamone, L. Sánchez-Rivera, I. Recio, F. Addeo, P. Ferranti, *Mol. Nutr. Food Res.* 2015, **59**, 948-956.
14 E. Cruz-Huerta, S. Fernández-Tomé, M. C. Arques, L. Amigo, I. Recio, A. Clemente, B. Hernández-Ledesma, *Food Funct.* 2015, **6**, 2626-2635.
15 I. Hubatsch, E. G. E. Ragnarsson, P. Artursson, *Nat. Prot.* 2007, **2**, 2111-2119.
16 M. J. García-Nebot, I. Recio, B. Hernández-Ledesma, *Food Chem. Toxicol.* 2014, **65**, 155-161.
17 A. Quirós, A. Dávalos, M. A. Lasunción, M. Ramos, I. Recio, *Int. Dairy J.* 2008, **18**, 279-286.
18 J. J. W. Broeders, J. C. H. van Eijkeren, B. J. Blaauboer, J. L. M. Hermens, *Chem. Res. Toxicol.* 2012, **25**, 1442-1451.
19 M. M. Contreras, A. I. Sancho, I. Recio, C. Mills, *Food Dig.* 2012, **3**, 16-24.
20 S. Wang, P. Liu, S. –M- Lu, Z. –Q. Ling, *Int. J. Biol. Sci.* 2016, **12**, 746-756.
21 V. P. Dia, E. G. de Mejia, *Cancer Lett.* 2010, **295**, 44-53.
22 S. Fernández-Tomé, S. Ramos, I. Cordero-Herrera, I. Recio, L. Goya, B. Hernández-Ledesma, *Food Res. Int.* 2014, **62**, 793-800.
23 K. Ohsawa, H. Satsu, K. Ohki, M. Enjoh, T. Takano, M. Shimizu, *J. Agric. Food Chem.* 2008, **56**, 854-858.
24 L. Ding, Y. Zhang, Y. Jiang, L. Y. Wang, B. Liu, J. B. Liu, *J. Agric. Food Chem.* 2014, **62**, 3177-3182.
25 D. Regazzo, D. Mollé, G. Gabai, D. Tomé, D. Dupont, J. Leonil, R. Boutrou, *Mol. Nutr. Food Res.* 2010, **54**, 1428-1435.
26 S. Howell, A. J. Kenny, A. J. Turner, *Biochem. J.* 1992, **284**, 595-601.
27 R. Mentlein, *Reg. Peptides,* 1999, **85**, 9-24.
28 M. Miguel, A. Dávalos, M. A. Manso, G. de la Peña, M. A. Lasunción, R. López-Fandiño, *Mol. Nutr. Food Res.* 2008, **52**, 1507-1513.
29 L. Ding, L. Y. Wang, Y. Zhang, J. B. *J. Agric. Food Chem.* 2015, **63**, 8143-8150.
30 E. Sienkiewicz-Szlapka, B. Jarmolowska, S. Krawczuk, E. Kostyra, H. Kostyra, K. Bielikowicz, *Int. Dairy J.* 2009, **19**, 252-257.
31 M. Iwan, B. Jarmolowska, K. Bielikowicz, E. Kostyra, H. Kostyra, M. Kaczmarski, *Peptides,* 2008, **29**, 1042-1047.
32 L. Lei, H. Sun, D. Liu, L. Liu, S. Li, *J. Agric. Food Chem.* 2008, **56**, 3582-3586.
33 M. Foltz, A. Cerstiaens, A. van Meensel, R. Mols, P. C. van der Pijl, G. Duchateau, P. Augustijns, *Peptides,* 2008, **29**, 1312-1320.
34 M. Shimizu, D. O. Son, *Curr. Pharm. Des.* 2007, **13**, 885-895.
35 B. S. Vig, T. R. Stouch, J. K. Timoszyk, Y. Quan, D. A. Wall, R. L. Smith, T. N. Faria, *J. Med. Chem.* 2006, **49**, 3636-3644.
36 N. Salamat-Miller, T. P. Johnston, *Int. J. Pharm.* 2005, **294**, 201-216.
37 M. Satake, M. Enjoh, Y. Nakamura, T. Takano, Y. Kawamura, S. Arai, M. Shimizu, M. *Biosci. Biotechnol. Biochem.* 2002, **66**, 378-384.
38 L. Ding, L. Y. Wang, Z. P. Yu, T. Zhang, J. B. Liu, *Int. J. Food Sci. Nutr.* 2016, **67**, 111-116.
39 F. Antunes, F. Andrade, D. Ferreira, H. M. Nielsen, B. Sarmento, *Curr. Drug Metab.* 2013, **14**, 4-20.
40 P. Artursson, J. Karlsson, *Biochem. Biophys. Res. Commun.* 1991, **29**, 880-885.
41 K. C. Cheng, C. Li, A. S. Uss, *Exp. Opin. Drug Metab. Toxicol.* 2008, **4**, 581-590.
42 B. Press, D. Di Grandi, *Curr. Drug Metab.* 2008, **9**, 893-900.
43 G. Picariello, G. Iacomino, G. Mamone, P. Ferranti, O. Fierro, C. Gianfrani, A. Di Luccia, F. Addeo, *Food Chem.* 2013, **139**, 203-212.
44 A. F. Galvez, N. Chen, J. Macasieb, B. O. de Lumen, *Cancer Res.* 2001, **61**, 7473-7478.
45 V. P. Dia, E. G. de Mejia, *Mol. Nutr. Food Res.* 2011, **55**, 623-634.
46 B. Hernández-Ledesma, C. –C. Hsieh, *Crit. Rev. Food Sci. Nutr.* 2015, in press.
47 B. Hernández-Ledesma, C. –C. Hsieh, B. O. de Lumen, *Mol. Nutr. Food Res.* 2011, **55**, 989-998.
48 H. –C. Chang, D. Lewis, C. –Y. Tung, L. Han, S. M. P. Henriquez, L. Voiles, I. P. Lupov, D. Pelloso, A. L. Sinn, K. E. Pollok, B. O. de Lumen, F. Li, J. S. Blum, S. Srivastava, M. J. Robertson, *Cancer Immunol. Immunother.* 2014, **63**, 283-295.
49 E. J. McConnell, B. Devapatla, K. Yaddanapudi, K. R. Davis, *Oncotarget,* 2015, **6**, 4649-4662.
50 A. Cam, M. Sivaguru, E. G. de Mejia, *PLoS ONE,* 2013, **8**, e72115.
51 A. F. Galvez, L. Huang, M. M. J. Magbanua, K. Dawson, R. L. Rodriguez, *Nutr. Cancer,* 2011, **63**, 623-636.

Estudio de los mecanismos celulares protectores del péptido lunasina responsables de su actividad quimiopreventiva.

2.3. Publicación III: **Bioactive peptide lunasin targets colorectal cancer HCT-116 cells and their tumorsphere-derived cancer stem-like cell subpopulation.**

MANUSCRIPT

2.4. Publicación IV: ***In vitro*** **chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells.**

FOOD RESEARCH INTERNATIONAL, 62 (2014) 793-800

Bioactive peptide lunasin targets colorectal cancer HCT-116 cells and their tumorsphere-derived cancer stem-like cell subpopulation

Samuel Fernández-Tomé[a], Fei Xu[b], Blanca Hernández-Ledesma[a], Isidra Recio[a], Hang Xiao[b*]

[a] Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain

[b] Department of Food Science, University of Massachusetts, Amherst, MA, USA

* Corresponding author: H. Xiao

Phone: +01 413-545-2281

e-mail: hangxiao@foodsci.umass.edu

ABSTRACT

The involvement of cancer stem-like cells (CSC) in the tumor pathogenesis has profound implications for cancer therapy and chemoprevention. Lunasin is a bioactive peptide with proved protective activities against cancer and other chronic diseases. The present study focused on the cytotoxic effect of peptide lunasin in colorectal cancer HCT-116 cells, both the bulk tumor and the CSC subpopulations. Lunasin inhibited the proliferation and the tumorsphere-forming capacity of HCT-116 cells. Flow cytometry results demonstrated that the inhibitory effects were related to an induction of apoptosis and cell cycle-arrest at G1 phase. Moreover, lunasin caused an increase in the sub-GO/G1 phase of bulk tumor cells, linked to the apoptotic events found. Inmunoblotting analysis further showed that lunasin induced apoptosis evidenced by activation of caspase-3 and cleavage of PARP, and modestly controlled cell cycle progress by increasing the protein expression of the cyclin-dependent kinase inhibitor p21. Together these results provide new evidence on the chemopreventive activity of peptide lunasin on colorectal cancer by modulating both the parental and the CSC tumorigenic subsets of HCT-116 cells.

KEYWORDS: colorectal cancer, cancer stem cells, chemoprevention, bioactive peptide, lunasin

1. INTRODUCTION

Current statistics on colorectal cancer (CRC) have ranked this disease as the third leading cause of cancer-related deaths and the third most common diagnosed cancer in both men and women in the United States (Siegel and others 2014). In recent years, a great deal of research has been focused on CRC pathogenesis. Meanwhile, the existence of tumor-initiating cells or cancer stem-like cells (CSC) in this solid tumor has been established (O'Brien and others 2007; Ricci-Vitiani and others 2007; Anderson and others 2011). According to the CSC theory, a minor population of tumor cells is responsible for the driving of tumorigenesis (Reya and others 2001). These stem cells, like those in adult tissue, undergo unlimited proliferation and asymmetrically division into more differentiated cells leading to the neoplastic growth and maintenance (Visvader and Lindeman 2008). In addition, it has been suggested that this CSC subpopulation might be potentially responsible for the tumor invasion, metastasis, recurrence, and resistance to therapy (Zhou and others 2009; Clevers 2011). Therefore, the potential of preventive strategies need to be evaluated not only against CRC cells, representing the bulk of the tumor mass (non-CSC), but also against colon CSC.

Accumulating evidence and epidemiological studies have revealed an inverse correlation between soybean consumption and the risk of CRC development (Spector and others 2003; Yang and others 2009), that can be in part due to the chemopreventive effects of the bioactive compounds described in this legume. Several soybean components including isoflavones (Kim and others 2012), saponins (Tsai and others 2010), and bioactive proteins and peptides, such as lectins and protease inhibitors (de Mejia and others 2003; Clemente and others 2010) have been shown to exert protective activities against the growth of CRC cells. Identified in soybean, lunasin is a 43-amino acid peptide which chemopreventive properties have been recently reviewed (Fernández-Tomé and Hernández-Ledesma 2016). It has been demonstrated that lunasin is able to cause cytotoxicity in four different human CRC cell lines, HCT-116, HT-29, KM12L4, and RKO, and their oxaliplatin-resistant variants (Dia and de Mejia 2011a). Studies on the mechanisms of action involved in this antiproliferative activity have been mostly carried out in HT-29 and KM12L4 cells, in

which Dia and de Mejia (2010, 2011a, b) evaluated lunasin´s effects on apoptosis-induction, cell cycle progression and CRC-related biomarkers. Moreover, García-Nebot and others (2014) have demonstrated the protective role played by lunasin in differentiated Caco-2 cells, as a model of human enterocytes, exposed to oxidizing agents through promoting cell viability and counteracting the rise in reactive oxygen species levels. This notably antioxidant protection at intestinal level is also a noteworthy aspect, pointing lunasin as a promising chemopreventive agent against CRC.

The emergence of the CSC model has profound implications on cancer chemoprevention and the search of natural components targeting these cells has been markedly prompted (Kawasaki and others 2008). Some dietary compounds and phytochemicals have been shown to potentially interact toward the pathways involved in the renewal and proliferation of CSC (Li and others 2011; Kim and others 2012; Oh and others 2016). Despite the fact that food proteins and peptides have received increasing attention for their efficacy preventing the different stages of cancer, including initiation, promotion, and progression (de Mejia and Dia 2010; Hernández-Ledesma and Hsieh 2015), their protective role against CSC has been scarcely studied.

Accordingly, this study aimed to evaluate the cytotoxicity of peptide lunasin in human CRC HCT-116 cells, and to investigate its potential chemoprotective activity against colon CSC. Antiproliferative and tumorsphere forming inhibitory activities were evaluated, as well as lunasin´s effects on apoptosis induction, cell cycle progression, and carcinogenesis-related protein biomarkers.

2. MATERIALS AND METHODS

2.1. Materials

Peptide lunasin was synthesized by Chengdu KaiJie Biopharm Co., Ltd. (Chengdu, Sichuan, P. R. China). Its purity (>95%) was confirmed by liquid chromatography (HPLC) coupled to mass spectrometry (HPLC-MS).

2.2. Cell lines

The human CRC cell line HCT-116 was obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and maintained in RPMI medium (ATCC) supplemented with 5% heat inactivated fetal bovine serum (FBS; Mediatech, Herndon, VA, USA), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 ºC, kept sub-confluent, and medium was changed every other day. All cells were assayed within 5-25 passages. Enrichment culture of tumor-derived colon CSC was performed by incubating parental HCT-116 cells in serum-free medium (SFM) composed of DMEM/F-12 medium supplemented with 2% B-27 supplement, 20 ng/mL recombinant human epidermal growth factor, 10 ng/mL fibroblast growth factor-basic (Life Technologies, Grand Island, NY, USA), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 10 µg/mL insulin (Sigma-Aldrich) in ultra low-attachment plates (Corning, Lowell, MA, USA) at 37 ºC. Plated under these anchorage-independent conditions in supplemented-SFM, tumor cells form floating spheres reported to represent the growth of CSC (Kanwar and others 2010; O'Brien and others 2012).

2.3. Cell proliferation assay

HCT-116 cells were seeded in 96-well plates (11×10^3 cells/mL). After 24 h incubation, cells were treated with different concentrations of lunasin ranging from 5 to 80 µM. After 72 h treatment, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Treatment medium was replaced by 200 µL of fresh medium containing 0.5 mg/mL MTT (Sigma-Aldrich). After 1 h incubation at 37 ºC, MTT-containing medium was removed and the reduced formazan dye was solubilized by adding 100 µL of dimethyl sulfoxide to each well. After gently mixing, the absorbance was read at 570 nm using a microplate reader (Elx800TM, BioTek Instrument, Winooski, VT, USA). The results were expressed as percentage of the control, considered as 100%. Experiments were carried out in triplicate with at least three replicates per concentration.

2.4. Tumorsphere formation assay

To examine the effect of lunasin on the formation of tumorspheres, HCT-116 cells were grown in SFM and plated as single cells in ultra low-attachment 24-well plates (6×10^3 cells/mL). Right after seeding, cells were treated with different concentrations of lunasin ranging from 5 to 80 μM, and incubated at 37 ºC for 10 days. After that time, tumorspheres were formed and transferred to 6-well dishes in differentiating medium (RPMI supplemented with 5% FBS and 1% antibiotics). Under these conditions, tumorspheres were adhered after 24 h incubation. Then, cells were stained with crystal violet solution (0.2% crystal violet in 2% ethanol) for 20 min at room temperature, photographed and counted. Results were presented as percentage of tumorspheres forming cells compared to control, considered as 100%. Analyses were performed in triplicate with at least three replicates per concentration.

2.5. Detection of apoptosis

Apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining using an apoptotic detection kit (BioVision, Mountain View, CA, USA) according to manufacturer's instruction, followed by flow cytometry. HCT-116 cells (4×10^4 cells/mL) and colon CSC (3×10^3 cells/mL) were seeded in 6-well plates and treated (20-80 μM lunasin) as described above. After 72 h treatment, HCT-116 cells were collected as described by Qiu and others (2010). In the case of colon CSC, after 7 days treatment, floating tumorspheres in medium were collected in ice-cold flow cytometry tubes. After centrifugation (2000 × *g*, 2 min), single-cell suspensions were generated by incubation with 0.5 mL trypsin (0.25% trypsin-ethylenediaminetetraacetic acid, Sigma-Aldrich) and 1 mL medium for 5 min at 37 ºC, and gentle pippeting. Afterwards, in both cell cultures, cell suspensions were centrifuged (2000 × *g*, 2 min) and washed twice with 0.5 mL ice-cold phosphate buffer saline (PBS). Then, cells were suspended in 0.3 mL binding buffer containing Annexin V and PI, and incubated for 15 min at room temperature in the dark. Apoptotic cells were identified using a BD LSR II cell analyzer (BD Biosciences, San Jose, CA, USA) as Annexin V-positive cells, being

Annexin V-positive/PI-negative and Annexin V-positive/PI-positive cells identified as early apoptotic and late apoptotic cells, respectively. At least 10000 events were recorded to assess the percentage of apoptotic cells. Analyses were performed in duplicate with at least three replicates per concentration, and results were presented as the increased number in apoptotic cell populations, compared to control cells.

2.6. Cell cycle analyses

HCT-116 cells and colon CSC were treated as described for the apoptosis detection assay. After 72 h treatment, HCT-116 cells were collected as described by Qiu and others (2010). In the case of colon CSC, after 7 days treatment, cells were collected as described for apoptosis detection assay with some modifications. Briefly, floating tumorspheres in medium were collected, centrifuged, and single-cell suspensions were generated, washed with ice-cold PBS, and then fixed in 1 mL of 70% ethanol and kept at -20 ºC overnight. After centrifugation (2000 × *g*, 2 min), cells were washed with 0.5 mL PBS, and incubated with 0.3 mL PBS solution containing RNAse (10%; Sigma-Aldrich) and PI (1%; BioVision) for 25 min at room temperature in the dark. Cell cycle distribution was analyzed with at least 8000 events recorded using a BD LSR II cell analyzer (BD Biosciences), and data were processed using ModFit LT software. Analyses were performed in duplicate with at least three replicates per concentration, and results were presented as percentage of cells in G1, S, and G2-phases.

2.7. Immunoblotting

HCT-116 cells (3.5×10^4 cells/mL) were seeded in 10-cm cell culture dishes. Colon CSC were seeded exactly same as described for apoptosis assay. After 72 h treatment (20-80 μM lunasin), HCT-116 cells were collected and whole-cell lysates were prepared as previously described (Xiao and others 2009). In the case of colon CSC, after 7 days-treatment (20-80 μM

lunasin), cells were collected following the same procedure with some modifications. Briefly, floating tumorspheres in medium were collected, centrifuged, and washed with ice-cold PBS. Then, cells were incubated on ice for 30 min in RIPA lysis buffer containing a protease inhibitor cocktail (Boston BioProducts, Ashland, MA, USA), and processed as previously described (Xiao and others 2009). Supernatants were collected and protein content was quantified by the bicinchoninic acid method (Pierce, Rockford, IL, USA) using bovine serum albumin as standard protein. Equal amount of proteins (50-70 µg) were resolved over 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were incubated with different monoclonal primary antibodies overnight at 4 ºC, according to manufacturer's instructions. Primary antibodies for cleaved caspase-3 (Asp175), full-length PARP, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ were from Cell Signaling Technology (Beverly, MA, USA). Antibody for β-actin was from Sigma-Aldrich. After 1 h incubation with the appropriate secondary antibodies [goat anti-mouse IgG, and goat anti-rabbit IgG IRDye (LI-COR Biosciences, Lincoln, NE, USA)], proteins of interest were visualized using enhanced chemiluminescence (Boston Bioproducts). Analyses were conducted for three independent replicates.

2.8. Statistical analysis

Data were evaluated using one-way ANOVA followed by Bonferroni post hoc test, and expressed as the mean ± standard variation (SD) of the different experiments carried out. GraphPad Prism 5.0 software (San Diego, CA, USA) was used to perform statistical analyses. Differences with a P value < 0.05 (*), P value < 0.01 (**) or P value < 0.001 (***) were considered significant.

3. RESULTS AND DISCUSSION

3.1. Inhibitory effect of lunasin on cell viability and tumorsphere formation

A growing amount of evidence asserts that epithelial cancers, including CRC are driven by a small subset of self-renewing CSC different from the cellular bulk of the tumor (Anderson and others 2011). In this study, the effect of lunasin was thus assessed against both non-CSC and CSC populations. As shown in Figure 1, the human HCT-116 cell line was grown in monolayer as parental CRC cells (Figure 1A) and used for the expansion of tumor-derived CSC as colonspheres (Figure 1B).

We first examined the growth of HCT-116 cells exposed to lunasin. HCT-116 cells were treated with serial concentrations of lunasin (5-80 µM) for 72 h and the number of viable cells was assessed by the MTT assay. As shown in Figure 1C, lunasin caused a dose-dependent inhibition of proliferation. Treatment with 10 µM lunasin was able to induce a significant reduction on cellular growth (12.9%, $P < 0.01$) compared to control cells. The cytotoxic effect increased up to the highest concentration used (37.3%, $P < 0.001$). However, to calculate the IC_{50} value, expressed as the peptide concentration needed to inhibit 50% cell number, an additional experiment was carried out with lunasin at 160 µM. This concentration showed a 64.1% inhibition ($P < 0.001$), being the IC_{50} value of 107.5 µM. It had been previously demonstrated that lunasin is able to induce cytotoxicity to HCT-116, HT-29, KM12L4, and RKO CRC cells, with IC_{50} values of 26.3 µM, 61.7 µM, 13.0 µM, and 21.6 µM, respectively (Dia and de Mejia 2011a). While these authors used purified lunasin (~ 90%) from defatted soybean flour, in our study we have assessed the effects of synthetic lunasin. The higher IC_{50} value found in our study might be due to differences in the secondary and tertiary structures between plant-purified lunasin and the synthetic peptide. Additionally, other compounds present in the natural preparation could be responsible for the observed change in the inhibitory potency. In this regard, synthetic lunasin has been shown to suppress the growth of breast cancer MDA-MB-231 cells with a reported IC_{50} value of 181.0 µM (Hernández-Ledesma and others 2011).

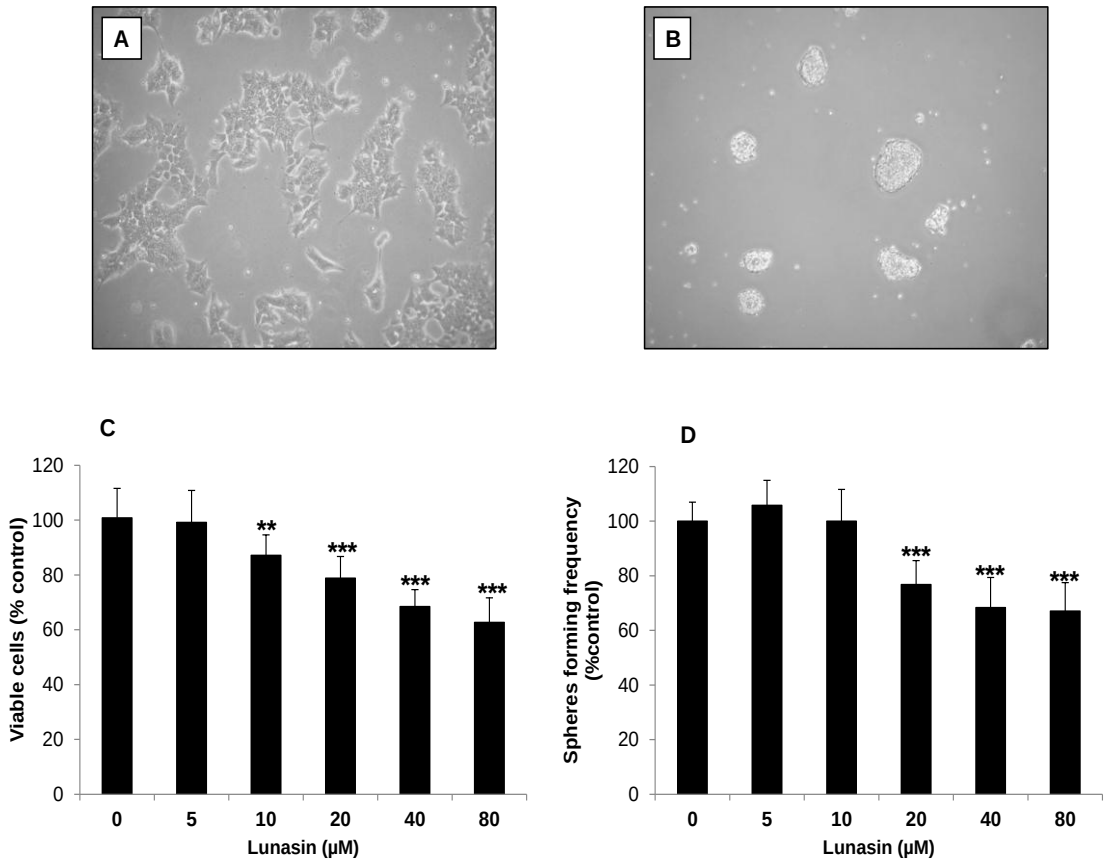


Figure 1. Cell culture and cytotoxic effect of lunasin on colorectal cancer (CRC) cells. Representative images of (A) HCT-116 cells in adherent conditions and (B) enrichment culture of tumor-derived colon stem-like cells (CSC) formed from the parental HCT-116 cell line under anchorage-independent conditions. (C) HCT-116 cells were treated with lunasin (5-80 μM) for 72 h, and cell viability was determined by the MTT assay. (D) Colon CSC were treated with lunasin (5-80 μM) for 10 days, and tumorspheres were stained with crystal violet solution and counted. Results, expressed as percentage of control cells, are means ± SD of the replicates of experiments carried out. ** ($P < 0.01$), *** ($P < 0.001$) significantly different from control.

Since colonsphere subpopulations were demonstrated to exert a key role in the CRC pathogenesis, the culture of tumor-derived spheroids has been widely used for the evaluation of chemotherapy drugs and chemopreventive agents (Anderson and others 2011). The sphere formation assay is extensively applied as *in vitro* method for the derivation and characterization of stem-like cancer cells with intrinsic self-renewal and tumorigenic properties (Kanwar and others 2010). To evaluate whether lunasin protects against colon CSC, we first determined its effect on the

proliferation of colonspheres. Colon CSC were cultured as non-adherent spheres under anchorange-independent conditions and treated with lunasin for 10 days. As shown in Figure 1D, lunasin at the lowest range assessed (5-10 μM) was not able to suppress tumorsphere-forming capacity. Likewise, Pabona and others (2013) have showed that while isoflavone genistein (40 nM) reduces the number of mammosphere-forming units in malignant breast cancer MCF-7 cells, peptide lunasin (2 μM) is not able to recapitulate this inhibitory protection. However, as represented in Figure 1D, this peptide in the range of 20-80 μM exerted an inhibitory effect ca. 30%. Lunasin´s IC_{50} value on HCT-116-derived CSC was 161.0 μM, indicating that colonspheres are less sensitive to this peptide than parental HCT-116 cells. These results are in agreement with the reported higher resistance of CSC to anti-cancer therapies (Zhou and others 2009; Kim 2014). Similarly, Yang and others (2013) have recently showed that fatty acid DHA exerts higher antiproliferative potency on adherent CRC SW620 cells than on their tumorspheres-derived CSC subpopulation. Nevertheless, in the study of McConnell and others (2015), it has been recently found that peptide lunasin presents a higher anti-proliferative activity against non-small cell lung cancer cells when they were assessed under anchorage-independent growth conditions, compared to anchorage-dependent conditions. Therefore, lunasin´s modulatory chemoprevention might notably depend on the culture conditions and the cell line used.

3.2. Apoptosis analysis of lunasin-treated CRC cells

Tumor cell populations expand in number through several molecular processes such as the capability of evading programmed cell death by presenting an elevated apoptotic threshold (Hanahan and Weinberg 2011). In order to determine whether the inhibitory effect of lunasin on HCT-116 cells was through interacting with the apoptotic pathways, adherent and colonspheres-derived cells were incubated with lunasin, and apoptosis detection was assessed by flow cytometry-based Annexin V/PI assay. Based on the lunasin´s inhibitory effects on HCT-116 cell viability and

colonsphere forming-frequency, the range of 20-80 µM for this peptide was chosen as the optimal treatment concentration for subsequent experiments.

Figure 2 presents the apoptotic state of adherent HCT-116 cells after control- or lunasin-treatment for 72 h. The apoptotic populations of cells treated with the peptide were significantly increased with a dose-dependent trend (Figure 2A). Lunasin at 20, 40 and 80 µM induced a 1.3-fold, 1.7-fold and 1.8-fold increase of total apoptotic cells, respectively, compared to controls. In the case of lunasin at 40 and 80 µM, both the late and the early apoptotic populations were also significantly enhanced. These findings might be explained by a weak apoptosis-induction effect in 20 µM cells, corresponding to the lower MTT activity found at this dose. The apoptosis-involved inhibitory role of lunasin against CRC HCT-116 cells was further addressed by the immunoblotting study of the molecular proteins PARP and caspase-3. PARP is responsible for the regulation of many cellular functions, such as key events supporting cell viability and DNA repair (Satoh and Lindahl 1992). PARP degradation has been shown to facilitate cellular disassembly and serve as a marker of cells undergoing apoptosis, with this protein being the main cleavage target on the activity of the apoptotic trigger caspase-3 (Oliver and others 1998). As shown in Figure 2B, lunasin activated the cleavage of caspase-3 up to 190% (40 µM), a hallmark of apoptosis, and consequently decreased the protein level of full-length PARP by 30% (40 µM) and 60% (80 µM). In this line, Dia and de Mejia (2010, 2011a) found that lunasin was able to activate the apoptotic mitochondrial pathway in CRC HT-29 and KM12L4 cells, as evidenced by the modulation of Bcl-2/Bax family of proteins, nuclear clusterin, cytochrome c, and caspases-activity. Similar apoptosis-related properties have been reported for this peptide against the growth of leukemia L1210 cells (de Mejia and others 2010), and breast cancer MCF-7 (Pabona and others 2013) and MDA-MB-231 (Hsieh and others 2011) cells.

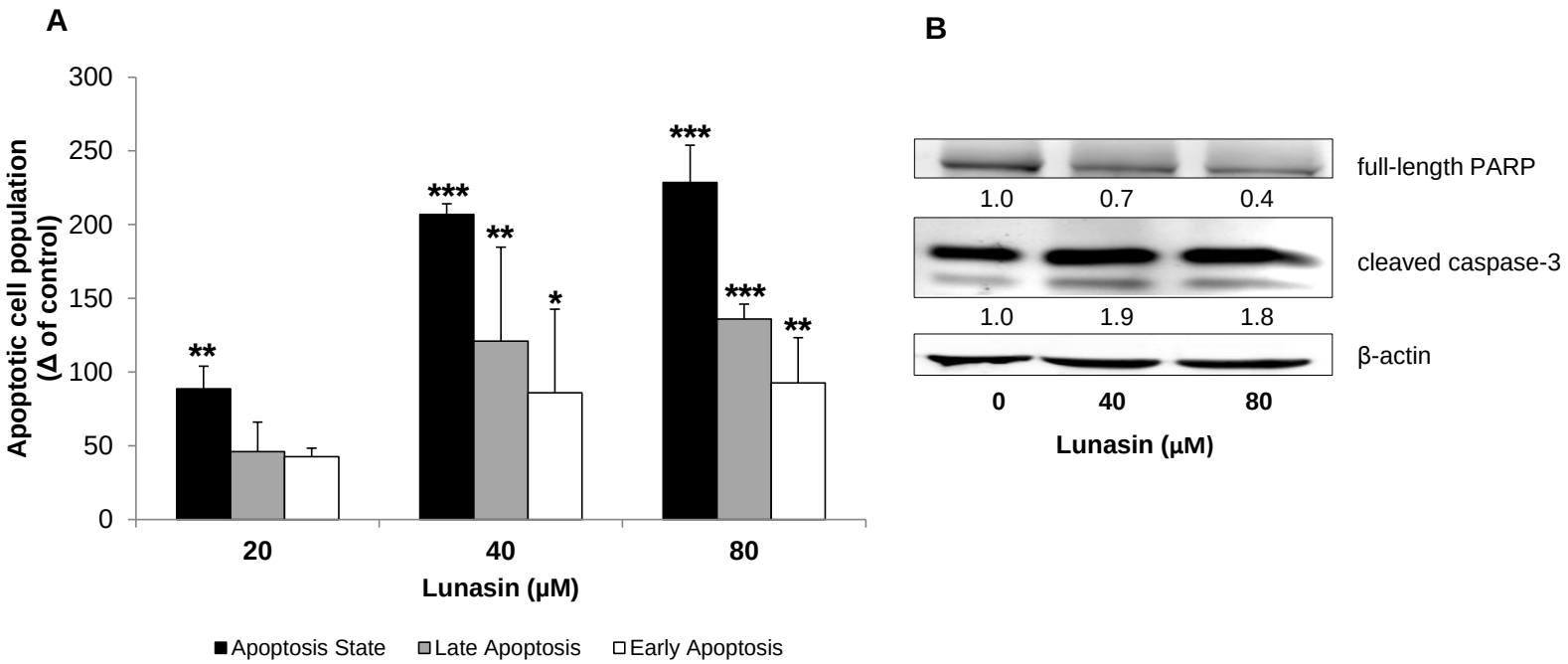


Figure 2. Effect of lunasin on the apoptosis state of HCT-116 cells. Cells were treated with lunasin at the indicated concentrations for 72 h, and harvested for apoptosis analyses and Western immunoblotting. (A) Flow cytometry-based Annexin V/PI double labelling of apoptotic cells. Apoptotic cells were identified as Annexin V-positive cells, being Annexin V-positive/PI-negative and Annexin V-positive/PI-positive cells identified as early apoptotic and late apoptotic cells, respectively. Results, presented as the increased number in apoptotic cell populations compared to control cells, are means ± SD of the replicates of experiments carried out. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) significantly different from control. (B) Expression of full length PARP and cleaved caspase-3 proteins determined by Western Blot. The numbers underneath of the blots represent band intensity (normalized to β-actin), measured by Image J software. β-actin was served as an equal loading control. The immunoblots are shown as representative images of different experiments with similar results.

We next aimed to determine whether this apoptosis-inducing property was also involved in the suppression of the spheroid-forming capacity of HCT-116 cells. Colonspheres were treated with lunasin for 7 days and apoptosis detection was examined as shown in Figure 3. Results from the flow cytometry study after staining of colon CSC with Annexin-V/PI showed that lunasin led to an induction in the cellular apoptotic state (Figure 3A and 3B). The raise in the number of apoptotic cells was not significantly promoted at lunasin 20 µM, however lunasin at 40 and 80 µM exerted a 2.0-fold apoptosis-induction effect, mostly in the late apoptotic cellular subset. As shown in Figure 3C, the implication of an apoptosis mechanism responsible for the inhibitory effect of lunasin peptide against the expansion of the HCT-116-derived CSC subpopulation was further demonstrated by immunobloting. Cleaved caspase-3 activity was induced by lunasin treatment, with this activation being markedly companied by a down-regulation of PARP level in a dose-dependent manner. Therefore, in the present study, it has been suggested that lunasin has similar effects in the apoptosis-induction of both CRC HCT-116 parental and tumor-derived colonsphere cells. In this sense, other food/natural compounds and phytochemicals have been demonstrated to exert similar inhibitory effects against the cellular expansion of the CSC subpopulation of CRC (Lin and others 2011; Yang and others 2013; Massey and others 2014), and pancreatic and prostate cancer cells (Ottinger and others 2012; Soner and others 2014) by induction of the apoptotic pathways.

3.3. Effect of lunasin on cell cycle progression of CRC cells

To provide further insights into the growth inhibitory effects exerted by lunasin in HCT-116 cells, analyses on cell cycle distribution were performed on both parental and colonspheres stem cells after treatment with lunasin for 72 h and 7 days, respectively. Deregulation of cell cycle control and potential to replicate without limit are one of the hallmarks of cancer, with all these events being highly regulated by internal checkpoints that ensure the proper cellular division (Hanahan and Weinberg 2011).

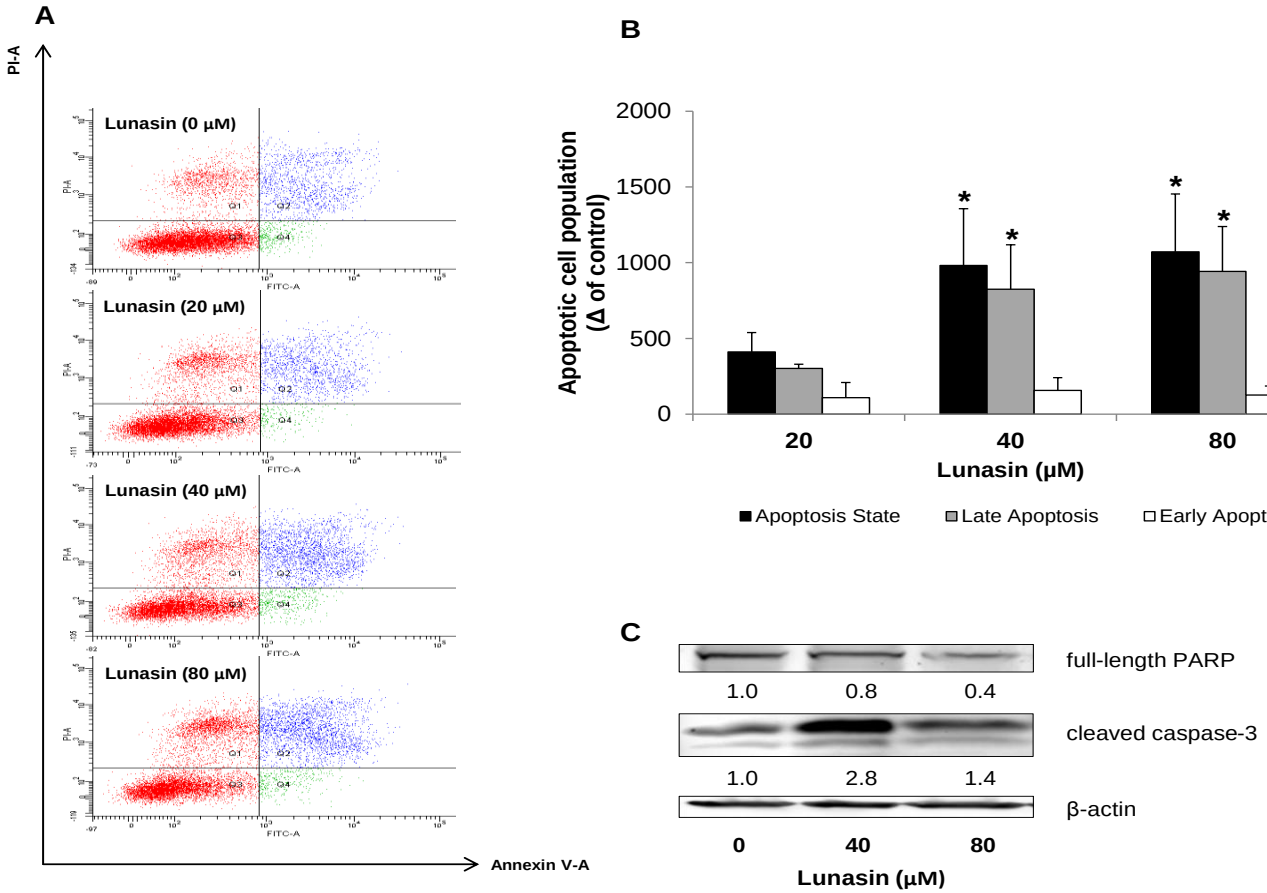


Figure 3. Effect of lunasin on the apoptosis state of colon CSC. Cells were treated with lunasin at the indicated concentrations for 7 days, and harvested for apoptosis analyses and Western immunoblotting. (A) Flow cytometry-based Annexin V/PI double labelling of apoptotic cells. (B) Apoptotic cells were identified as Annexin V-positive cells, being Annexin V-positive/PI-negative and Annexin V-positive/PI-positive cells identified as early apoptotic and late apoptotic cells, respectively. Results, presented as the increased number in apoptotic cell populations compared to control cells, are means ± SD of the replicates of experiments carried out. * ($P < 0.05$) significantly different from control. (C) Expression of full length PARP and cleaved caspase-3 proteins determined by Western Blot. The numbers underneath of the blots represent band intensity (normalized to β-actin), measured by Image J software. β-actin was served as an equal loading control. The immunoblots are shown as representative images of different experiments with similar results.

As shown in Figure 4A, control adherent HCT-116 cells (G1: 66.5 ± 1.7%) were found to significantly increase their G1-cellular phase after lunasin´s treatment (20 µM lunasin: 70.2 ± 0.3%, $P < 0.05$; 40 µM lunasin: 70.5 ± 0.7%, $P < 0.05$; 80 µM lunasin: 72.0 ± 1.2%, $P < 0.01$). Interestingly, as represented in Figure 4B, lunasin-treated cells also resulted in a marked accumulation of the sub-G0/G1 cell population, compared to control cells. Cells at the sub-G0/G1 fraction contain less amount of DNA than G1 cells, suggesting DNA degradation potentially caused by apoptotic events (Nagata and others 2003). This effect had also been demonstrated for peptide lunasin in leukemia L1210 cells (de Mejia and others 2010), and is in agreement with our results on apoptosis-induction in HCT-116 cells (Figure 2). On the other hand, our findings differ with other studies showing the capability of this peptide to arrest cell cycle at S-phase in breast cancer MDA-MB-231 cells (Hsieh and others 2011), and at G2-phase in leukemia L1210 cells (de Mejia and others 2010) and CRC HT-29 and KM12L4 cells (Dia and de Mejia 2010, 2011a). However, other arginine-glycine-aspartic acid (RGD-motif)-containing peptides, such as lunasin, have been also reported to result in a G0/G1-phase arrest in cancer cells (Yang and others 2006). Moreover, regarding to the colon CSC subpopulation (Figure 5A), lunasin at 80 µM also led to an enhancement of G1-arrest (74.0 ± 0.6%, $P < 0.001$), accompanied with a reduction in the S-cellular subset (14.3 ± 0.9%, $P < 0.05$), compared to control cells (G1: 69.4 ± 0.6%; S: 16.4 ± 0.9%). This effect might be related to the antiproliferative and pro-apoptotic activities above indicated, however 20-40 µM-treated colonspheres showed a similar trend but in a weaker manner lacking of statistical significance.

To further explain lunasin´s effect on cell cycle progression, evaluation of the expression of the cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ was performed by Western Blot. While treatment of CRC cells with lunasin showed no effect on the level of p27 marker (data not shown), it resulted in a modest increase in the molecular expression of p21 protein up to 140% and 120% in adherent HCT-116 cells (Figure 4C) and colonspheres CSC (Figure 5B), respectively. Dia and de Mejia (2010, 2011a) also reported lunasin´s capability to induce the expression of the CDK- inhibitor p21 in the CRC HT-29 and KM12L4 cells, and linked this effect with a decreased cell

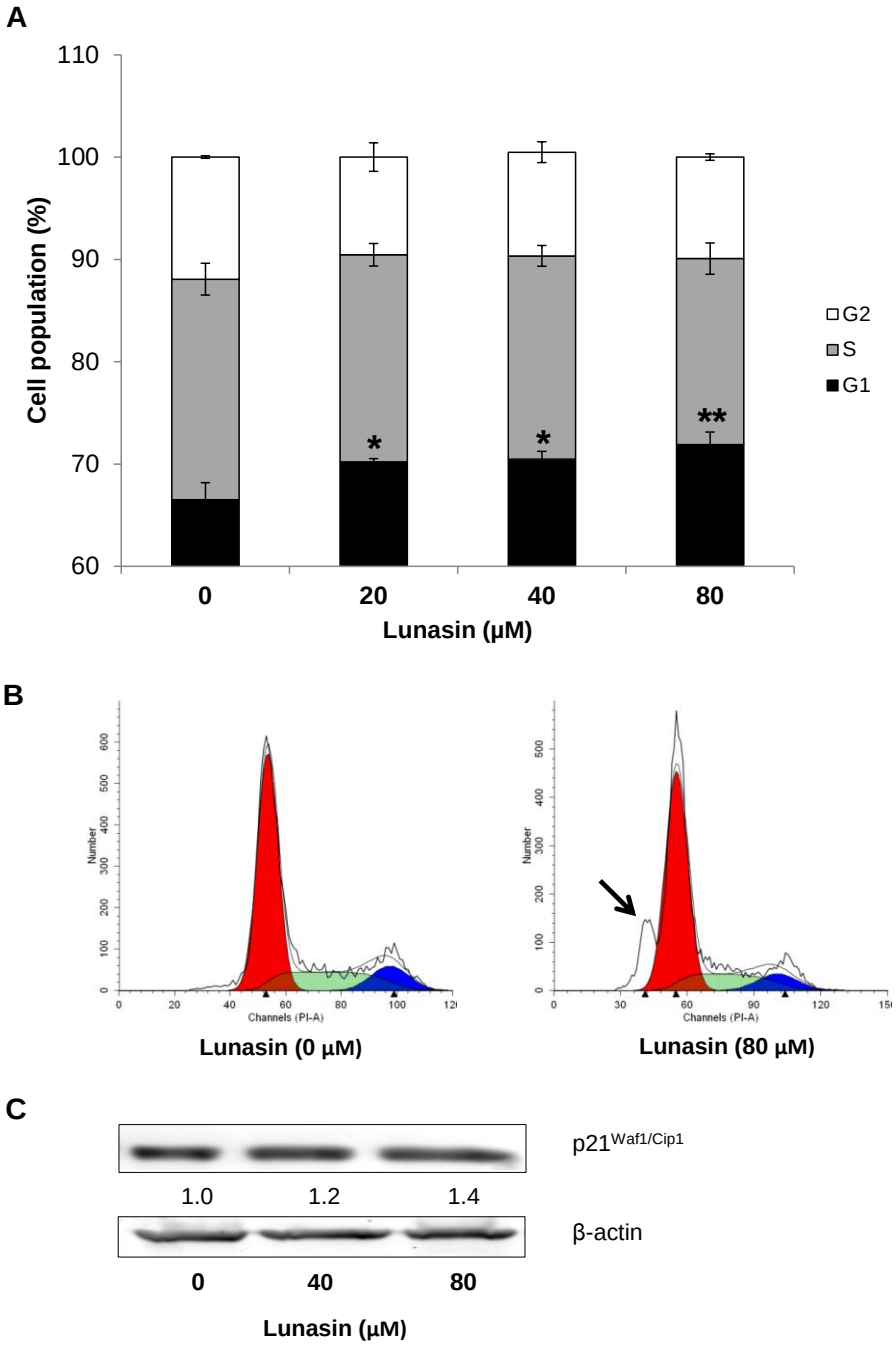


Figure 4. Effect of lunasin on cell cycle progression of HCT-116 cells. Cells were treated with lunasin at the indicated concentrations for 72 h, and harvested for cell cycle analyses and Western immunoblotting. (A) Cell cycle distribution was assessed by flow cytometry using PI staining. Results, presented as percentage of cells in G1, S, and G2 phases, are means ± SD of the replicates of experiments carried out. * ($P < 0.05$), ** ($P < 0.01$) significantly different from control. (B) Representative images of lunasin-induced increase in the sub-GO/G1 cell population (black arrow). (C) Expression of $p21^{Waf1/Cip1}$ protein determined by Western Blot. The numbers underneath of the blots represent band intensity (normalized to β-actin), measured by Image J software. β-actin was served as an equal loading control. The immunoblots are shown as representative images of different experiments with similar results.

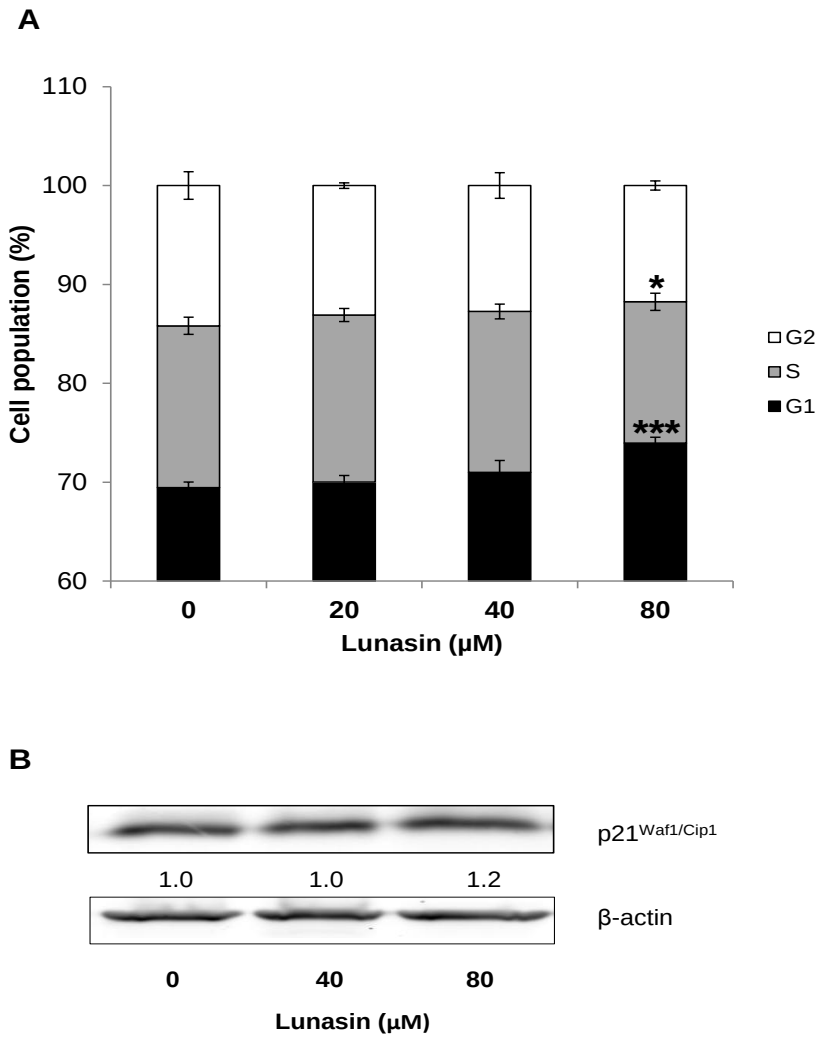


Figure 5. Effect of lunasin on cell cycle progression of colon CSC. Cells were treated with lunasin at the indicated concentrations for 7 days, and harvested for cell cycle analyses and Western immunoblotting. (A) Cell cycle distribution was assessed by flow cytometry using PI staining. Results, presented as percentage of cells in G1, S, and G2 phases, are means ± SD of the replicates of experiments carried out. * ($P < 0.05$), *** ($P < 0.001$) significantly different from control. (B) Expression of $p21^{Waf1/Cip1}$ protein determined by Western Blot. The numbers underneath of the blots represent band intensity (normalized to β-actin), measured by Image J software. β-actin was served as an equal loading control. The immunoblots are shown as representative images of different experiments with similar results.

proliferation, cell cycle arrest, and up-regulation of the pro-apoptotic markers caspase-3 and nuclear clusterin isoform.

In order to provide more evidence on the cancer-preventive role of bioactive peptide lunasin against the CRC malignancy some studies have been carried out. In the highly metastatic KM12L4

cell line, Dia and de Mejia (2011a, b) demonstrated that lunasin is able to internalize into the cell and sit within the nucleus, to modify the expression of human extracellular matrix and cell adhesion genes by binding to $\alpha_5\beta_1$ integrin, and also to inhibit the FAK/ERK/NF-κB signaling pathway. The *in vivo* effect of this peptide was suggested in the CRC liver metastasis mice model by Dia and de Mejia (2013), although disagreements between intraperitoneally- and orally-administered findings made it hard to establish a definitive lunasin´s role on preventing the CRC liver metastasis. Additionally, several studies have already demonstrated the chemopreventive properties of lunasin peptide by modulating expression of different proteins and genes involved in signaling pathways related to the complex pathogenesis of tumor cells (Fernández-Tomé and Hernández-Ledesma 2016).

4. CONCLUSION

In the present study, our cellular model allowed us accomplishing the ideal approach for the evaluation of cancer-preventive agents by targeting both the parental and the stem-like tumorigenic populations. The protective mechanisms on lunasin-treated cells can be postulated in terms of inhibition of cell growth and tumorsphere-forming activity, induction of apoptosis, and regulation of cell cycle progression. The recent CSC hypothesis has supposed a challenge on the search of chemotherapeutic agents that efficiently target fast diving cancer cells as well as CSC responsible for the growth and maintenance of the tumorigenic bulk mass. The protective potential of bioactive peptides against the CSC subpopulation deserves additional studies. The promising results of this work clearly need to be further addressed to elucidate the molecular basis of the chemopreventive activity of this peptide on CSC, to study its potential on stem-related markers and signaling pathways, such as Wnt/β-catenin, Hedgehog and Notch, and especially to confirm this role by using *in vivo* models of CSC self-renewal.

ACKNOWLEDGMENTS

This work has received financial support from project AGL2015-66886-R from the Spanish Ministry of Economy and Competitiveness (MINECO). S. F. -T. and B. H. -L. acknowledge MINECO for their FPI fellowship (AGL2011-24643) and "Ramón y Cajal" post-doctoral contract, respectively.

REFERENCES

Anderson EC, Hessman C, Levin TG, Monroe MM, Wong MH. 2011. The role of colorectal cancer stem cells in metastatic disease and therapeutic response. Cancers 3:319-39.

Clemente A, Moreno FJ, Marín-Manzano MC, Jiménez E, Domoney C. 2010. The cytotoxic effect of Bowman-Birk isoinhibitors, IBB1 and IBBD2, from soybean (*Glycine max*) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases. Mol Nutr Food Res 54:396-405.

Clevers H. 2011. The cancer stem cell: premises, promises and challenges. Nat Med 17:313-19.

de Mejia EG, Bradford T, Hasler C. 2003. The anticarcinogenic potential of soybean lectin and lunasin. Nutr Rev 61:239-46.

de Mejia EG, Dia VP. 2010. The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. Cancer Metast Rev 29:511-28.

de Mejia EG, Wang W, Dia VP. 2010. Lunasin, with an arginine-glycine-aspartic acid motif, causes apoptosis to L1210 leukemia cells by activation of caspase-3. Mol Nutr Food Res 54:1-9.

Dia VP, de Mejia EG. 2010. Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. Cancer Lett 295:44-53.

Dia VP, de Mejia EG. 2011a. Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. Mol Nutr Food Res 55:623-34.

Dia VP, de Mejia EG. 2011b. Lunasin potentiates the effect of oxaliplatin preventing outgrowth of colon cancer metastasis, binds to $\alpha_5\beta_1$ integrin and suppresses FAK/ERK/NF-κB signaling. Cancer Lett 313:167-80.

Dia VP, de Mejia EG. 2013. Potential of lunasin orally-administered in comparison to intraperitoneal injection to inhibit colon cancer metastasis *in vivo*. J Cancer Ther 4:34-43.

Fernández-Tomé S, Hernández-Ledesma B. 2016. An update on lunasin research, a bioactive seed peptide for health promotion. In: Govil JN, editor. Recent Progress in Medicinal Plants. Vol. 43 Phytotherapeutics II, Studium Press LLC, New Dehli, India. p 331-352.

García-Nebot MJ, Recio I, Hernández-Ledesma B. 2014. Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. Food Chem Toxicol 65:155-61.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. Cell 144:646-74.

Hernández-Ledesma B, Hsieh CC, de Lumen BO. 2011. Relationship between lunasin´s sequence and its inhibitory activity of histones H3 and H4 acetylation. Mol Nutr Food Res 55:989-98.

Hernández-Ledesma B, Hsieh CC. 2015. Chemopreventive role of food-derived proteins and peptides: A review. Crit Rev Food Sci. In press. DOI: 10.1080/10408398.2015.1057632

Hsieh CC, Hernández-Ledesma B, de Lumen BO. 2011. Cell proliferation inhibitory and apoptosis-inducing properties of anacardic acid and lunasin in human breast cancer MDA-MB-231 cells. Food Chem 125:630-6.

Kanwar SS, Yu YJ, Nautiyal J, Patel BB, Majumdar APN. 2010. The Wnt/beta-catenin pathway regulates growth and maintenance of colonospheres. Mol Cancer 9:212.

Kawasaki BT, Hurt EM, Mistree T, Farrar WL. 2008. Targeting cancer stem cells with phytochemicals. Mol Interv 8:174-84.

Kim GN, Song JH, Kim ES, Choi HT, Jang HD. 2012. Isoflavone content and apoptotic effect in HT-29 cancer cells of a soy germ extract. Food Chem 130:404-7.

Kim TI. 2014. Chemopreventive drugs: Mechanisms via inhibition of cancer stem cells in colorectal cancer. World J Gastroentero 20:3835-46.

Kim YS, Farrar W, Colburn NH, Milner JA. 2012. Cancer stem cells: potential target for bioactive food components. J Nutr Biochem 23:691-8.

Li Y, Wicha MS, Schwartz SJ, Sun D. 2011. Implications of cancer stem cell theory for cancer chemoprevention by natural dietary compounds. J Nutr Biochem 22:799-806.

Lin L, Liu Y, Li H, Li PK, Fuchs J, Shibata H, Iwabuchi Y, Lin J. 2011. Targeting colon cancer stem cells using a new curcumin analogue, GO-Y030. Brit J Cancer 105:212-20.

Massey AR, Reddivari L, Vanamala J. 2014. The dermal layer of sweet sorghum (*Shorgum bicolor*) stalk, a byproduct of biofuel production and source of unique 3-deoxyanthocyanidins, has more antiproliferative and proapoptotic activity than the pith in p53 variants of HCT116 and colon cancer stem cells. J Agr Food Chem 62:3150-9.

McConnell EJ, Devapatla B, Yaddanapudi K, Davis KR. 2015. The soybean-derived peptide lunasin inhibits non-small cell lung cancer cell proliferation by suppressing phosphorylation of the retinoblastoma protein. Oncotarget 6:4649-62.

Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H. 2003. Degradation of chromosomal DNA during apoptosis. Cell Death Differ 10:108-16.

O'Brien CA, Kreso A, Ryan P, Hermans KG, Gibson L, Wang YD, Tsatsanis A, Gallinger S, Dick JE. 2012. ID1 and ID3 regulate the self-renewal capacity of human colon cancer-initiating cells through p21. Cancer Cell 21:777-92.

O'Brien CA, Pollett A, Gallinger S, Dick JE. 2007. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. Nature 445:106-10.

Oh J, Hlatky L, Jeong YS, Kim D. 2016. Therapeutic effectiveness of anticancer phytochemicals on cancer stem cells. Toxins 8:199.

Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G, Menissier-de Murcia J. 1998. Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis - Lesson from an uncleavable mutant. J Biol Chem 273:33533–9.

Ottinger S, Klöppel A, Rausch V, Liu L, Kallifatidis G, Gross W, Gebhard MM, Brümmer F, Herr I. 2012. Targeting of pancreatic and prostatic cancer stem cell characteristics by *Crambe crambe* marine sponge extract. Int J Cancer, 130:1671-81.

Pabona JMP, Dave B, Su Y, Montales MTE, de Lumen BO, de Mejia EG, Rahal OM, Simmen RCM. 2013. The soybean peptide lunasin promotes apoptosis of mammary epithelial cells via induction of tumor suppressor PTEN: similarities and distinct actions from soy isoflavone genistein. Genes Nutr 8:79-90.

Qiu P, Dong P, Guan H, Li S, Ho CT, Pan MH, McClements DJ, Xiao H. 2010. Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells. Mol Nutr Food Res 54:S244-52.

Reya T, Morrison SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer, and cancer stem cells. Nature 414:105-11.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445:111-5.

Satoh MS, Lindahl T. 1992. Role of poly(ADP-ribose) formation in DNA repair. Nature 356:356-8.

Siegel R, DeSantis C, Jemal A. 2014. Colorectal cancer statistics, 2014. CA-A Cancer J Clin 64:104-17.

Soner BC, Aktug H, Acikgoz E, Duzagac F, Guven U, Ayla S, Cal G, Oktem G. 2014. Induced growth inhibition, cell cycle arrest and apoptosis in $CD133^{+}/CD44^{+}$ prostate cancer stem cells by flavopiridol. Int J Mol Med 34:1249-56.

Spector D, Anthony M, Alexander D, Arab L. 2003. Soy consumption and colorectal cancer. Nutr Cancer 47:1-12.

Tsai CY, Chen YH, Chien YW, Huang WH, Lin SH. 2010. Effect of soy saponin on the growth of human colon cancer cells. World J Gastroentero 16:3371-6.

Visvader JE, Lindeman GJ. 2008. Cancer stem cells in solid tumors: accumulating evidence and unresolved questions. Nat Rev Cancer 8:755-68.

Xiao H, Yang CS, Li S, Jin H, Ho CT, Patel T. 2009. Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. Mol Nutr Food Res 53:398-406.

Yang G, Shu XO, Li HL, Chow WH, Cai H, Zhang XL, Gao YT, Zheng W. 2009. Prospective cohort study of soy food intake and colorectal cancer risk in women. Am J Clin Nutr 89:577-83.

Yang T, Fang S, Zhang HX, Xu LX, Zhang ZQ, Yuan KT, Xue CL, Yu HL, Zhang S, Li YF, Shi HP, Zhang Y. 2013. N-3 PUFAs have antiproliferative and apoptotic effects on human colorectal cancer stem-like cells in vitro. J Nutr Biochem 24:744-53.

Yang W, Meng L, Wang H, Chen R, Wang R, Ma X, Xu G, Zhou J, Wang Y, Lu Y, Ma D. 2006. Inhibition of proliferative and invasive capacities of breast cancer cells by arginine-glycine-aspartic acid peptide *in vitro*. Oncol Rep 15:113-7.

Zhou BBS, Zhang HY, Damelin M, Geles KG, Grindley JC, Dirks PB. 2009. Tumor-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 8:806-23.

Food Research International 62 (2014) 793–800

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

In vitro chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells

Samuel Fernández-Tomé [a], Sonia Ramos [b], Isabel Cordero-Herrera [b], Isidra Recio [a], Luis Goya [b,*], Blanca Hernández-Ledesma [a,**]

[a] Departamento de Bioactividad y Análisis de Alimentos, Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM + CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain
[b] Departamento de Metabolismo y Nutrición, Instituto de Ciencia y Tecnología de Alimentos y Nutrición ICTAN (CSIC), José Antonio Novais, 10, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 24 February 2014
Accepted 13 April 2014
Available online 30 April 2014

Keywords:
Antioxidant defenses
Biomarkers for oxidative stress
Dietary antioxidants
Peptide lunasin

ABSTRACT

Lunasin is a peptide with proven properties against cancer and cardiovascular diseases. Relevant amounts of lunasin have been found in liver of rats fed lunasin-enriched diets, indicating its potential bioactive effect in this tissue. This study investigated the stability of lunasin in human liver HepG2 cells, and its chemoprotective effect against oxidative stress induced by *tert*-butylhydroperoxide. Pre-treatment of cells with lunasin (0.5–10 μM) significantly prevented the increased reactive oxygen species (ROS) generation (122% compared to 190% in stressed cells), and glutathione peroxidase and catalase activities, as well as the depletion of reduced glutathione. By restraining ROS overproduction, lunasin evoked a decline in carbonyl groups, and a significant recovery from cell death by apoptosis. These findings suggest that lunasin, at physiological concentrations, might confer a significant chemoprotective effect against oxidative stress-associated liver disorders. In addition, fragments released after hydrolysis of lunasin by cell enzymes might contribute to the observed antioxidant effects.

Introduction

Cells are naturally provided with an extensive array of protective enzymatic and non-enzymatic antioxidants that counteract the potentially injurious oxidizing agents. But even this multifunctional protective system cannot completely prevent the deleterious effects of reactive oxygen species (ROS), and consequently, molecules damaged by oxidation accumulate in cells. Large amounts of ROS have been shown to participate in the pathogenesis of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer (Ramos, 2008). Restoration or activation of improperly working or repressed antioxidant machinery as well as suppression of abnormally amplified inflammatory signaling can provide important strategies for chemoprevention. Therefore, determination of anti-inflammatory and/or antioxidant properties has been proposed as a good indicator for screening anti-cancer agents (Federico, Morgillo, Tuccillo, Clardiello, & Loguercio, 2007).

There is substantial evidence that antioxidant food components have a protective role against oxidative stress-induced atherosclerosis, degenerative and age-related diseases, cancer and aging. Food-derived peptides are promising natural antioxidants without marked adverse effects. In addition to their potential as safer alternatives to synthetic antioxidants used to avoid or retard oxidation reactions in foods, antioxidant peptides can also act reducing the risk of numerous oxidative stress-associated disorders (Meisel, 2004). Lunasin is a 43-amino acid peptide identified in soybean and other seeds and plants which chemopreventive properties have been recently reviewed (Hernández-Ledesma, Hsieh, & de Lumen, 2013). This peptide has demonstrated, by using cell cultures and animal models, to act as an anticarcinogenic agent against skin, prostate, colon, and breast cancer (Hernández-Ledesma, de Lumen, & Hsieh, 2013). Recently, lunasin has been commercialized in the US by its benefits on the cardiovascular system through reduction of low density lipoprotein cholesterol concentration (Gálvez, 2012). Moreover, its promising anti-inflammatory and antioxidant activities reported in the recent years might contribute to lunasin chemoprotective action. Lunasin has been shown to inhibit inflammation in cultured RAW 264.7 macrophages through suppression of the nuclear factor (NF)-κB pathway (Cam, Sivaguru, & González de Mejia, 2013; González de Mejía & Dia, 2009; Hernández-Ledesma, Hsieh, & de Lumen, 2009a). Additionally, in vitro assays have revealed the ability of this peptide to scavenge peroxyl radicals, and to block Fenton reaction by chelating iron ferrous ions, protecting DNA from oxidative damage (García-Nebot, Recio, & Hernández-Ledesma, 2014; Hernández-Ledesma et al., 2009a; Jeong, de Lumen, & Jeong, 2010).

Studies on bioavailability carried out in mice and rats have demonstrated that, after its oral ingestion, lunasin appears in an intact and active form in different organs and tissues, such as blood, liver, and kidney, among others (Hsieh, Hernández-Ledesma, Jeong, Park, & de Lumen, 2010; Jeong et al., 2009). In humans, the presence of lunasin has been

* Corresponding author. Tel.: +34 915445607; fax: +34 915493627.
** Corresponding author. Tel.: +34 910017970; fax: +34 910017905.
E-mail addresses: luisgoya@ictan.csic.es (L. Goya), b.hernandez@csic.es (B. Hernández-Ledesma).

http://dx.doi.org/10.1016/j.foodres.2014.04.054
0963-9969/

also reported in plasma, indicating that this peptide might reach different target tissues, and exert its biological activity (Dia, Torres, de Lumen, Erdman, & González de Mejía, 2009).

The liver is particularly susceptible to toxic and oxidative insults since the portal vein brings blood to this organ after intestinal absorption. Therefore, studies dealing with the effects of chemopreventive compounds at a cellular level in cultured hepatic cells are essential. Human HepG2, a well differentiated transformed cell line, is a reliable model for cultured hepatocyte-type cells used for biochemical, pharmacological and nutritional studies since it retains hepatocyte morphology and most of its functionality in culture (Alía, Ramos, Mateos, Bravo, & Goya, 2006; Mateos, Goya, & Bravo, 2006).

The aims of the study were to evaluate the stability of lunasin in human liver HepG2 cells as a model for cultured hepatocytes, and to investigate the potential chemo-protective effect of this peptide against oxidative stress chemically induced by a potent pro-oxidant, *tert*-butyl hydroperoxide (*t*-BOOH). Cell integrity and several biomarkers of oxidative damage were evaluated to estimate the effect of lunasin on cell survival and on the response of the antioxidant defense systems of HepG2 cells to *t*-BOOH.

Materials and methods

Reagents

Peptide lunasin (>95% of purity) was synthesized by Chengdu KaiJie Biopharm Co., Ltd. (Chengdu, Sichuan, P. R. China). *t*-BOOH, glutathione reductase, reduced glutathione (GSH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), *o*-phthalaldehyde (OPT), dichlorofluorescin (DCFH), dinitrophenylhydrazine (DNPH), trifluoroacetic acid (TFA), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, gentamicin, penicillin G, streptomycin, Triton-X100, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (Madrid, Spain). Sodium dodecyl sulfate (SDS) was from Panreac Química (Madrid, Spain). N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from BD Pharmingen (Madrid, Spain). Bradford reagent was from BioRad Laboratories S.A. (Madrid, Spain). The rest of the chemicals used were of HPLC grade.

Cell culture

Human HepG2 cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in Dulbecco's Modified Eagle Medium (DMEM)–F12 medium from Biowhittaker (Lonza, Madrid, Spain), supplemented with 2.5% (v/v) Biowhittaker fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin G, and streptomycin. Cells were changed to FBS-free medium the day before the assay (Mateos et al., 2006).

Cell treatment conditions

Cells were incubated for 20 h with peptide lunasin dissolved in FBS-free DMEM–F12 at final concentrations ranging from 0.5 to 10 μM. To evaluate both direct and protective effects against oxidative stress, the incubation period was followed by a 3 h treatment with culture medium (direct effect) or oxidant chemical *t*-BOOH (400 μM). Crystal violet (CV) staining, GSH concentration, ROS generation and glutathione peroxidase (GPx) and catalase (CAT) activities were evaluated in both direct and protective experiments. Besides, the protective effect of lunasin against *t*-BOOH-induced oxidative damage to proteins and apoptotic cellular signals was evaluated by carbonyl groups and caspase-3 assays, respectively.

Crystal violet assay

Cell viability was evaluated following the CV assay described by Granado-Serrano and co-workers (Granado-Serrano et al., 2007). HepG2 cells were seeded in 96-well plates (1×10^4 cells per well) and incubated overnight. Then, cells were treated with lunasin for 20 h as described above, washed with PBS, and incubated with CV (0.2% in ethanol) for 20 min at room temperature. Finally, cell lysis was carried out with 1% SDS, and the absorbance was read at 570 nm in a microplate reader (FL600, Bio-Tek, Winooski, VT, USA). Results were pooled from different plates to obtain an average of $n = 12$, and presented as percentage of viable cells compared to control, considered as 100%.

Morphological analysis

HepG2 cells were exposed to increasing concentrations (0.5–10 μM) of lunasin for 20 h, then treated with DMEM (controls) or DMEM supplemented with *t*-BOOH for 3 h, and cell images were taken using an inverted phase contrast microscope at 20× magnification.

Determination of intracellular ROS levels

Intracellular ROS levels were quantified following the method described by Alía and co-workers, using DCFH as fluorescent probe (Alía, Ramos, Mateos, Bravo, & Goya, 2005). HepG2 cells were cultured in 24-well multiwell plates (2×10^5 cells per well), incubated overnight, and then, treated with lunasin as described above. After 19.5 h incubation with the peptide, a solution of DCFH was added to each well (5 μM final concentration), and cells were incubated in the dark for 30 min at 37 °C. Afterwards, cells were washed with PBS, incubated with culture medium (direct effects), or subjected to chemical-induced oxidative stress with *t*-BOOH (protective effects). The production of intracellular ROS was followed over 3 h measuring the fluorescence intensity at $\lambda_{excitation}$ and $\lambda_{emission}$ of 485 nm and 530 nm, respectively, in a microplate reader (FL600, Bio-Tek). The results were pooled from different plates to obtain an average of $n = 8$, and expressed as percentage of the control (cells in a basal state), considered as 100%.

Quantification of concentration of GSH and determination of GPx and CAT activity

Cells were seeded onto 100-mm Petri dishes, incubated overnight, treated with lunasin (for both direct and protective assays), exposed to *t*-BOOH (for protective assays), and then, collected following the methodology described by Quéguineur and co-workers (Quéguineur et al., 2012). The obtained supernatants, corresponding to the cellular content of HepG2 cells, were subjected to the determination of the concentration of GSH and the activity of GPx and CAT enzymes. The content of GSH was evaluated by a fluorometric assay as previously described (Quéguineur et al., 2012). Briefly, 50 μL of each sample were transferred in triplicate to a 96-multiwell plate, and the reaction mixture containing 15 μL of 1 M NaOH, 175 μL of PBS/EDTA, and 10 μL of a solution of OPT (10 mg/mL) was added. After 20 min incubation, the fluorescence was read ($\lambda_{excitation}/\lambda_{emission} = 340/460$ nm) in a microplate reader (FL600, Bio-Tek). Results were pooled from different plates to obtain an average of $n = 6$, and interpreted considering those of a standard GSH curve similarly prepared within each of the experiments run.

GPx catalyzes the oxidation of GSH to oxidized glutathione, using *t*-BOOH as a substrate, reaction coupled to the decomposition of NADPH to β-nicotinamide adenine dinucleotide by glutathione reductase. Thus, disappearance of NADPH reliably estimates GPx activity, which can be measured by following the decrease in absorbance at 340 nm (Alía et al., 2006). CAT activity was determined by following the breakdown of the peroxide H_2O_2 to H_2O, monitored as a decrease in absorbance at 240 nm (Granado-Serrano et al., 2007). Results for both GPx and CAT activities were pooled from different plates to obtain an average of $n = 6$, and 4, respectively, and referred to the total protein concentration of the cytosolic samples, measured by the Bradford reagent (Bio-Rad).

Evaluation of carbonyl groups

Oxidative damage to proteins by reactive species, particularly ROS, was evaluated by measuring the content of carbonyl groups in cell supernatants according to the method of Richert and co-workers (Richert, Wehr, Stadtman, & Levine, 2002). Absorbance was measured at 360 nm, and carbonyl content results (experiments run to obtain an average of $n = 4$) were expressed as nmol of carbonyl groups per mg of total protein, using an extinction coefficient of 22,000 nmol/L/cm.

Determination of caspase-3 activity

Caspase-3 activity was measured according to the fluorometric assay previously described (Herrera et al., 2001). After treatment with lunasin for 20 h and chemical induction with *t*-BOOH for 3 h, cells were collected, and lysed in a buffer containing 5 mM Tris, 20 mM EDTA, and 0.5% Triton-X100. Then, the reaction mix containing 30 μg cell protein, 20 mM HEPES, 10% glycerol, 2 mM DTT, and 20 μM Ac-DEVD-AMC was incubated in the dark for 2 h. Fluorescence was measured at $\lambda_{excitation}$ and $\lambda_{emission}$ of 380 nm and 440 nm, respectively, in a microplate reader (FL600, Bio-Tek), and enzymatic activity results (experiments run to obtain an average of $n = 4$) were expressed as units of caspase-3 per μg of total protein.

Stability of lunasin

HepG2 cells were plated in 60 mm-diameter plates at a density of 1.5×10^6 cells per plate, and incubated at 37 °C overnight. Then, cells were treated with 10 μM lunasin dissolved in FBS free DMEM–F12, and incubated at 37 °C for 20 h, taking aliquots of the medium at 0, 2, 6, 12, and 20 h of incubation. These aliquots were subjected to liquid chromatography (HPLC) coupled to tandem mass spectrometry (HPLC–MS/MS) on an Agilent 1100 HPLC System (Agilent Technologies, Waldbronn, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany), and equipped with an electrospray ionization source as previously described (Contreras et al., 2010). The column used was a Mediterranea Sea_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain), the injection volume was 50 μL, and the flow was set at 0.2 mL/min. Peptides were eluted with a linear gradient of solvent B (acetonitrile:TFA 1000:0.27 v/v) in A (water:TFA 1000:0.37 v/v) going from 0% to 45% in 130 min. Data obtained were processed and transformed to spectra representing mass values using the Data Analysis program (version 4.0, Bruker Daltonik). To process the MS/MS spectra and to perform peptide sequencing BioTools (version 3.1, Bruker Daltonik) was used.

Statistics

Data were analyzed by a one-way ANOVA followed by the Bonferroni Multiple Comparison test, and expressed as the mean ± standard variation (SD). GraphPad Prism 5.0 software (San Diego, CA, USA) was used to perform statistical analyses. Differences with a P value < 0.05 ($^{*,\#}$), P value < 0.01 ($^{**,\#\#}$) or P value < 0.001 ($^{***,\#\#\#}$) were considered significant.

Results and discussion

Chemoprotective effects of lunasin on cell viability and redox status

In order to evaluate the effect of lunasin at a physiological level, in the present study, a range of concentrations between 0.5 and 10 μM was selected. Previous studies on lunasin's bioavailability have demonstrated that, because of the protection against gastric and pancreatic enzymes exerted by naturally occurring protease inhibitors such as the Bowman–Birk inhibitor (BBI), a high percentage of daily ingested peptide remains intact during its passage through the gastrointestinal tract, reaching target organs and tissues in an active form (Hsieh et al., 2010). The presence of lunasin and BBI has been confirmed in different soybean products, such as soymilk, tofu, soybean cake and fermented soybean products (Hernández-Ledesma, Hsieh, & de Lumen, 2009b). Daily consumption of 25 g of soy protein, recommended by the Food and Drug Administration (FDA) to reduce coronary disease (FDA, 1999), supplies lunasin in quantity ranged from 110 mg (21.9 μmol) to 1760 mg (350.2 μmol). Selected concentrations in our study were not far from reality since steady-state concentrations of 99.3 μg/g tissue were isolated from liver of rats fed lunasin-enriched rye diets for 4 weeks, and lunasin extracted from those livers has been demonstrated to be active (Jeong et al., 2009).

Before testing the chemo-protective effect of lunasin, it was important to evaluate the effect of this peptide per se, in basal conditions, ensuring that no direct damage is caused to the cell by the compound. Thus, the direct effect of lunasin on HepG2 cell viability was evaluated. The CV assay, based on the growth rate reduction reflected by the colorimetric determination of the stained cells, was used to determine the cell viability. As shown in Fig. 1A, treatment of HepG2 cells with lunasin for 20 h evoked no decreases in CV staining, indicating that the concentrations selected for the study (0.5–10 μM) did not damage cell integrity during the period of incubation. Higher concentrations of lunasin were also assessed, observing that the percentage of viable cells was not affected by 25 μM lunasin (111.20% ± 4.80) and 50 μM lunasin (111.07% ± 6.19).

The cell damage caused by ROS and other reactive species plays a crucial role in the induction and progression of several liver diseases such as hepatocarcinoma, viral and alcoholic hepatitis, and non-alcoholic steatosis. Consequently, there is an increasing interest in new therapeutic agents protecting the liver from such oxidative damage, with natural antioxidants being considered one of the most effective alternatives (Vitaglione, Morisco, Caporaso, & Fogliano, 2004). *t*-BOOH, a short-chain analog of lipid peroxide, is often used to induce acute oxidative stress in different *in vitro* and *in vivo* systems, and to evaluate the protective effects of antioxidants (Mersch-Sundermann, Knasmuller, Wu, Darroudi, & Kassie, 2004). In the case of hepatocytes, these cells metabolize *t*-BOOH to toxic peroxyl and alkoxyl radicals, initiating peroxidation of macromolecules, and thus, affecting the cell integrity and leading to chemical-induced hepatic oxidative damage. Therefore, treatment of HepG2 cells with *t*-BOOH is an excellent model of oxidative stress in cell culture systems (Alía et al., 2005). To evaluate whether lunasin protects HepG2 from oxidative stress induced by *t*-BOOH, we first determined its effect on the cell viability. As shown in Fig. 1A, the percentage of viable cells in HepG2 induced by 400 μM *t*-BOOH was 67.5% (compared to non-stressed cells), indicating that this chemical showed remarkable cytotoxicity ($P < 0.001$) on these cells. However, incubation of the cells with 0.5–10 μM lunasin for 20 h, prior to chemical oxidant treatment, significantly restored cell viability up to 99.8% ($P < 0.001$), although dose-dependent effects were not observed.

As shown in Fig. 2A and B, HepG2 cells treated with *t*-BOOH showed reduction in cell numbers and loss of cell-to-cell contact. These morphological changes in *t*-BOOH-induced HepG2 cells were attenuated by pre-treatment with lunasin at 0.5 μM (Fig. 2C), and 5 μM (Fig. 2D).

In order to understand whether the cytoprotective effect of lunasin might be attributed to the reduction of oxidative stress, the intracellular ROS generation was evaluated in HepG2 cells exposed to *t*-BOOH with and without pre-treatment with lunasin for 20 h. As shown in Fig. 1B, the level of intracellular ROS in HepG2 cells treated with *t*-BOOH alone was 190.0% compared to the non-treated cells (considered as 100%), indicating that 400 μM *t*-BOOH had a strong effect on ROS generation. When the cells were pre-treated with lunasin, intracellular ROS levels were significantly decreased up to 122% (compared to control) ($P < 0.001$), but no dose-dependent activity was observed. These results suggest that ROS generated during the period of oxidative stress were more efficiently quenched in cells pre-treated 20 h with lunasin, which could be a first explanation for the reduced cell damage and death shown. The intracellular ROS levels of non-stressed cells were

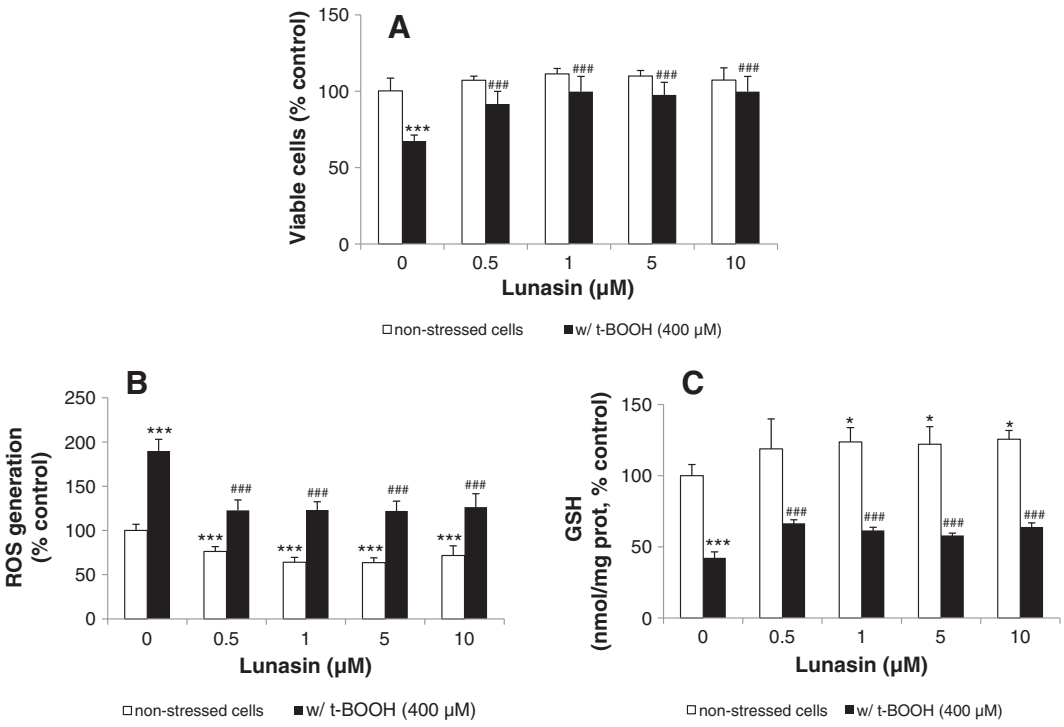


Fig. 1. Direct and protective effects of peptide lunasin on cell viability and intracellular ROS and GSH levels. HepG2 cells were pre-incubated with medium or medium supplemented with lunasin (final concentration ranged from 0.5 to 10 μM) for 20 h before treatment with medium (□: non-stressed cells) or medium supplemented with 400 μM *t*-BOOH for 3 h (■). (A) Cell viability was measured by the CV assay. Results are expressed as percent of viable cells, $n = 12$. (B) Intracellular ROS generation. Fluorescence units corresponding to intracellular ROS production are expressed as percent of data from non-stressed cells, $n = 8$. (C) Intracellular GSH levels. Results of fluorescent analysis are calculated as nmoles of GSH per mg of protein, and represented as percentage of non-stressed cells, $n = 6$. $^{*}(P < 0.05)$, $^{***}(P < 0.001)$, significantly different from control non-stressed cells, and $^{\#\#\#}(P < 0.001)$, significantly different from control *t*-BOOH-induced cells.

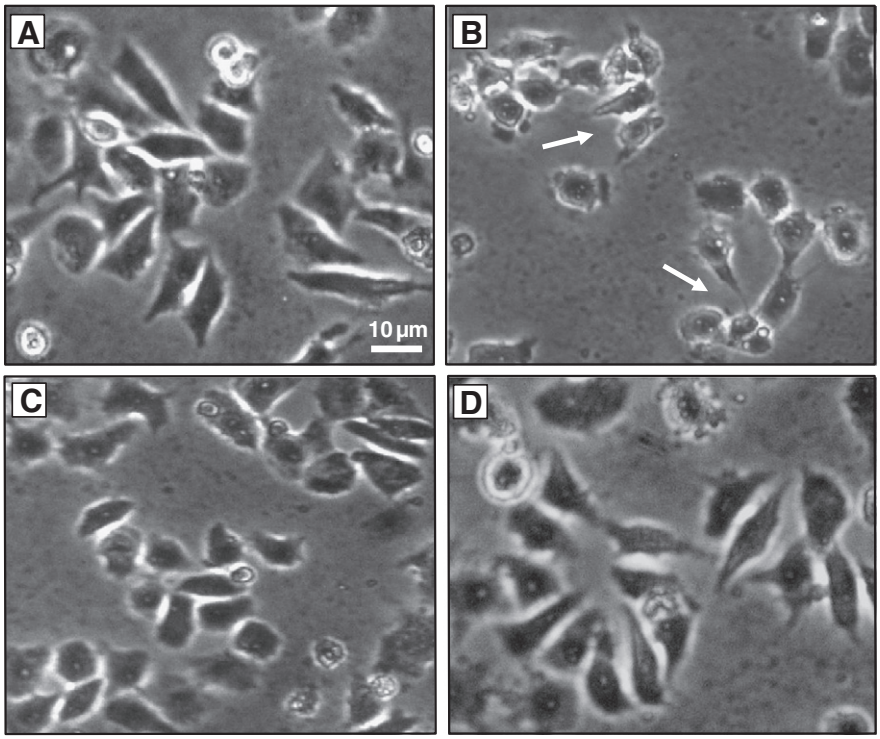


Fig. 2. Morphological analysis of HepG2 cells. HepG2 cells were pre-incubated with medium or medium supplemented with lunasin (final concentration 0.5–10 μM) for 20 h before treatment with medium (non-stressed cells) or medium supplemented with 400 μM *t*-BOOH for 3 h. Representative images of (A) non-stressed cells pre-incubated with medium, (B) *t*-BOOH-induced cells pre-incubated with medium, and *t*-BOOH-induced cells pre-incubated with (C) 0.5 μM lunasin and (D) 5 μM lunasin. HepG2 cells pre-incubated with medium and treated with *t*-BOOH showed reduction in cell numbers and loss of cell-to-cell contact (arrows), compared with non-stressed cells pre-incubated with medium, and *t*-BOOH-induced cells pre-incubated with lunasin. Size bar: 10 μm.

also decreased by treatment with lunasin, reaching 63.6% (compared to control cells) when 5 μM lunasin was used (Fig. 1B). These findings confirm the ability of this peptide as an effective scavenger of ROS in cell cultures. Previous studies have demonstrated that lunasin is a potent antioxidant in different *in vitro* assays, including reducing power, and ABTS, peroxyl and superoxide radical scavenging activities (García-Nebot et al., 2014; Hernández-Ledesma et al., 2009a; Jeong et al., 2010).

Lunasin at concentrations higher than 1 μM significantly increased the cytosolic levels of GSH ($P < 0.05$) (Fig. 1C). GSH is a tripeptide found in all mammalian cells and considered the main non-enzymatic antioxidant defense within the cell. It appears in high concentration in the liver where it plays a crucial role protecting against oxidative stress through elimination of toxic ROS, and mitigation of macromolecule peroxidation and cell injury (Mari, Morales, Colell, García-Ruiz, & Fernández-Checa, 2009). It is usually assumed that enhanced levels of GSH prepare the hepatocytes against a potential oxidative insult, whereas its depletion reflects an intracellular oxidation state (Alía et al., 2005). The effect of lunasin on GSH levels, together with the decreased ROS generation, reflects a diminished intracellular oxidation which could be expected to place the cell in favorable conditions to face a potential oxidative insult. Treatment of HepG2 cells with *t*-BOOH induced a significant decrease in the concentration of GSH ($P < 0.001$), as it was previously demonstrated (Goya, Mateos, & Bravo, 2007). However, this depletion of the GSH store was partly prevented by pre-treatment with lunasin (Fig. 1C). These findings indicate that increased levels of GSH in the lunasin-treated cells before exposure to the oxidative damage greatly helped to prevent the dramatic depletion of intracellular GSH during the oxidative stress, an effect of lunasin that had not been reported previously. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents an enormous advantage for cell survival.

Influence of lunasin on antioxidant enzyme activity

In non-stressed HepG2 cells, lunasin did not provoke any change in the activity of GPx and CAT (data not shown). These results indicate that in spite of the direct effects of lunasin on ROS levels and GSH content, the enzymatic defense system in the cells was balanced. The increase in the activity of GPx and CAT observed after exposure to *t*-BOOH (Fig. 3A and B), clearly indicates a positive response of the cell defense system to face the increasing generation of ROS evoked by the oxidative insult (Alía et al., 2006; Goya, Martín, Ramos, Mateos, & Bravo, 2009). It has been demonstrated that GPx and CAT play a crucial role as the first line of the antioxidant defense system against ROS generated during oxidative stress (Ray & Husain, 2002). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will place the cell in a favorable condition to deal with a new insult. Therefore, changes in their activity are considered as biomarkers of the antioxidant cellular response. It has been previously shown that flavonoid quercetin (Alía et al., 2006), olive oil phenol hydroxytyrosol (Goya et al., 2007), organic selenium derivatives (Cuello et al., 2007), and seaweed metabolite phloroglucinol (Quéguineur et al., 2012), among others, protect HepG2 cell integrity by preventing the severely increased activity of antioxidant enzymes induced by *t*-BOOH. In the present study, we show, for the first time, that a 20 h treatment of human HepG2 cells with lunasin prevents the permanent increase in the activity of both glutathione-related GPx and glutathione-independent CAT induced by oxidative stress (Fig. 3A and B). Thus, the restrained ROS production during the stressful challenge in lunasin-treated HepG2 cells reduces the need for peroxide detoxification through GPx and CAT. Although a potential direct effect of lunasin on antioxidant enzymes gene expression throughout the antioxidant response element cannot be ruled out, the protective mechanism of lunasin can be illustrated in terms of regulation of the specific activity of antioxidant defense enzymes.

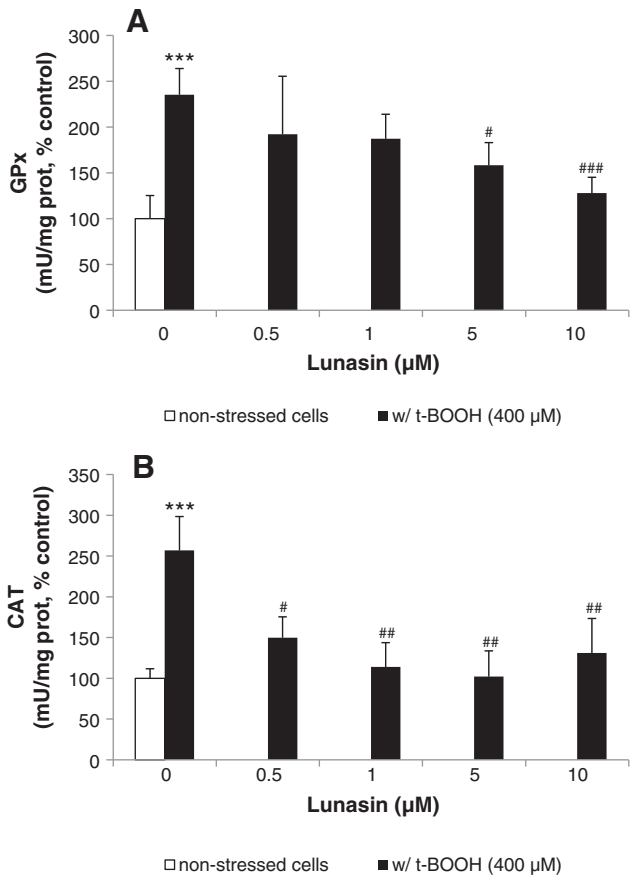


Fig. 3. Protective effects of lunasin on enzymes GPx and CAT activity. HepG2 cells were pre-incubated with medium or medium supplemented with lunasin (final concentration ranged from 0.5 to 10 μM) for 20 h before oxidation induction with 400 μM *t*-BOOH for 3 h (■). Results of enzymes (A) GPx and (B) CAT activities are calculated as mUnits per mg of protein and represented as percentage of data from non-stressed cells (□). Represented values are means ± SD of $n = 6$ (GPx) and $n = 4$ (CAT). $^{***}(P < 0.001)$, significantly different from control non-stressed cells, and $^{\#}(P < 0.05)$, $^{\#\#}(P < 0.01)$, and $^{\#\#\#}(P < 0.001)$, significantly different from control *t*-BOOH-induced cells.

Lunasin-induced reduction of protein oxidation

Since protein carbonyl concentration has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of protein oxidation in biological and medical sciences (Mateos & Bravo, 2007). Fig. 4A shows that 3 h-treatment of HepG2 with 400 μM *t*-BOOH evoked a significant increase in the cellular concentration of protein carbonyl groups of about 3-times compared to non-stressed cells, indicating an intense oxidative damage to cell proteins. However, pre-incubation of the cells with lunasin at concentrations ranged from 0.5 to 10 μM for 20 h reduced the protein carbonyl levels down to levels measured in non-stressed cells. This fact indicates the ability of this peptide to diminish the level of protein oxidation resulting from chemical induction with *t*-BOOH. Other food compounds including plant polyphenols, beta carotene, lutein, seaweed metabolite phloroglucinol and biscuit melanoidins have been also reported to prevent protein oxidation (Alía et al., 2006; Goya et al., 2007; Martín et al., 2010; Murakami, Hirakawa, Inui, Nakano, & Yoshida, 2002; Quéguineur et al., 2012). However, to date, no dietary peptide had demonstrated to exert this protective effect.

Lunasin prevents apoptosis by reducing stress-induced caspase-3

Oxidative stress-induced hepatic cell injury results not only from direct chemical interactions by altering cellular macromolecules such

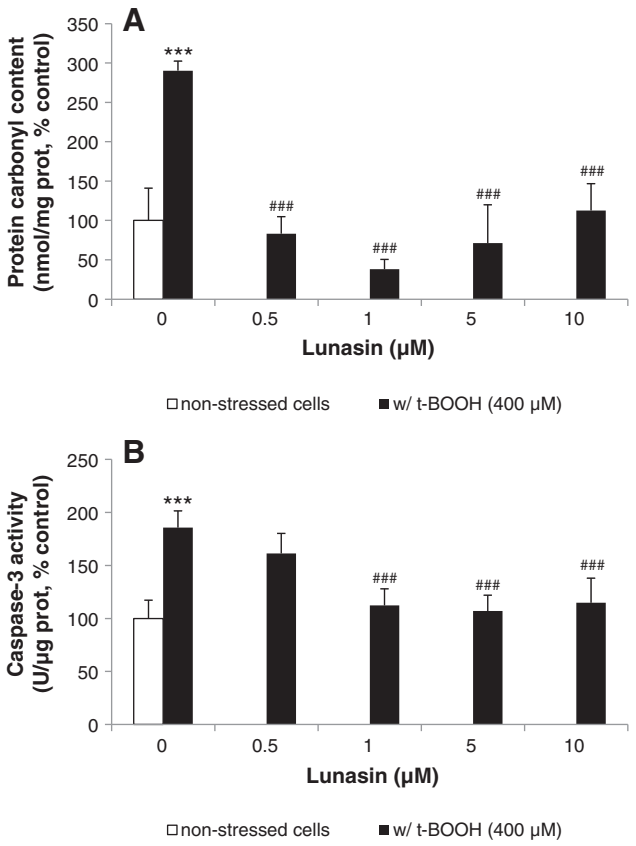


Fig. 4. Protective effects of lunasin on protein carbonyl content and caspase-3 activity. HepG2 cells were pre-incubated with medium or medium supplemented with lunasin (final concentration ranged from 0.5 to 10 μM) for 20 h before oxidation induction with 400 μM *t*-BOOH for 3 h (■). (A) Protein carbonyl content. Results are expressed as nmol per mg protein and represented as percentage of non-stressed cells (□). (B) Caspase-3 activity. Results are calculated as Units per μg of protein and expressed as percent of control data. Values are means ± SD of 4 different samples per condition. $^{***}(P < 0.001)$, significantly different from control non-stressed cells, and $^{###}(P < 0.001)$, significantly different from control *t*-BOOH-induced cells.

as DNA, proteins and lipids, but also from alterations in key mediators of stress signals and stress-dependent apoptosis reactions (Singh & Czaja, 2008). In this regard, ROS generation has been described as a critical upstream activator of the caspase cascade that ends up with stimulation of downstream key effectors such as caspase-3 and subsequent development of apoptosis (Singh & Czaja, 2008). Besides, GSH depletion is a common feature of apoptotic cells, and its role as a critical regulator in the signaling pathways leading to the progression of apoptosis has been reported (Franco & Cidlowski, 2009, 2012). As shown in Fig. 4B, the caspase-3 activity was significantly increased after 400 μM *t*-BOOH treatment for 3 h ($P < 0.001$), compared with the non-stressed cells. Previous studies had also demonstrated this pro-apoptotic effect of chemical *t*-BOOH on HepG2 cells through activation of caspase-3 and, ultimately, cell death (Martín et al., 2010). Consistent with the above-mentioned ROS scavenging effect of lunasin, this peptide in the range of 1–10 μM effectively reduced caspase-3 activity in *t*-BOOH-induced HepG2 cells ($P < 0.001$) although no dose–response relationship was observed, indicating that increases in lunasin's concentration did not improve the caspase-3 activity reduction caused by this peptide. The demonstrated effects indicated the preventive capacity of lunasin against apoptotic effects induced by disruption of the redox steady-state.

Stability of peptide lunasin in HepG2 cultures

In order to study the stability of lunasin after its addition to HepG2 cells and incubation for 20 h, the medium without and with lunasin (10 μM) at different incubation hours (0, 2, 6, 12, and 20 h) was analyzed by HPLC–MS/MS. As an example, Fig. 5A shows the extracted ion chromatograms of the molecular ion of lunasin m/z 1257.5 (charge +4) obtained after 6, 12, and 20 h-incubation of cells with lunasin. The lunasin's peak area was measured (Fig. 5B), and it was observed that the content of this peptide in the medium notably decreased with the incubation time. After 12 h-incubation, only 29% of initial lunasin remained in the medium, and after 20 h, less than 1% was observable. In the present study, the analysis by HPLC–MS/MS also allowed the identification of lunasin-derived fragments that could be released during incubation of lunasin with HepG2 cells (Fig. 5A). Five lunasin-fragments were observed; all of them corresponding to the C-terminal region of the peptide, from the amino acid residue 25. The identified peptides' peak areas were also measured (Fig. 5B). After 2 h-incubation, fragments f(25-43) and f(26-43) were already detectable in the medium. Other three peptides were released at 6 and 12 h-incubation. It has to be highlighted that after 20 h, extracellular lunasin only represented the 0.6% of total identified peptides whereas peptide f(25-43) represented 76.5%. Therefore, it can be postulated that the most abundant fragment, f(25-43), can be, at least, in part, responsible for the activity observed, and further studies with this lunasin-derived peptide are already in progress. In spite the important hydrolysis observed, it cannot be excluded that part of the lunasin could also internalize into HepG2 cells. It is also important to note that all the identified fragments contain the C-terminal part of the lunasin, which had previously been proposed as the active site of lunasin (Hernández-Ledesma, de Lumen, & Hsieh, 2013). The hydrolysis of lunasin observed in contact with the HepG2 cells was different to that observed in our previous study with differentiated human intestinal Caco-2 cells (García-Nebot et al., 2014), where most of lunasin added to the culture medium remained intact after 24 h incubation with these cells.

In the present study, analyses, focused on evaluating the antioxidant activity of peptide lunasin in HepG2 cells, were carried out after 20 h pre-treatment with this peptide. The protective mechanism on cells submitted to an oxidative stress that can be illustrated in terms of regulation of the cellular redox status, i.e. peptide treatment restrained ROS production and maintained GSH concentration during the stress which reduced the necessity of peroxide detoxification through GPx and CAT was demonstrated. Additionally, a controlled ROS generation reduced oxidative damage to proteins and restrained activity of the apoptotic pathway resulting in improved cell viability. The final findings found on lunasin's stability in HepG2 cells indicated that this peptide was markedly hydrolyzed at the selected time (20 h), and thus, in addition to the remaining peptide, the fragments released during its hydrolysis might be the actual fragments responsible for the observed effects. All the peptides identified at 20 h corresponded to the active sequence of lunasin described to date. Also, they contained the motif RGD known to be crucial for the interaction of proteins or peptides with its cell surface receptor (Ruoslahti & Pierschbacher, 1986). Therefore, these structural characteristics make these five peptides promising chemo-protective peptides against oxidative stress in liver HepG2 cells. In conclusion, our results point out for the first time a direct antioxidant action of lunasin or its derived fragments on hepatocytes exposed to oxidizing species. It indicates the possibility that these peptides may significantly contribute to preserve the integrity of liver tissues against oxidative damage related disorders. Since this study has been carried out with synthetic lunasin which secondary and tertiary structures could differ to that of plant-purified lunasin, confirmation of the effects should be needed to demonstrate the chemo-protective potential of natural lunasin.

Acknowledgments

This work was supported by projects AGL2010-17579, AGL2011-24643, and CSD2007-00063 from Programa Consolider-Ingenio from the Spanish Ministry of Education and Science (CICYT), FEDER-

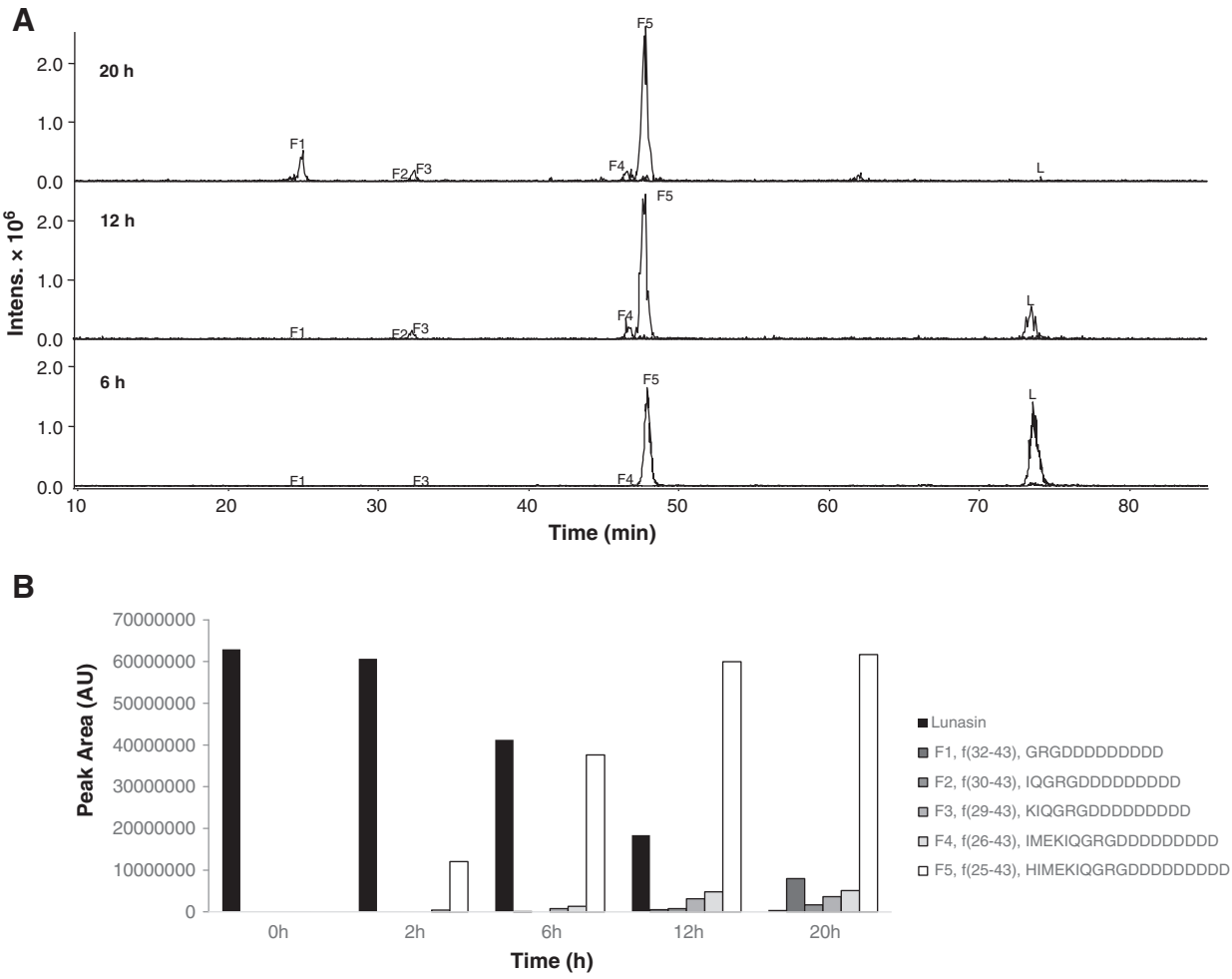


Fig. 5. Stability of peptide lunasin in medium added to HepG2 cells. (A) Extracted ion chromatogram (EIC) of the molecular ion of lunasin (L) *m/z* 1257.5 (charge +4), f(32-43) (F1) *m/z* 1324.5 (charge +1), f(30-43) (F2) *m/z* 1565.5 (charge +1), f(29-43) (F3) *m/z* 1693.8 (charge +1), f(26-43) (F4) *m/z* 1034.1 (charge +2), and f(25-43) (F5) *m/z* 1102.7 (charge +2) in serum free DMEM-F12 medium incubated with 10 μM lunasin and collected after 6 h, 12 h, and 20 h-incubation. (B) Relative amount (expressed as peak area) of lunasin and its derived fragments (F1–F5) in serum free DMEM-F12 medium incubated with lunasin for 0, 2, 6, 12, and 20 h.

INNTERCONECTA-GALICIA (ENVELLEFUN), and FP7-SME-2012-315349. The authors are participants in the FA1005 COST Action INFOGEST on food digestion. I. C. -H. and S. F. -T. acknowledge the Ministry of Economy and Competitiveness (MINECO) for their FPI fellowships, and B. H. -L. acknowledges MINECO for her "Ramón y Cajal" contract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2014.04.054.

References

Alía, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2005). Response of the antioxidant defence system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology*, *19*, 119–128.

Alía, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2006). Quercetin protects human hepatoma cell line (HepG2) against oxidative stress induced by *tert*-butyl hydroperoxide. *Toxicology and Applied Pharmacology*, *212*, 110–118.

Cam, A., Sivaguru, M., & González de Mejia, E. (2013). Endocytic mechanism of internalization of dietary peptide lunasin into macrophages in inflammatory condition associated with cardiovascular disease. *PloS One*, *8*, e72115.

Contreras, M. M., Gómez-Sala, B., Martín-Álvarez, P., Amigo, L., Ramos, M., & Recio, I. (2010). Monitoring the large-scale production of the antihypertensive peptides RYLGY and AYFYPEL by HPLC–MS. *Analytical and Bioanalytical Chemistry*, *397*, 2825–2832.

Cuello, S., Ramos, S., Mateos, R., Martín, M. A., Madrid, Y., Cámara, C., et al. (2007). Selenium methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tert-butylhydroperoxide. *Analytical and Bioanalytical Chemistry*, *389*, 2167–2178.

Dia, V. P., Torres, S., de Lumen, B. O., Erdman, J. W., & González de Mejía, E. (2009). Presence of lunasin in plasma of men after soy protein consumption. *Journal of Agricultural and Food Chemistry*, *57*, 1260–1266.

FDA Talk Paper (1999). *FDA approves new health claim for soy protein and coronary heart disease*. FDA, United States Department of Health and Human Services, October 26, 1999. Washington, DC: United States Government Printing Office.

Federico, A., Morgillo, F., Tuccillo, C., Clardiello, F., & Loguercio, C. (2007). Chronic inflammation and oxidative stress in human carcinogenesis. *International Journal of Cancer*, *121*, 2381–2386.

Franco, R., & Cidlowski, J. A. (2009). Apoptosis and glutathione: Beyond an antioxidant. *Cell Death and Differentiation*, *16*, 1303–1314.

Franco, R., & Cidlowski, J. A. (2012). Glutathione efflux and cell death. *Antioxidants and Redox Signaling*, *17*, 1694–1713.

Gálvez, A. F. (2012). Abstract 10693: Identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease. *Circulation*, *126* (A10693).

García-Nebot, M. J., Recio, I., & Hernández-Ledesma, B. (2014). Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. *Food Chemical and Toxicology*, *65*, 155–161.

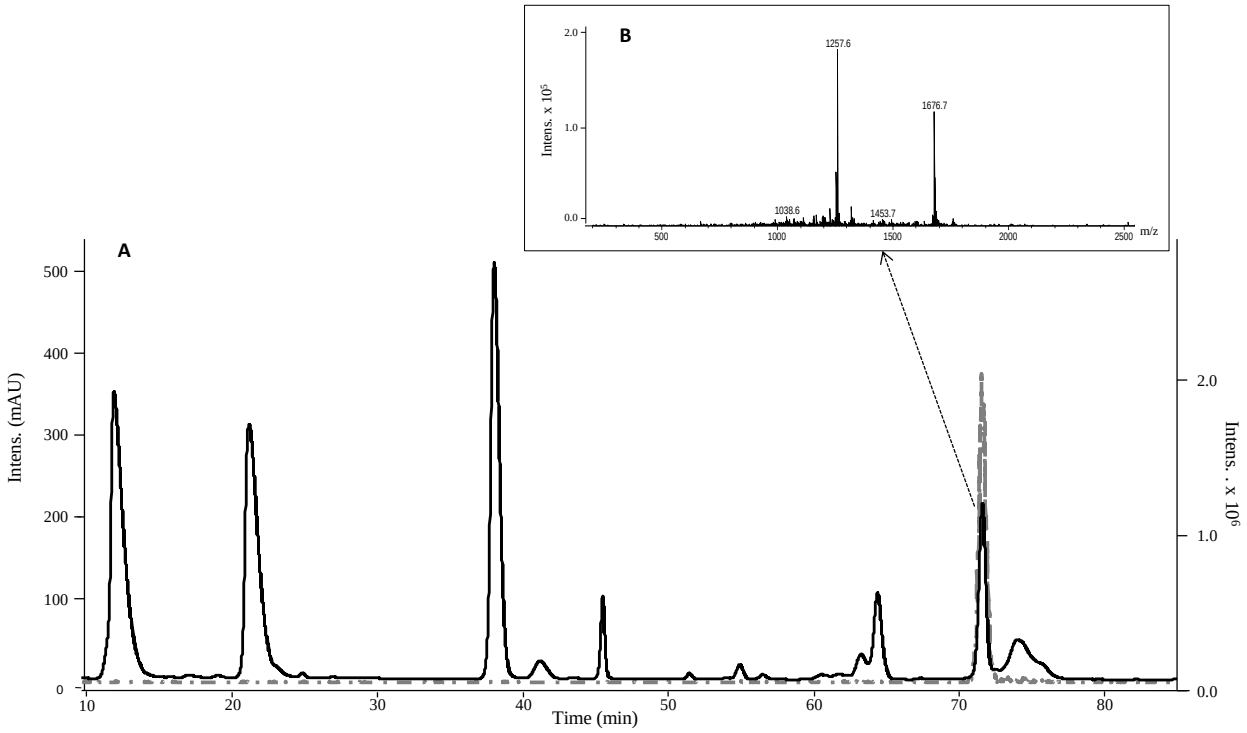
González de Mejía, E., & Dia, V. P. (2009). Lunasin and lunasin-like peptide inhibit inflammation through suppression of NFkB pathway in the macrophage. *Peptides*, *30*, 2388–2398.

Goya, L., Martín, M. A., Ramos, S., Mateos, R., & Bravo, L. (2009). A cell culture model for the assessment of the chemopreventive potential of antioxidant compounds. *Current Nutrition and Food Science*, *5*, 56–64.

Goya, L., Mateos, R., & Bravo, L. (2007). Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *European Journal of Nutrition*, *46*, 70–78.

Granado-Serrano, A. B.., Martín, M. A., Izquierdo-Pulido, M., Goya, L., Bravo, L., & Ramos, S. (2007). Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the

regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line (HepG2). *Journal of Agricultural and Food Chemistry*, *55*, 2020–2027.
Hernández-Ledesma, B., de Lumen, B. O., & Hsieh, C. -C. (2013). 1997–2012: Fifteen years of research on peptide lunasin. In B. Hernández-Ledesma, & C. -C. Hsieh (Eds.), *Bioactive food peptides in health and disease* (pp. 23–44). Rijeka, Croatia: InTech-Open Access Publisher.
Hernández-Ledesma, B., Hsieh, C. -C., & de Lumen, B. O. (2009a). Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications*, *390*, 803–808.
Hernández-Ledesma, B., Hsieh, C. -C., & de Lumen, B. O. (2009b). Lunasin and Bowman–Birk protease inhibitor (BBI) in US commercial soy foods. *Food Chemistry*, *115*, 574–580.
Hernández-Ledesma, B., Hsieh, C. -C., & de Lumen, B. O. (2013). Chemopreventive properties of peptide lunasin: A review. *Protein & Peptide Letters*, *20*, 424–432.
Herrera, B., Fernández, M., Alvarez, A., Roncero, C., Benito, M., Gil, J., et al. (2001). Activation of caspases occurs downstream from radical oxygen species production, Bcl-xL down-regulation, and early cytochrome C release in apoptosis induced by transforming growth factor beta in rat fetal hepatocytes. *Hepatology*, *34*, 548–556.
Hsieh, C. -C., Hernández-Ledesma, B., Jeong, H. J., Park, J. H., & de Lumen, B. O. (2010). Complementary roles in cancer prevention: Protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PloS One*, *5*, e8890.
Jeong, H. J., Lee, J. R., Jeong, J. B., Park, J. H., Cheong, Y. K., & de Lumen, B. O. (2009). The cancer preventive seed peptide lunasin from rye is bioavailable and bioactive. *Nutrition and Cancer*, *61*, 680–686.
Jeong, J. B., de Lumen, B. O., & Jeong, H. J. (2010). Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage from suppressing the generation of hydroxyl radical via blocking Fenton reaction. *Cancer Letters*, *293*, 58–64.
Mari, M., Morales, A., Colell, A., García-Ruiz, C., & Fernández-Checa, J. C. (2009). Mitochondrial glutathione, a key survival antioxidant. *Antioxidants and Redox Signalling*, *11*, 2685–2700.
Martín, M. A., Granado-Serrano, A. B.., Ramos, S., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2010). Cocoa flavonoids up-regulate antioxidant enzymes activity via ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *Journal of Nutritional Biochemistry*, *21*, 196–205.
Martín, M. A., Ramos, S., Mateos, R., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2010). Protection of human HepG2 cells against oxidative stress induced by the flavonoid epicatechin. *Phytotherapy Research*, *24*, 503–509.
Mateos, R., & Bravo, L. (2007). Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *Journal of Separation Science*, *30*, 175–191.
Mateos, R., Goya, L., & Bravo, L. (2006). Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic and ferulic acids) by HepG2 cells as a model of the human liver. *Journal of Agricultural and Food Chemistry*, *54*, 8724–8732.
Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors*, *21*, 55–61.
Mersch-Sundermann, V., Knasmuller, S., Wu, X. J., Darroudi, F., & Kassie, F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology*, *198*, 329–340.
Murakami, C., Hirakawa, Y., Inui, H., Nakano, Y., & Yoshida, H. (2002). Effects of epigallocatechin 3-O-gallate on cellular antioxidative system in HepG2 cells. *Journal of Nutritional Science and Vitaminology*, *48*, 89–94.
Quéguineur, B., Goya, L., Ramos, S., Martín, M. A., Mateos, R., & Bravo, L. (2012). Phloroglucinol: Antioxidant properties and effects on cellular oxidative markers in human HepG2 cell line. *Food Chemical and Toxicology*, *50*, 2886–2893.
Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Molecular Nutrition & Food Research*, *52*, 507–526.
Ray, G., & Husain, S. A. (2002). Oxidants, antioxidants and carcinogenesis. *Indian Journal of Experimental Biology*, *40*, 1213–1232.
Richert, S., Wehr, N. B., Stadtman, E. R., & Levine, R. L. (2002). Assessment of skin carbonyl content as a noninvasive measure of biological age. *Archives of Biochemistry and Biophysics*, *397*, 430–432.
Ruoslahti, E., & Pierschbacher, M. D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell*, *44*, 517–518.
Singh, R., & Czaja, M. J. (2008). Regulation of hepatocyte apoptosis by oxidative stress. *Journal of Gastroenterology and Hepatology*, *23*, 501–502.
Vitaglione, P., Morisco, F., Caporaso, N., & Fogliano, V. (2004). Dietary antioxidant compounds and liver health. *Critical Reviews in Food Science and Nutrition*, *44*, 575–586.



Supplementary Figure. (A) UV-chromatogram (black line) of the serum free DMEM-F12 medium incubated with 10 micromolar (µM) lunasin and collected at the starting point of incubation, and extracted ion chromatogram (EIC) (grey line) of the molecular ion of lunasina *m/z* 1257.5 (charge +4). (B) Mass spectrum of the selected chromatographic peak in (A). Other peaks showed in (A) correspond to DMEM-F12 medium components.

Estudio de los mecanismos implicados en la actividad protectora de péptidos lácteos sobre la capa mucosa del tracto intestinal.

2.5. Publicación V: **Novel peptides derived from α_{s1}-casein with opioid activity and mucin stimulatory effect on HT29-MTX cells.**

JOURNAL OF FUNCTIONAL FOODS, 25 (2016) 466-476

2.6. Publicación VI: ***Short communication:*** **Effect of a casein hydrolyzate on mucin secretion and gene expression in the zucker rat intestine.**

MANUSCRIPT

Journal of Functional Foods 25 (2016) 466–476

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Novel peptides derived from α_{s1}-casein with opioid activity and mucin stimulatory effect on HT29-MTX cells

S. Fernández-Tomé [a], D. Martínez-Maqueda [a], R. Girón [b], C. Goicoechea [b], B. Miralles [a], I. Recio [a,*]

[a] Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

[b] Farmacología y Nutrición, Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos, Unidad Asociada al IQM y al CIAL del CSIC, Avda. Atenas, s/n, 28922 Alcorcón, Madrid, Spain

ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form 15 June 2016
Accepted 21 June 2016
Available online

Keywords:
Opioid peptide
μ-Opioid receptor
Molecular dynamics
Mucin
Goblet cells

ABSTRACT

The opioid effect of α_{s1}-casein fragments related to ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, which had previously shown mucin-stimulatory activity in human goblet cells, was investigated. Peptides ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} showed opioid agonistic activity in guinea pig ileum, and in mouse vas deferens but to a lower extent. Peptides were partly hydrolysed during the assay and several of the resulting fragments lost the N-terminal Tyr residue. Docking of peptides ^{144}YFYPEL^{149} (active) and ^{144}YFYP^{147} (inactive) into the active site of the opioid receptor model showed remarkable differences regarding the flexibility at the third intracellular loop of the receptor and the interaction with Pro at the peptide C-terminus that forced residues Arg^{148} and Glu^{166} from the receptor to move towards the interior of the binding pocket. The study on human cells HT29-MTX has shown that ^{144}YFYPEL^{149} is the minimum fragment able to stimulate MUC5AC expression.

1. Introduction

During food digestion, proteins render a large variety of different peptides. Some of these sequences are structurally similar to endogenous physiologically active peptides, and therefore these food-derived sequences can interact with the same receptors in the organism, exerting an agonist or antagonist activity. One of the best examples where this applies, are food-derived opioid peptides, i.e., exogenous opioid receptor ligands with agonistic activity. From milk, peptides derived from β-casein, referred as β-casomorphins, were the first food protein-derived opioid receptor ligands whose sequences were identified (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). They have been found in *in vivo* digestion products from humans and minipigs (Barbé et al., 2014; Boutrou et al., 2013). The common structural characteristics of both exogenous and endogenous opioid peptides are the presence of a tyrosine residue at the N-terminus and the presence of other aromatic residue, phenylalanine or tyrosine in the third or fourth position (Meisel,

* *Corresponding author.* Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain.
E-mail address: i.recio@csic.es (I. Recio).
http://dx.doi.org/10.1016/j.jff.2016.06.023
1756-4646/

1997). In case of the α_{s1}-casein derived peptides or α-casein exorphins, the active sequence can contain an additional arginine residue at the N-terminus. Although caseins are source of many peptides showing agonist or antagonist action on different opioid receptors, opioid peptides from other protein sources have been also described, such as whey proteins (Antila et al., 1991) and haemoglobin (Zhao, Garreau, Sannier, & Piot, 1997) from animal sources, and gluten, rice, or soy from plant proteins (Yoshikawa, 2015).

Biological activities observed for these food-derived opioid sequences include analgesia and modulation of social behaviour after parenteral or intracerebral administration to animals. Orally administered food-derived opioid peptides have demonstrated to influence postprandial metabolism by stimulating secretion of insulin and somatostatin, prolongation of gastrointestinal transit time, stimulation of food intake, and effects on the immune system, among others (for reviews regarding biological activity, see Meisel & Fitzgerald, 2000; Rutherfurd-Markwick, 2012; Teschemacher, 2003; Teschemacher, Koch, & Brantl, 1997). Moreover, it was found that the opioid peptide β-casomorphin-7 (^{60}YPFPGPI^{66}) induced intestinal mucin release through a nervous pathway and opioid receptor activation in *ex-vivo* preparations of rat jejunum (Claustre et al., 2002; Trompette et al., 2003). In human (HT29-MTX) and rat (DHE) intestinal mucin-producing cells, this peptide increased secretion and expression of mucin, and these effects were prevented with the μ-opioid antagonist cyprodime (Zoghbi et al., 2006). Similar effects were reported for the μ-opioid ligands, α-lactorphin (Martínez-Maqueda et al., 2012) and β-lactorphin (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013b). However, other β-casein-derived peptides whose structures do not fulfil the requirements of opioid ligands have also demonstrated regulation of mucin production in HT29-MTX cells and in animals, such as the peptide β-casein f(94–123) found in yogurts and the derived fragments (94–108) and (117–123) (Plaisancié et al., 2013, 2015).

Our group had shown the mucin secretory activity of various bovine α_{s1}-casein-derived peptides with favourable structures to bind opioid receptors due to the presence of Tyr at the N-terminus or in the second position and other Tyr in the third or fourth position. From these peptides, fragments ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149} and a casein hydrolysate containing both sequences produced an increased mucin secretion and MUC5AC gene expression in HT29-MTX cells (Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013a). Interestingly, these two peptides had been identified in *in vivo* gastric and duodenal human digests after milk ingestion (Chabance et al., 1998). However, despite the favourable structure of these sequences and their potential to interact with opioid receptors located at the intestinal tract, the opioid activity of these peptides has not been previously demonstrated. The objective of this work was to investigate the opioid effect of the bovine α_{s1}-casein fragment ^{143}AYFYPEL^{149}, and four derived peptides comprising the core structure for opioid activity YFY in guinea pig ileum and mouse vas deferens preparations. A molecular docking of two peptides into the active site of the μ-opioid receptor was carried out to identify the key residues responsible for the affinity to the receptor. In addition, a preliminary study of these sequences on MUC5AC gene overexpression in HT29-MTX cells is shown.

2. Materials and methods

2.1. Peptides

α_{s1}-casein fragments ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146,} and the β-casein fragment ^{60}YPFPGPI^{66} were synthesized in house using conventional solid-phase FMOC synthesis with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK). In the peptide purification protocol, the treatment with acetic acid was included to replace trifluoroacetic acid which affects the pH of the peptide solutions in the bioassays. Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS).

2.2. Isolated preparations from guinea pig ileum and mouse vas deferens

Female guinea pigs weighing 300–450 g and male CD-1 mice weighing 25–30 g were used. Myenteric plexus–longitudinal muscle strips (MP–LM) from guinea pig ileum and the mouse vas deferens were isolated as described by Ambache (1954) and Hughes et al. (1975), respectively. Tissues were suspended in a 10 mL organ bath containing 5 mL of Krebs solution (NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 mM). This solution was continuously gassed with 95% O_2 and 5% CO_2. Tissues were kept under 1 g (guinea pig ileum) or 0.5 g (mouse vas deferens) of resting tension at 37 °C and were electrically stimulated through two platinum ring electrodes. MP–LM strips were stimulated with rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency, and mouse vas deferens with trains of 15 rectangular pulses of 70 V, 15 Hz and 2 ms duration each minute. The isometric force was monitored by computer using a MacLab data recording and analysis system. In both assays the interval between applications of increasing concentrations was optimized to obtain a stable signal, and it was 9 min for α_{s1}-casein peptides and 3 min when control opioid agonists were tested. To evaluate the opioid-agonistic activity of peptides in the guinea pig ileum, cumulative concentration–response curves with five doses in the range 6.1×10^{-8}–1.0×10^{-5} M were constructed in a step by step manner as follows: after 15-min stabilization of MP–LM strips in organ bath, electrical stimulation was applied, and the peptide's effect on the electrically induced contractions was evaluated once the response reached a plateau. Morphine was used as μ-opioid agonist positive control. To corroborate that the inhibitory effect of the peptides was mediated through interaction with opioid receptors, one dose of naloxone (1.0×10^{-6} M, Sigma, St. Louis, MO, USA), a non-selective opioid antagonist, was added to the organ bath at the end of each experiment. In mouse vas deferens preparations, non-cumulative concentration–response curves with four doses in the range 5.5×10^{-7}–1.0×10^{-5} M were tested. Once tissue stabilization was achieved, the first peptide dose was added and evaluated. The tissue was then washed, and subsequent doses were applied. In this preparation, [D-Pen(2),D-Pen(5)]-enkephalin (DPDPE) was used as δ-opioid agonist, and one dose of naltrindole (1.0×10^{-9}–1.0×10^{-7} M, Sigma), a δ-selective opioid antagonist, was added after the experiments to evaluate selective interaction with δ-opioid

receptors. Results were expressed as % of inhibition, taking the mean amplitude of the last five contractions before the addition of the peptides as 100%. In guinea pig ileum, when the effect of peptidase inhibitors (captopril, amastatin and phosphoramidon 1.0×10^{-6} M, Sigma) was evaluated, they were added 5 min before the beginning of the peptide's concentration–response curve (Akahori et al., 2008). Each tissue was employed only once, with at least six biological replicates per condition. Experimental protocols used in this investigation were approved by the Ethical Committee of Rey Juan Carlos University. Data were analysed using GraphPad Prism 5.03 software by a one-way ANOVA, followed by the Bonferroni multiple comparison test. Differences between each dose with a P value < 0.05 were considered significant.

2.3. *Analysis by HPLC–MS/MS*

In order to evaluate the stability of the peptides during the activity assay on MP–LM preparations, they were added at 5.0×10^{-6} M to the organ bath solution, and aliquots were withdrawn at time 0, 4, and 9 min, frozen immediately and kept at –20 °C until analysis. Analysis of the peptides was performed using an Acquity ultrahigh performance liquid chromatography system (Waters, Milford, MA, USA) connected to microToF II Quadrupole-Time-of-Flight mass spectrometer (Bruker Daltonik, Bremen, Germany). A Waters Acquity UPLC BEH C_{18} column 100 mm length, 2.1 mm internal diameter, 1.7 µm packing was used. A binary elution gradient, based on water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (v/v), and operated at a flow rate of 0.2 mL/min, was adopted. A gradient elution programme based on solvent B increase from 0% to 70% in 30 min was used. Column reconditioning at the initial mobile phase composition was accomplished in 15 min. ESI-MS acquisitions were carried out in positive ion mode. The LC–MS system was controlled by the HyStar 3.2 software (Bruker). The main parameters of the ESI interface and of the quadrupole were: dry temperature 180 °C; dry gas flow 4.0 L/min; nebulizer gas pressure 0.4 bar; capillary voltage 4.5 kV; quadrupole ion energy 5.0 eV. The ToF analyser was calibrated on a daily basis in MS mode, using the m/z ratios of adduct ions arising from sodium formate as reference.

2.4. *Molecular modelling receptor-peptide*

Homology model of the µ-opioid receptor (UniProtKB – P35372 (OPRM_HUMAN)) was built by standard homology modelling techniques using MODELER version 9.7. (Eswar et al., 2006), as described in the Supplementary material. The structure of the human δ- and µ-opioid receptors (Protein Data Bank entries 4RWA and 5C1M) (Fenalti et al., 2015; Huang et al., 2015) was used as template. The structure of the bifunctional δ-opioid antagonist and µ-opioid agonist tetrapeptide Dmt-Tic-Phe-Phe-NH_2 (Protein Data Bank entry 4RWA) was used as template for docking the peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} into the active site of the opioid receptor model.

2.5. *Cell culture*

HT29-MTX, a human colon adenocarcinoma-derived mucin-secreting goblet cell line, was provided by Dr. Thécla Lesuffleur (Lesuffleur et al., 1993). The cell line was grown in plastic 75-cm^2 culture flasks in Dulbecco's Modified Eagle's medium supplemented with 10% foetal bovine serum and 10 mL/L penicillin–streptomycin solution (all from Gibco, Paisley, UK) at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged weekly using trypsin/EDTA 0.05% (Gibco). The culture medium was changed every two days. To study the effect of peptides, cells were seeded at a density of 5×10^5 cells per well in 12-well culture plates (Nunc, Roskilde, Denmark). The cell line was used between passages 12 and 19. Experiments were performed 21 days after confluence. The culture medium was replaced, 24 h before the studies, by serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones. After serum-free medium removal, the monolayer was washed twice with PBS. Serum-free medium with or without peptide (5.0×10^{-5}, 1.0×10^{-4}, and 5.0×10^{-4} M) was added to the cells and incubated at 37 °C for 2 to 24 h in a 5% CO_2 humidified atmosphere. The supernatants were collected, frozen and stored at –80 °C. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany) according to manufacturer's instruction.

2.6. *Quantitative RT-PCR assays (qRT-PCR)*

qRT-PCR amplification was carried out using a Lightcycler 480 (Roche, Mannheim, Germany) in 384 wells microplates (Roche). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instruction. For MUC5AC (accession no. AJ001402), target gene, primers 2870–2889/3109–3091 were used. For reference genes human cyclophilin (accession no. Y00052) and human β-actin (accession no. NM_001101) primers 280–304/445–421 and 879–896/1076–1053, respectively, were used (Tai et al., 2008; Zoghbi et al., 2006). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 5 µL 2x SYBR Green real-time PCR Master Mix (Roche), 0.25 µL of a 10 µM of gene-specific forward and reverse primers, 0.27 µL of cDNA (5.06 ng), and 4.23 µL of water. Amplification was initiated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Control PCRs were included to confirm the absence of primer dimer formation (no-template control) and to verify that there was no DNA contamination (without RT enzyme). All real-time PCR assays amplified a single product as determined by melting curve analysis. Amplification efficiencies of the target and reference genes were performed, and the relative expression levels of the target gene were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Human cyclophilin and human β-actin were tested as reference gene. Human cyclophilin gene was then chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at least three times in triplicate. Data were analysed using GraphPad Prism 5.03 software by a two-way ANOVA. For a better comparison of the concentration versus control data for each time, data were analysed by one-way ANOVA, followed by the Newman–Keuls test. Differences between means and controls with a P value < 0.05 (*) or P value < 0.01 (**) were considered significant.

3. Results

3.1. Activity of α_{s1}-casein fragment ^{143}AYFYPEL^{149} and derived peptides on guinea pig ileum and mouse vas deferens preparations

As illustrated in Fig. 1, the inhibition of the electrically induced contractions in MP–LM preparations in cumulative curves of five doses showed a significant increase upon addition of the α_{s1}-casein peptides ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} between the first and the third dose but the curve did not show any further increase. The other peptides, ^{143}AYFYPEL^{149}, ^{144}YFYP^{147} and ^{144}YFY^{146}, did not show inhibitory effect on this preparation. The maximum inhibition responses for ^{144}YFYPEL^{149} (35.1 ± 5.6%) and ^{144}YFYPE^{148} (31.8 ± 2.5%) were reached at a dose 5.5×10^{-7} M and it was followed by a smooth decrease. As positive controls, β-casomorphin-7 (YPFPGPI) and morphine were used. β-casomorphin-7 showed an increasing trend up to 1.0×10^{-5} M with a maximum response of 23.3 ± 5.1%, while the morphine inhibition curve increased up to 1.6×10^{-6} M reaching a 60.6 ± 5.8% of inhibition on the electrically stimulated contractions of the MP–LM preparations. In this latter case, the plateau observed for the highest concentration (5.0×10^{-6} M) could be due to receptor saturation. However, the decreasing trend of the curves obtained for the α_{s1}-casein-derived peptides from the third dose led us to consider the possibility of peptide degradation in the organ bath. In order to check peptide stability during the experiments, aliquots of the organ bath were withdrawn at time 0, 4 and 9 min and analysed by HPLC–MS/MS. The chromatographic areas of the extracted molecular ion corresponding to the assayed peptides showed a decrease with the time (Supplementary Fig. S1). The observed decrease in the area of intact peptide was accompanied with the increase of derived peptide fragments, which could be also identified by their fragmentation pattern. In this way, from peptide ^{143}AYFYPEL^{149} increasing concentrations of fragments ^{144}YFYPEL^{149}, ^{145}FYPEL^{149}, and ^{146}YPEL^{149} were detected over time; from peptide ^{144}YFYPEL^{149}, fragments ^{145}FYPEL^{149}, ^{146}YPEL^{149}, and ^{144}YFYP^{147} were found; and from ^{144}YFYPE^{148}, the formation of

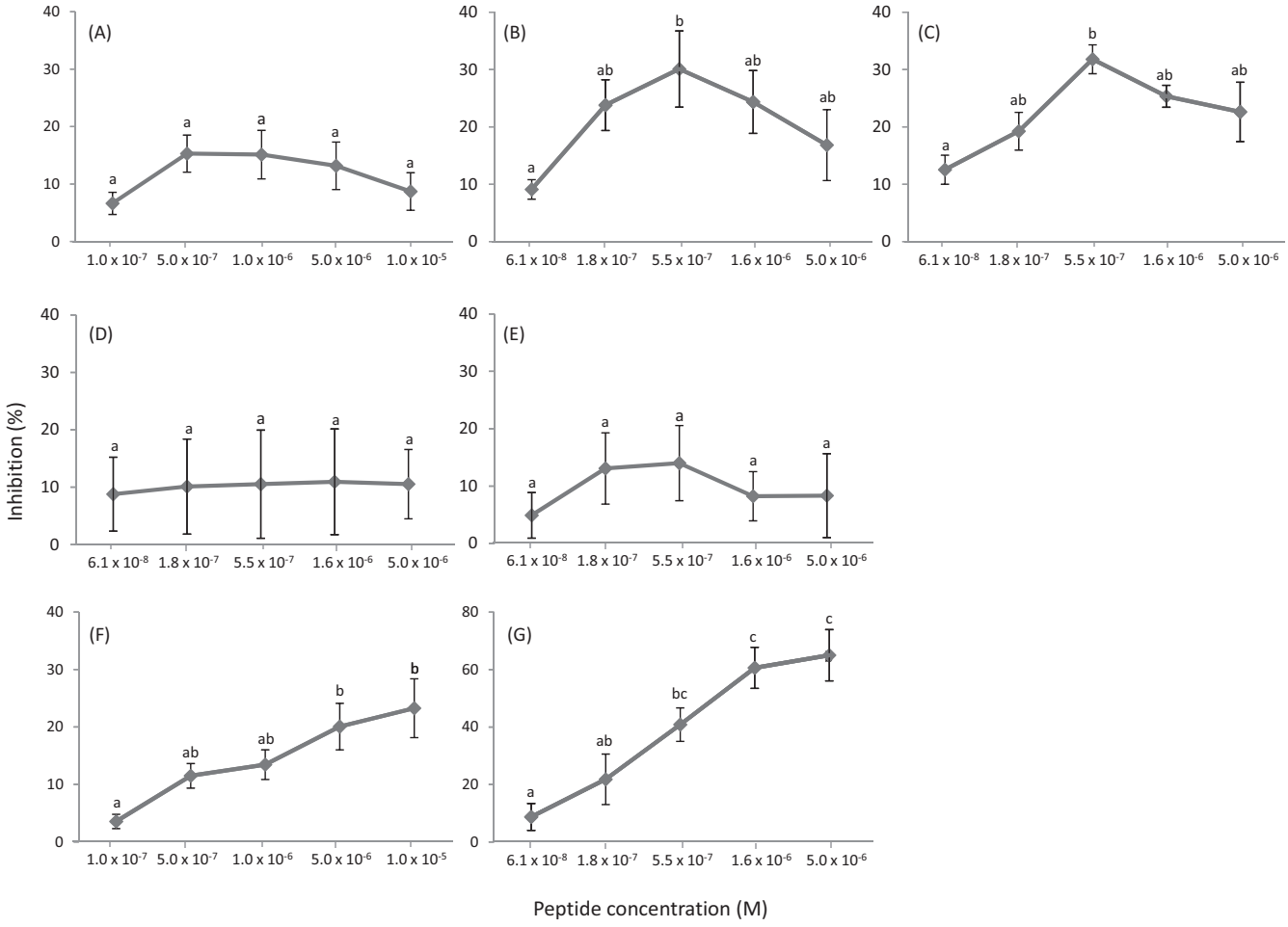


Fig. 1 – Inhibition of the electrically induced contractions in guinea pig ileum preparations at increasing concentrations (cumulative curve) of α_{s1}-casein fragment ^{143}AYFYPEL^{149} (A), α_{s1}-casein fragment ^{144}YFYPEL^{149} (B), α_{s1}-casein fragment ^{144}YFYPE^{148} (C), α_{s1}-casein fragment ^{144}YFYP^{147} (D), α_{s1}-casein fragment ^{144}YFY^{146} (E), β-casein fragment (β-casomorphin-7, ^{60}YPFPGPI^{66}) (F), and morphine (G). For clarity, food-derived peptides and morphine results are represented in the Y-axis up to 40 and 80% inhibition, respectively. Each point represents the mean % ± SEM (n = 6). Different letters denote statistically significant differences between concentrations ($P < 0.05$).

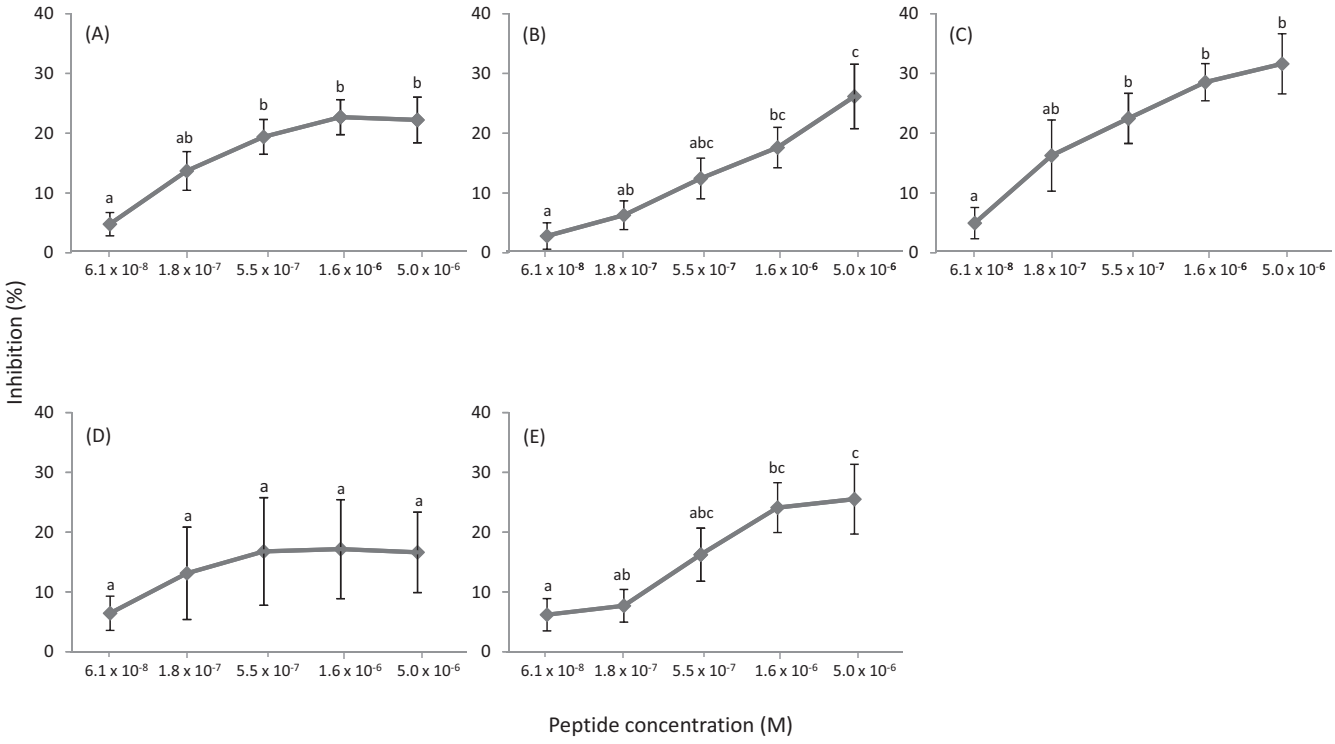


Fig. 2 – Inhibition of the electrically induced contractions in guinea pig ileum preparations in the presence of the peptidase inhibitors (captopril, amastatin, and phosphoramidon) at increasing concentrations (cumulative curve) of α_{s1}-casein fragment ^{143}AYFYPEL^{149} (A), α_{s1}-casein fragment ^{144}YFYPEL^{149} (B), α_{s1}-casein fragment ^{144}YFYPE^{148} (C), α_{s1}-casein fragment ^{144}YFYP^{147} (D), and α_{s1}-casein fragment ^{144}YFY^{146} (E). Each point represents the mean % ± SEM (n = 6). Different letters denote statistically significant differences between concentrations ($P < 0.05$).

^{145}FYPE^{148} could be followed. No peptide fragments were detected for ^{144}YFYP^{147} or ^{144}YFY^{146}, although a concentration decrease ca. 34% and 29% was found from 0 to 9 min, respectively. For these two short peptides, it is possible that although peptide degradation could occur, the derived peptide fragments could not be detected due to the short size of the fragments. It has to be noted that some of the new peptide fragments generated by the action of tissue peptidases preserved the cluster ^{144}YFY^{146}, but in other cases the N-terminal Tyr residue was lost, and therefore presumably their potential opioid effect. Since the lost in opioid effect along the accumulative dose–response curve could be attributed to the peptide degradation over time, the assays were repeated in the presence of three peptidase inhibitors.

Fig. 2 shows the inhibition curves for peptides ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} when peptidase inhibitors captopril, amastatin and phosphoramidon were used. The inhibition curves for all peptides, except ^{144}YFYP^{147}, showed an increasing trend with the peptide concentration and a significant dose-effect was found for various peptides, such as ^{144}YFYPEL^{149} and ^{144}YFY^{146}. Peptide ^{144}YFYPE^{148} reached the highest mean inhibitory value, 31.6 ± 4.6%, although the inhibition percentages determined for ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, and ^{144}YFY^{146} were not significantly different from this, being 22.2 ± 4.1, 26.2 ± 4.9 and 25.5 ± 5.3%, respectively. The suppressive effect of these peptides on the electrically stimulated contractions in MP–LM preparations was reverted by the use of naloxone at 1.0×10^{-6} M, once the maximum concentration of the peptide was tested. In addition, the stability of the peptides over time in the presence of peptidase inhibitors was also evaluated, as previously. As expected, derived peptide fragments were not detected or their intensities were considerably lower (data not shown).

In the mouse vas deferens preparation, α_{s1}-casein-derived peptides showed lower activity. Peptides ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} induced a maximum inhibitory effect of the contractions of 8.8 ± 3.4, 6.9 ± 1.6 and 13.4 ± 2.8%, respectively, at the highest dose assayed (1.0×10^{-5} M), with a dose-effect trend only found for ^{144}YFYPE^{148}. The positive control, DPDPE, also showed a dose-dependent activity, with the maximum inhibitory effect found at 6×10^{-8} M (55.3 ± 10.2%). By the addition of naltrindole (1×10^{-9} M), a selective δ-opioid receptor antagonist, DPDPE effect was partly reversed (36.0 ± 7.9%), while the weak activity found for the peptides was not affected.

3.2. *Molecular modelling of the μ-opioid receptor bound to α_{s1}-casein peptides*

To better understand the opioid activity of these α_{s1}-casein peptides and aiming to elucidate a structure–activity relationship, molecular dynamics simulations of peptides binding at μ-opioid receptor were performed. As representative examples, peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} were chosen for these experiments to explain the lack of activity of the shorter form, according to the results on guinea pig ileum preparations.

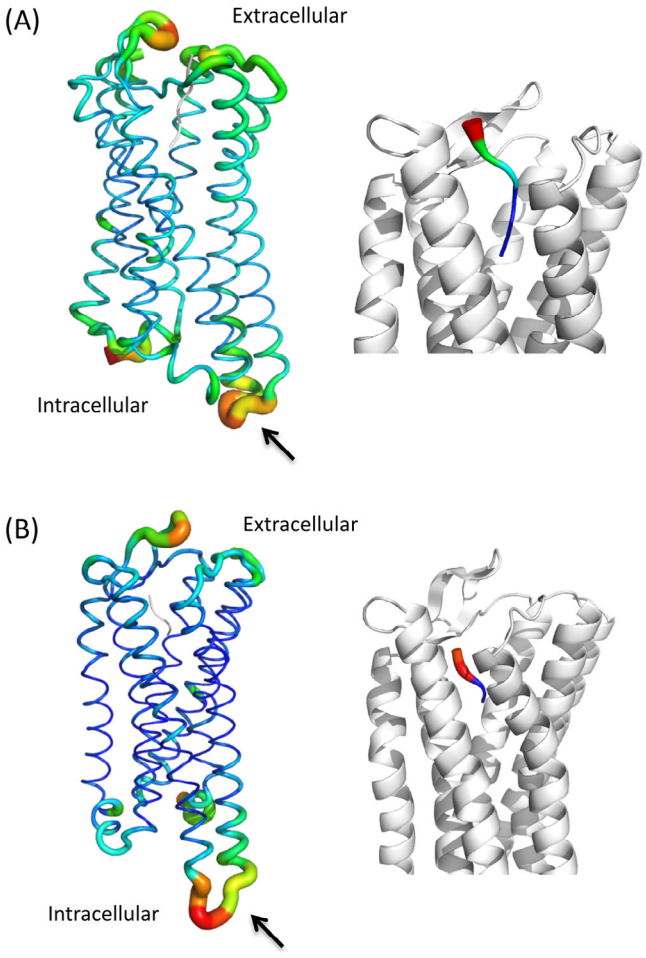


Fig. 3 – Mean residue fluctuations of the μ-opioid receptor and the α_{s1}-casein fragment ^{144}YFYPEL^{149} (A) and the α_{s1}-casein fragment ^{144}YFYP^{147} (B). Structures of opioid receptor and peptides are represented as ribbons where the most moving regions are thicker and coloured in red-orange, while static regions are thinner and coloured in blue. Third loop between receptor residues 195–200 is marked with an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The structural stability of the complex receptor-peptide was evaluated by monitoring the Root-Mean-Square Deviation (RMSD) between different structures sampled along the simulation. The fluctuations of the individual residues from the receptor and the peptides were studied. In both cases, intra and extra loops of the receptor exhibited the greatest variability, most noteworthy loops between residues 145–150, 195–200, 240–250 and the C-terminal domain of the receptor (Fig. 3A). However, when peptide ^{144}YFYP^{147} was assayed the loop between residues 195–200 started the simulation with a retracted loop but it became stretched maintaining this position for the rest of the simulation (Fig. 3B). While peptide ^{144}YFYPEL^{149} stays stable at the active site at its first three residues and fluctuates due to the repositioning of its last two residues, movements of peptide ^{144}YFYP^{147} are mainly due to residues Tyr at the third position and Pro at the C-terminus, as consequence of the new C-terminal group.

As shown in Table 1 and Supplementary Fig. S2 the interaction energy of the different amino acid residues of the peptides at the active site of μ-receptor was calculated. The position of peptide ^{144}YFYPEL^{149} at the base of active site is stabilized by strong van der Waals, hydrogen bond, and charge–charge interactions. This is clearly seen at the final model where the interactions of the residues of the receptor Asp^{84}, Tyr^{263}, His^{234}, and Trp^{230} interact with Tyr at the peptide N-terminus stabilizing this position (Fig. 4A). Peptide Phe and Tyr residues, at the second and third position, respectively, showed strong hydrogen bonds and van der Waals interactions between their aromatic rings and compatible μ-receptor residues that also help N-terminal Tyr to maintain its right configuration (Fig. 4B and 4C). At the top of the cavity, peptide Glu and Leu establish charge–charge and hydrogen bond interactions, building a network of electrostatic interactions between the peptide, some water molecules, and the last residues of the cavity (Fig. 4D). In the case of peptide ^{144}YFYP^{147}, the behaviour of the N-terminal Tyr at the right side of the binding pocket resembles that described above for ^{144}YFYPEL^{149} (Fig. 4E). However the interaction between the Tyr at the third position and the Gln^{61} residue from the receptor changes from van der Waals-type to hydrogen bond-type (Table 1). Moreover, an additional hydrogen bond is established with Tyr^{85}, instead of Tyr^{12} as before. Consequently, as shown in Fig. 4G, the orientation of the second Tyr residue from ^{144}YFYP^{147} differs from that of peptide ^{144}YFYPEL^{149}. In addition, the C-terminal Pro residue from ^{144}YFYP^{147} played a remarkable role in receptor-peptide relation, not described in the case of ^{144}YFYPEL^{149}, by establishing a strong charge–charge-type interaction with Arg^{148}, which modifies the configuration of this location (Table 1 and Fig. 4H).

The interactions of peptide-receptor residues were monitored during the simulation time (45 ns). In case of peptide ^{144}YFYPEL^{149}, interactions with N-terminal Tyr were stable during the simulation, whereas for the rest of residues interactions became stronger as the simulation progressed (Supplementary Fig. S3). However, main interactions of peptide ^{144}YFYP^{147} seem to follow a different behaviour. Whereas Tyr at the peptide N-terminus and Asp^{84} maintained a quite stable interaction, this peptide residue lost interaction strength with Tyr^{85} after 10 ns, and with Tyr^{263} and His^{234} at the last steps of the simulation. It is remarkable that the second peptide Tyr residue began to show a tough interaction with Tyr^{85}, Asp^{84}, and Ile^{81} at the opioid receptor after 20 ns. Similarly, C-terminal Pro increased interaction with Arg^{148} and Thr^{155} at the end of the simulation. All these modifications might be most likely due to a change in the peptide-receptor orientation.

3.3. *Activity of bovine α_{s1}-casein-derived peptides ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} on MUC5AC expression*

Because peptides ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149} had previously demonstrated stimulatory effect on mucin secretion and MUC5AC gene expression (Martínez-Maqueda et al., 2013a), the effect of the shorter derived peptides on HT29-MTX cells was also evaluated. The relative expression of MUC5AC was determined by qRT-PCR after exposure of HT29-MTX cells to the peptides. Table 2 shows the maximum relative MUC5AC

Table 1 – Binding free energy for μ-opioid receptor-peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} complexes decomposed by protein and peptide residues (vdW: Van der Waals; HB: hydrogen bond; q–q: charge–charge).

Peptide (YFYPEL)	μ-Opioid receptor	E (kcal/mol)	Interaction type	Peptide (YFYP)	μ-Opioid receptor	E (kcal/mol)	Interaction type
Tyr	ASP84	–13,75	q-q	Tyr	ASP84	–15,69	q–q
	TYR263	–5,35	HB		TYR263	–5,43	HB + vdW
	HIS234	–4,12	HB		HIS234	–3,05	HB
	ILE233	–1,79	vdW		ILE259	–1,75	vdW
	TRP230	–1,65	vdW (π-π)		ILE233	–1,54	vdW
	MET88	–1,63	vdW		MET88	–1,50	vdW
	ILE259	–1,62	vdW		TRP230	–1,33	vdW
Phe	TRP255	–3,36	HB		TYR85	–1,05	vdW
	LYS170	–1,44	vdW	Phe	VAL237	–2,23	vdW
	VAL237	–1,32	vdW		TRP255	–1,75	vdW
	ILE259	–1,12	vdW		LYS170	–1,69	vdW
	TYR85	–0.91	VdW		ILE259	–1,07	vdW
Tyr	TYR12	–2,34	HB		VAL173	–1,07	vdW
	GLN61	–2,18	vdW	Tyr	GLN61	–3,58	HB
	TRP255	–1,87	vdW		ILE81	–2,94	vdW
	ILE259	–1,64	vdW		TYR85	–2,07	HB
	HIS256	–1,10	vdW		LEU156	–1,25	vdW
Pro	GLU247	–1,68	vdW		VAL80	–1,16	vdW
Glu	LYS170	–10,65	HB + q–q		ASP84	–1,11	vdW
	GLU166	–1,28	vdW		CYS154	–1,10	vdW
Leu	ARG148	–3,38	HB + q–q	Pro	ARG148	–15,86	q–q
					THR155	–6,20	HB
					LEU156	–2,65	vdW
					GLU166	–1,13	vdW

expression level along 24 h of treatment with ^{144}YFYPE^{148}, ^{144}YFYP^{147}, and ^{144}YFY^{146} at three concentrations (5.0×10^{-5}, 1.0×10^{-4}, and 5.0×10^{-4} M). Although treatment with ^{144}YFY^{146} showed increased levels of MUC5AC after 24 h at the three concentrations, these values did not reach significance due to the high variability between replicates. For the other two peptides ^{144}YFYPE^{148} and ^{144}YFYP^{147}, no significant MUC5AC overexpression was observed compared with controls (untreated cells). From these two latter peptides, only ^{144}YFYPE^{148}, but not ^{144}YFYP^{147}, had demonstrated the ability to inhibit electrically stimulated contractions in MP–LM. According to these results, the assayed shorter forms exhibited different behaviour on the mucin gene expression in HT29-MTX cells than the longer peptides ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}.

4. Discussion

β-casomorphins were the first food-derived opioid peptides and were found in a casein hydrolysate in 1979 (Brantl et al., 1979).

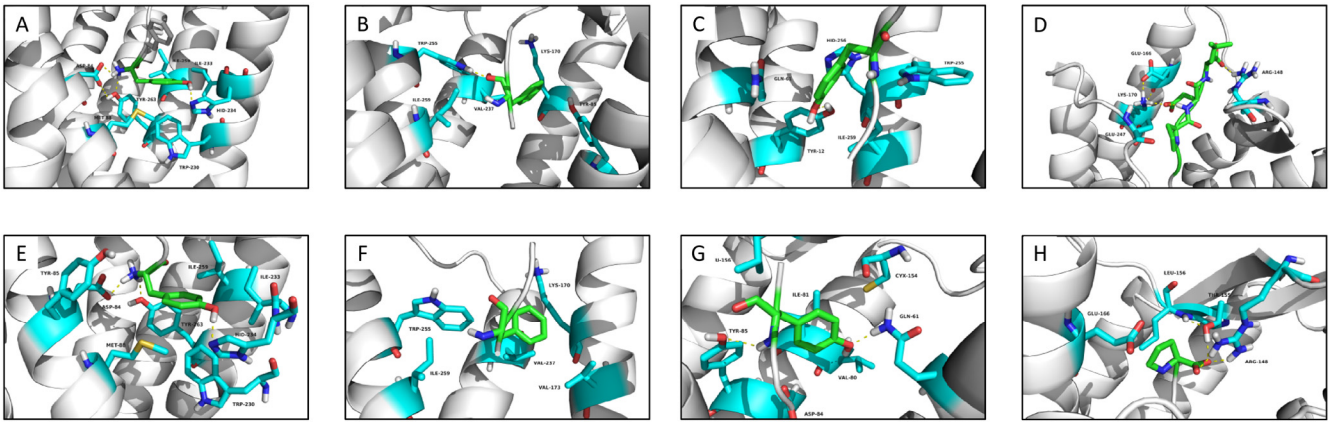


Fig. 4 – Final molecular dynamics structure of peptides ^{144}YFYPEL^{149} (A–D) and ^{144}YFYP^{147} (E–H) bound the μ-opioid receptor. Main residues involved in the interaction (showed as sticks) are highlighted in blue in the protein receptor, and those from the peptides in green. Interactions are shown by amino acid residue of each peptide ^{144}YFYPEL^{149} and ^{144}YFYP^{147}, respectively: N-terminal Tyr (A and E); Phe (B and F); Tyr at third position (C and G); Pro-Glu-Leu from ^{144}YFYPEL^{149} (D) and C-terminal Pro from ^{144}YFYP^{147} (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2 – qRT-PCR analysis of MUC5AC mRNA level in HT29-MTX cells after addition of α_{s1}-casein peptides ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147}, and ^{144}YFY^{146}.

Peptide		Relative MUC5AC expression level			Time (h)
α_{s1}-casein fragment	Sequence	5.0×10^{-5} M	1.0×10^{-4} M	5.0×10^{-4} M	
143–149[a]	AYFYPEL	1.6 ± 0.1	1.7 ± 0.3*	1.5 ± 0.2	4
144–149[a]	YFYPEL	1.2 ± 0.1	1.4 ± 0.2	1.8 ± 0.2**	4
144–148	YFYPE	0.9 ± 0.1	1.5 ± 0.4	1.3 ± 0.2	8
144–147	YFYP	1.2 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	24
144–146	YFY	1.3 ± 0.6	1.8 ± 0.8	1.9 ± 1.0	24

[a] Martínez-Maqueda et al. (2013a).

Cells were treated at different concentrations (5.0×10^{-5}, 1.0×10^{-4}, and 5.0×10^{-4} M) and times (2, 4, 8, and 24 h), and the maximum relative MUC5AC expression level is shown. Data are normalized to control (untreated cells) expression level, using cyclophilin as reference gene. The results represent the mean ± SEM of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by two-way ANOVA applying the Bonferroni test: *$P < 0.05$, **$P < 0.01$.

Since then, different opioid peptides derived from milk proteins and other food proteins have been described. The present study was undertaken to examine if two α_{s1}-casein-derived sequences, ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, which share some structural characteristics with previously described opioid peptides, could also exert this effect. These two sequences, found in a peptic casein hydrolysate, had also demonstrated to stimulate mucin secretion and gene expression in HT29-MTX cells (Martínez-Maqueda et al., 2013a), and it had been suggested that this effect could be mediated by interaction with μ-opioid receptors (Zoghbi et al., 2006). The results of this study showed, for the first time, opioid activity in the guinea pig MP–LM preparation for various related α_{s1}-casein sequences, especially ^{144}YFYPEL^{149} and ^{144}YFYPE^{148}. Other related fragments, ^{143}AYFYPEL^{149} and ^{144}YFY^{146}, were able to exert this effect, but only when peptidase inhibitors were added to the preparation.

In view of their structures, opioid peptides have been divided into two groups designated as "typical" and "atypical" (Teschemacher et al., 1997). "Typical" opioid peptides exhibit the definite N-terminal sequence YGGF, characteristic of the endogenous opioid peptides: enkephalins, endorphins, and dynorphins. "Atypical" peptides are characterized by the presence of Tyr at the N-terminus which is a structural motif important in ligand-receptor binding, although this N-terminal residue is not present in nociception/orphanin FQ (Reinscheid et al., 1995). The α_{s1}-casein derived peptides studied in this work share some structural characteristics with previously described α_{s1}-casein exorphins. Loukas, Varoucha, Zioudrou, Streaty, and Klee (1983) identified various α_{s1}-casein opioid peptides, ^{90}RYLGYLE^{96}, ^{90}RYLGYL^{95} and ^{91}YLGYLE^{96}, from a digest of casein with pepsin. Similarly to α_{s1}-casein exorphins, peptides in this work were identified in a peptic casein hydrolysate and also possess two Tyr residues, in our case separated by a Phe residue instead by the dipeptide LG, as occurs in the peptides previously described. In addition, some α_{s1}-casein exorphins are characterized by the presence of an additional Arg residue at the N-terminus. Our results show by comparison of the activity of ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149} that the presence of an additional Ala residue at the N-terminus diminishes the effect on the guinea pig ileum preparation, when no peptidases inhibitors were used. However, in the presence of peptidases inhibitors ^{143}AYFYPEL^{149} exerted certain activity, and therefore the lack of activity in the absence of peptidase inhibitors might be explained by a rapid peptide degradation.

Peptides in this study, especially ^{144}YFYPEL^{149} and ^{144}YFYPE^{148}, showed naloxone-antagonizable inhibitory effect in the electrically stimulated guinea pig ileum preparation. This isolated tissue possesses both μ- and κ-opioid receptors that regulate the contractile response when stimulated by inhibiting acetylcholine release during electrical stimulation of the myenteric plexus neurons. Although this preparation also contains cannabinoid, adenosine and adrenergic receptors (Taylor, 2011), the reversion of the effect by naloxone, a competitive antagonist at μ, δ, and κ-opioid receptors, proved the participation of these receptors on the effect of the assayed peptides. However, lower effects were found in the mouse vas deferens. This tissue can be used to study all three opioid receptor subtypes, but the expression of μ- and κ-opioid receptors is lower in this preparation than in guinea pig ileum (Taylor, 2011), the δ-opioid being the predominant receptor subtype (Lord, Waterfield, Hughes, & Kosterlitz, 1977). The activity of the assayed peptides in mouse vas deferens was not reverted by the use of naltrindole, which suggests poor affinity for δ-opioid receptors. Likewise, bovine β-casomorphins have been also demonstrated to exert a higher opioid activity in the guinea pig ileum than in the mouse vas deferens assay (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981). On the contrary, the previously described α_{s1}-casein exorphins demonstrated a moderated effect on inhibition of adenyl cyclase in neuroblastoma × glioma hybrid cells, as well as inhibition of contractions in mouse vas deferens, suggesting selectivity for δ-opioid receptors (Loukas et al., 1983). The assayed peptides show structural homologies with β-casomorphins, with an aromatic residue at the first and third positions and a proline residue at the fourth position from the N-terminal. This is in agreement with the higher activity found in the guinea pig ileum preparation. Therefore, further studies with selective opioid antagonists such as β-funaltrexamine, naltrindole, and nor-binaltorphimine for μ-, δ-, and κ-opioid receptors, respectively, are required to evaluate the selectivity of the active peptides found in this study on different receptor subtypes.

Regarding activity, these peptides containing the tripeptide YFY were found to exert an agonist opioid effect on guinea pig ileum preparations, although moderate in strength. However,

it has to be pointed that under our experimental conditions, i.e., application of increasing concentrations at 9 min intervals, peptide β-casomorphin-7, which was used as positive control, did not reach the activity values previously reported in guinea pig ileum preparations, with IC_{50} values between 3.2×10^{-6} and 6.9×10^{-6} M (Jinsmaa & Yoshikawa, 1999; Koch, Wiedemann, & Teschemacher, 1985; Yoshikawa, Fumito, Takashi, & Hideo, 1986). The time intervals used in this study were optimized to ensure signal stability. In addition, the stability of the assayed peptides in the organ bath was studied by HPLC–MS/MS. It was demonstrated that these peptides were partly hydrolysed by the action of ileum peptidases within 9 min and some of the generated fragments lost the N-terminal Tyr residue characteristic of opioid peptides. In fact, it has been reported that endogenous opioid peptides, with homologous structure than the assayed peptides, are particularly sensitive to rapid hydrolysis by a number of peptidases that are present in the guinea pig ileum, as well as in other tissues (McKnight, Corbett, & Kosterlitz, 1983). From our results, it was also shown that the shape of the inhibition curve changed by the use of the three peptidase inhibitors but the maximum inhibitory activity did not significantly increase (Fig. 1 vs. Fig. 2). The change in the accumulative inhibition curves compared to experiments conducted in the absence of peptidase inhibitors confirmed that the decrease observed at higher doses in the absence of peptide inhibitors is caused by the peptide degradation during the assay. The combination of these peptidase inhibitors (captopril, amastatin and phosphoramidon 1.0×10^{-6} M) was not able to change the activity of morphine, DAMGO and metabolically stable synthetic opioid peptides in this preparation (Hiranuma et al., 1998). Aoki, Kajiwara, and Oka (1984) and McKnight et al. (1983) demonstrated that the peptidase inhibitor mixtures captopril 1.0×10^{-6}, bestatin 10.0×10^{-6}, and thiorphan 1.0×10^{-6} M, and captopril 10.0×10^{-6}, bestatin 10.0×10^{-6}, thiorphan 0.3×10^{-6}, and L-Leu-L-Leu 2.0×10^{-3} M, respectively, did not affect, alone nor in combination, the contraction of the guinea pig MP–LM.

In order to explain the different opioid activity found for these structurally similar peptides, molecular dynamic simulations were performed with ^{144}YFYPEL^{149} which showed opioid activity in guinea pig ileum, and ^{144}YFYP^{147} which was inactive. The homology model of μ-opioid receptor with the peptides docked at the active site was evaluated by the study of the energy stability and the identification of those responsible binding-residues at the cavity. The simulations with both peptides showed a notably energy variability in intra- and extracellular loops of the receptor at positions 145–150, 195–200, 240–250 and the C-terminal domain. This structural flexibility is in agreement with previous studies with the μ-opioid receptor (Serohijos et al., 2011). Similarly, it has been found that morphine leads to a greater flexibility of the third intra-cellular loop of the receptor, which is consistent with the critical role of this loop as the docking site of G-proteins binding (Waldhoer, Bartlett, & Whistler, 2004). Due to the flexibility observed in our simulation, it can be suggested that peptide ^{144}YFYPEL^{149} behaves as an opioid agonist. For peptide ^{144}YFYP^{147}, the flexible areas of the receptor were similar, but a remarkable difference was found at the movement of the loop 195–200, which is related to the activity of the receptor. In addition, while peptide ^{144}YFYPEL^{149} evoked a continuous change in this loop during the simulation, with peptide ^{144}YFYP^{147} it became fixed. Moreover, it has been found that both peptides establish two strong hydrogen bonds and a charge–charge interaction, in the same way as the morphinan agonist BU72 (Huang et al., 2015), between the N-terminal Tyr at the peptide and Tyr^{263}, His^{234}, and Asp^{84} residues. However, and in contrast with the ^{144}YFYPEL^{149} simulation, the second Tyr residue and Pro from ^{144}YFYP^{147} were found to create different interactions with the receptor, resulting in a clear change between the locations of both peptides at the active binding site. Besides, another fact that might contribute to the different behaviour is the orientation of Arg^{148} and Glu^{166} receptor residues. When peptide ^{144}YFYP^{147} was bound to the receptor, Arg^{148} and Glu^{166} residues had to move towards the interior of the binding pocket to interact with C-terminal Pro of ^{144}YFYP^{147}. This process would require a large amount of energy due to their charged character, and when exposed to the solvent, would be highly solvated. The energy needed to remove this solvation shell might render peptide ^{144}YFYP^{147} inactive.

Previously, Zoghbi et al. (2006) had reported the activity of β-casomorphin-7 on mucin production in HT29-MTX cells via μ-opioid interactions. Given the effect observed in guinea pig ileum preparations, it was expected that in addition to ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, peptide ^{144}YFYPE^{148} could also induce MUC5AC gene expression. However, although this peptide at 1.0×10^{-4} and 5.0×10^{-4} M showed increased expression levels over the control, it did not reach significance. There is sufficient literature that illustrates the lack of correlation between μ-opioid potency and effect on mucin secretion or gene overexpression. For instance, in a screening of several peptides with proved or probable μ- and δ-opioid activity, six out of eight peptides induced mucin secretion in HT29-MTX cells (Martínez-Maqueda et al., 2012). The evaluation of the effect on MUC5AC expression by α-lactorphin-amide and human β-casomorphin-5 revealed that only the first provoked a significant gene overexpression, despite both behaving as mucin secretors (Martínez-Maqueda et al., 2012), β-casomorphin-5 being more potent than α-lactorphin-amide in guinea pig preparations (IC_{50} values of 14×10^{-6} and 50×10^{-6} M, respectively) (Yoshikawa et al., 1986). Similarly, a recent report has shown that the μ-opioid peptide neocasomorphin-6 (YPVEPF) induced a significant rise in the transmembrane-associated mucin gene MUC4 but without effect on neither the expression of secreted MUC2 and MUC5AC nor the release of mucin-like glycoprotein (Plaisancié et al., 2015). The effect on mucin production by food protein hydrolysates and peptides appears to be mediated by more than one type of mechanism. It remains to be clarified whether any of these α_{s1}-casein peptide fragments might stimulate mucin secretion and/or induce mucin gene overexpression other than MUC5AC.

In conclusion, various α_{s1}-casein fragments containing the tripeptide YFY, especially ^{144}YFYPEL^{149} and ^{144}YFYPE^{148}, have shown for the first time opioid activity in guinea pig ileum preparations. Other related fragments, ^{143}AYFYPEL^{149} and ^{144}YFY^{146}, were able to exert this effect, but only when peptidase inhibitors were added to the preparation. This effect was antagonized by naloxone, which demonstrates the implication of opioid receptors. The susceptibility of these peptides to be hydrolysed by peptidases found in this tissue was demonstrated by HPLC–MS/MS. Several of the resulting fragments

lost the N-terminal Tyr residue, and therefore the potential ability to interact with opioid receptors. Similar to β-casomorphins, the assayed peptides exerted opioid activity on mouse vas deferens to a lower extent than in guinea pig ileum. The lack of activity of peptide ^{144}YFYP^{147} can be explained by the different position of the third intracellular loop at the receptor. In addition, it was found that interactions of Pro residue at ^{144}YFYPEL^{149} and ^{144}YFYP^{147} were established at different places of the receptor active site. The study of the MUC5AC expression in human goblet cells HT29-MTX has shown that bovine α_{s1}-casein ^{144}YFYPEL^{149} is the minimum fragment able to stimulate mucin production, whereas the smaller fragments ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} had no effect on MUC5AC expression, although activity on other mucin mRNA levels or on mucin secretion cannot be discarded.

Acknowledgements

This work has received financial support from projects AGL2015-66886-R from the Spanish Ministry of Economy and Competitiveness (MINECO), SAF2012-40075-C02-01 from the Spanish Ministry of Science and Innovation (MICINN), and FP7-SME-2012-315349 (FOFIND) financed by the European Commission (EC). S. F.-T. acknowledges MINECO for his FPI fellowship. The authors would like to thank Guadalupe Pablo for her excellent technical assistance.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.06.023.

REFERENCES

Akahori, K., Kosaka, K., Jin, X. L., Arai, Y., Yoshikawa, M., Kobayashi, H., & Oka, T. (2008). Great increase in antinociceptive potency of [Leu5]enkephalin after peptidase inhibition. *Journal of Pharmacology Sciences*, *106*, 295–300.

Ambache, N. (1954). Separation of the longitudinal muscle of the rabbit's ileum as a broad sheet. *Journal of Physiology*, *125*, 53P–55P.

Antila, P., Paakkari, I., Järvinen, A., Mattila, M. J., Laukkanen, M., Pihlanto-Leppälä, A., Mäntsälä, P., & Hellman, J. (1991). Opioid peptides derived from in-vitro proteolysis of bovine whey proteins. *International Dairy Journal*, *1*, 215–229.

Aoki, K., Kajiwara, M., & Oka, T. (1984). The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive "enkephalinase" in the potency of enkephalins in the guinea-pig ileum. *Japan Journal of Pharmacology*, *36*, 59–65.

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., & Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International*, *63*, 147–156.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition*, *97*, 1314–1323.

Brantl, V., Teschemacher, H., Bläsig, J., Henschen, A., & Lottspeich, F. (1981). Opioid activities of β-casomorphins. *Life Sciences*, *28*, 1903–1909.

Brantl, V., Teschemacher, H., Henschen, A., & Lottspeich, F. (1979). Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie*, *360*, 1211–1216.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollès, P., & Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie*, *80*, 155–165.

Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., & Plaisancié, P. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *283*, G521–G528.

Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., & Sali, A. (2006). Comparative protein structure modeling using Modeller. Chapter 5. In *Current protocols in bioinformatics*. New York: Wiley. Unit 5.6.

Fenalti, G., Zatsepin, N. A., Betti, C., Giguere, P., Han, G. W., Ishchenko, A., Liu, W., Guillemyn, K., Zhang, H., James, D., Wang, D., Weierstall, U., Spence, J. C., Boutet, S., Messerschmidt, M., Williams, G. J., Gati, C., Yefanov, O. M., White, T. A., Oberthuer, D., Metz, M., Yoon, C. H., Barty, A., Chapman, H. N., Basu, S., Coe, J., Conrad, C. E., Fromme, R., Fromme, P., Tourwe, D., Schiller, P. W., Roth, B. L., Ballet, S., Katritch, V., Stevens, R. C., & Cherezov, V. (2015). Structural basis for bifunctional peptide recognition at human delta-opioid receptor. *Nature Structural & Molecular Biology*, *22*, 265–268.

Hiranuma, T., Kitamura, K., Taniguchi, T., Kanai, M., Arai, Y., Iwao, K., & Oka, T. (1998). Protection against dynorphin-(1–8) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. *The Journal of Pharmacology and Experimental Therapeutics*, *286*, 863–869.

Huang, W., Manglik, A., Venkatakrishnan, A. J., Laeremans, T., Feinberg, E. N., Sanborn, A. L., Kato, H. E., Livingston, K. E., Thorsen, T. S., Kling, R. C., Granier, S., Gmeiner, P., Husbands, S. M., Traynor, J. R., Weis, W. I., Steyaert, J., Dror, R. O., & Kobilka, B. K. (2015). Structural insights into μ-opioid receptor activation. *Nature*, *524*, 315–321.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., & Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*, *258*, 577–579.

Jinsmaa, Y., & Yoshikawa, M. (1999). Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. *Peptides*, *20*, 957–962.

Koch, G., Wiedemann, K., & Teschemacher, H. (1985). Opioid activities of human β-casomorphins. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *331*, 351–354.

Lesuffleur, T., Porchet, N., Aubert, J. P., Swallow, D., Gum, J. R., Kim, Y. S., Real, F. X., & Zweibaum, A. (1993). Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. *Journal of Cell Science*, *106*, 771–783.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods: A Companion to Methods in Enzymology*, *25*, 402–408.

Lord, J. A. H., Waterfield, A. A., Hughes, J., & Kosterlitz, H. W. (1977). Endogenous opioid peptides: Multiple agonists and receptors. *Nature*, *267*, 495–499.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., & Klee, W. A. (1983). Opioid activities and structures of alpha-casein-derived exorphins. *Biochemistry*, *22*, 4567–4573.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., & Recio, I. (2013a). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *International Dairy Journal*, *32*, 13–19.

Martínez-Maqueda, D., Miralles, B., De Pascual-Teresa, S., Reverón, I., Muñoz, R., & Recio, I. (2012). Food-derived peptides stimulate mucin secretion and gene expression in intestinal cells. *Journal of Agricultural and Food Chemistry*, *60*, 8600–8605.

Martínez-Maqueda, D., Miralles, B., Ramos, M., & Recio, I. (2013b). Effect of beta-lactoglobulin hydrolysate and beta-lactorphin on intestinal mucin secretion and gene expression in human goblet cells. *Food Research International*, *54*, 1287–1291.

McKnight, A. T., Corbett, A. D., & Kosterlitz, H. W. (1983). Increase in potencies of opioid peptides after peptidase inhibition. *European Journal of Pharmacology*, *86*, 393–402.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers*, *43*, 119–128.

Meisel, H., & Fitzgerald, R. J. (2000). Opioid peptides encrypted in intact milk protein sequences. *The British Journal of Nutrition*, *84*, S27–S31.

Plaisancié, P., Boutrou, R., Estienne, M., Henry, G., Jardin, J., Paquet, A., & Léonil, J. (2015). β-casein(94–123)-derived peptides differently modulate production of mucins in intestinal goblet cells. *Journal of Dairy Research*, *82*, 36–46.

Plaisancié, P., Claustre, J., Estienne, M., Henry, G., Boutrou, R., Paquet, A., & Léonil, J. (2013). A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. *Journal of Nutritional Biochemistry*, *24*, 213–221.

Reinscheid, R. K., Nothacker, H.-P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J., & Civelli, O. (1995). Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor. *Science*, *270*, 792–794.

Rutherfurd-Markwick, K. J. (2012). Food proteins as a source of bioactive peptides with diverse functions. *The British Journal of Nutrition*, *108*, S149–S157.

Serohijos, A. W. R., Yin, S., Ding, F., Gauthier, J., Gibson, D. G., Maixner, W., Dokholyan, N. V., & Diatchenko, L. (2011). Structural basis for μ-opioid receptor binding and activation. *Structure (London, England: 1993)*, *19*, 1683–1690.

Tai, E. K. K., Helen, P. S. W., Emily, K. Y. L., William, K. K. W., Yu, L., Marcel, W. L. K., & Cho, C. H. (2008). Cathelicidin stimulates colonic mucus synthesis by up-regulating MUC1 and MUC2 expression through a mitogen-activated protein kinase pathway. *Journal of Cellular Biochemistry*, *104*, 251–258.

Taylor, D. A. (2011). In vitro opioid receptor assays. *Current Protocols in Pharmacology*, *55*(4.8), 4.8.1–4.8.34.

Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design*, *9*, 1331–1344.

Teschemacher, H., Koch, G., & Brantl, V. (1997). Milk protein-derived opioid receptor ligands. *Biopolymers*, *43*, 99–117.

Trompette, A., Claustre, J., Caillon, F., Jourdan, G., Chayvialle, J. A., & Plaisancie, P. (2003). Milk bioactive peptides and beta-casomorphins induce mucus release in rat jejunum. *The Journal of Nutrition*, *133*, 3499–3503.

Waldhoer, M., Bartlett, S. E., & Whistler, J. L. (2004). Opioid receptors. *The Annual Review of Biochemistry*, *73*, 953–990.

Yoshikawa, M. (2015). Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides*, *72*, 208–225.

Yoshikawa, M., Fumito, T., Takashi, Y., & Hideo, C. (1986). Opioid peptides from milk proteins. *Agricultural and Biological Chemistry*, *50*, 2419–2421.

Zhao, Q., Garreau, I., Sannier, F., & Piot, J. M. (1997). Opioid peptides derived from hemoglobin: Hemorphins. *Peptide Science*, *43*, 75–98.

Zoghbi, S., Trompette, A., Claustre, J., Homsi, M. E., Garzon, J., Jourdan, G., Scoazec, J.-Y., & Plaisancie, P. (2006). beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway. *American Journal of Physiology-Gastrointestinal Liver Physiology*, *290*, G1105–G1113.

Supplementary Materials and Methods

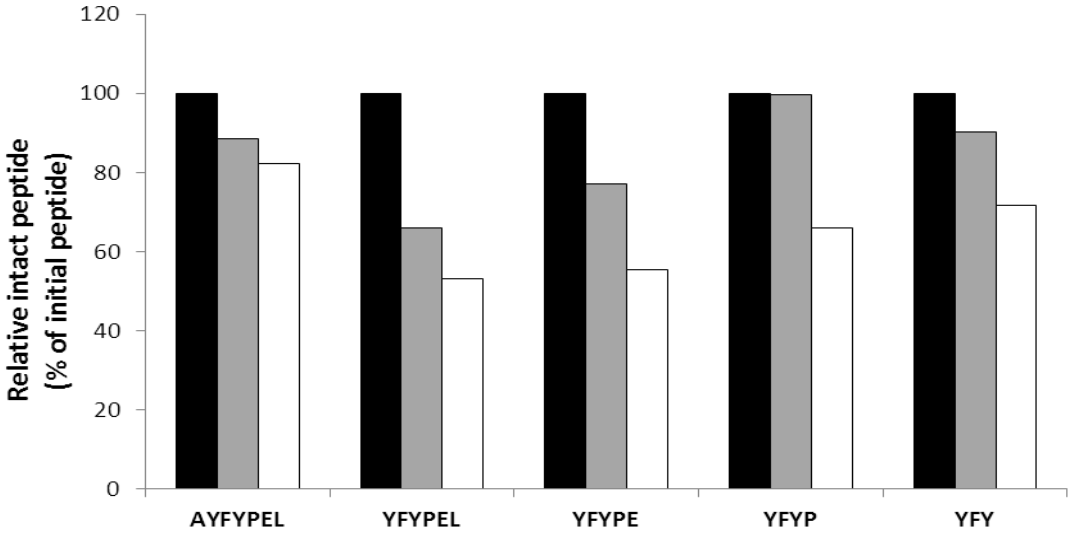
Molecular modelling receptor-peptide

Using the Membrane Builder module (Jo, Kim, & Im, 2007; Jo, Lim, & Im, 2009) in CHARMM-GUI (www.charmm-gui.org) (Jo, Kim, & Im, 2008), each protein was inserted in a pre-equilibrated box containing a POPC lipid bilayer, POPE lipid bilayer, cholesterol molecules and water, and a 0.15 M concentration of Na^+ and Cl^- ions. We performed molecular dynamics simulations using AMBER, the protocol was as followed: (i) energy minimization to relax the initial model (20 ps), (ii) heating (100k), holding lipid molecules and Cα carbons fixed (100 ps), (iii) heating (303k), holding lipid molecules and Cα carbons fixed (100 ps), (iv) holding, to equilibrate the simulation box (5 x 300 ps), (v) production at constant temperature and pressure (45 ns). All the simulations were performed at constant pressure (1 atm) and temperature (300 °K) with an integration time step of 2 fs. The SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1977) was used to constrain all the bonds involving H atoms at their equilibrium distances. Periodic boundary conditions and the particle mesh Ewald methods were applied to treat long-range electrostatic effects (Darden, York, & Pedersen, 1993). AMBER ff03 (Duan et al., 2003), Lipid11 (Skjevik, Madej, Walker, & Teigen, 2012), Lipid14 (Callum et al., 2014) and TIP3P (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) force fields were used in all cases. Finally, the effective binding free energies were estimated with the MM-GBSA (Still, Tempczyk, Hawley, & Hendrickson, 1990) approach that calculates the free energy of binding as a sum of a molecular mechanics (MM) interaction term, a solvation contribution through a generalized Born (GB) model, and a surface area (SA) contribution to account for the nonpolar part of the desolvation. A 12-6 Lennard-Jones term was used to model the MM contribution. For GB, the solute dielectric constant was set to 4, whereas that of the solvent was set to 80. The dielectric boundary was calculated using a solvent probe radius of 1.4 Å. The SA contribution was approximated as a linear relationship to the change in solvent accessible surface area (SASA). All the trajectories and analysis were performed using the AMBER 12 computer program and associated modules (Case et al., 2012). Free energy

decomposition interaction matrices, obtained from the same snapshots, were represented in an energy-dependent color gradient using Matrix2png (Pavlidis & Noble, 2003).

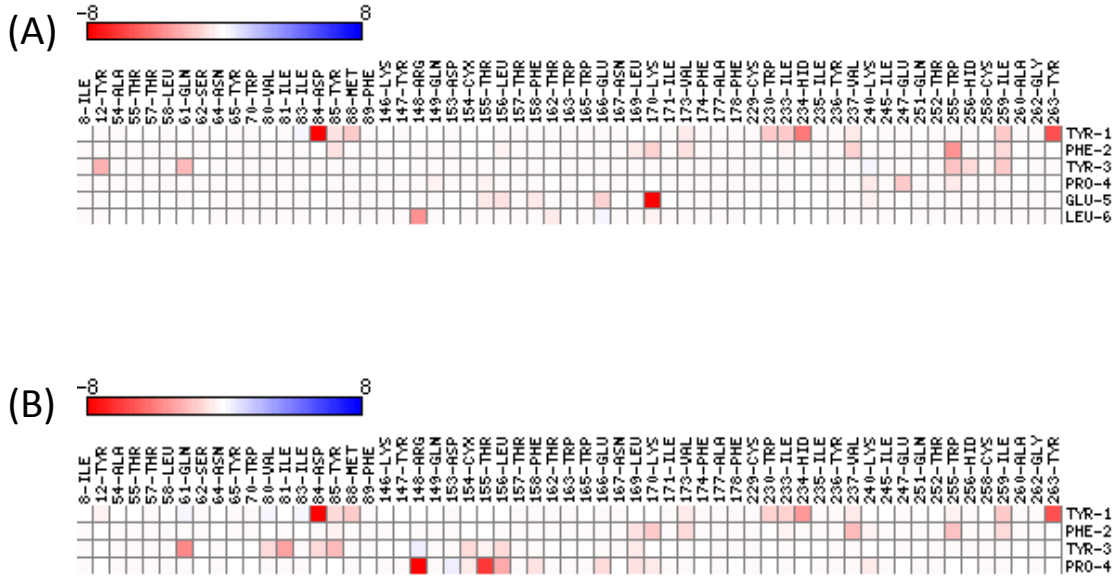
Supplementary Results

Supplementary Figure 1



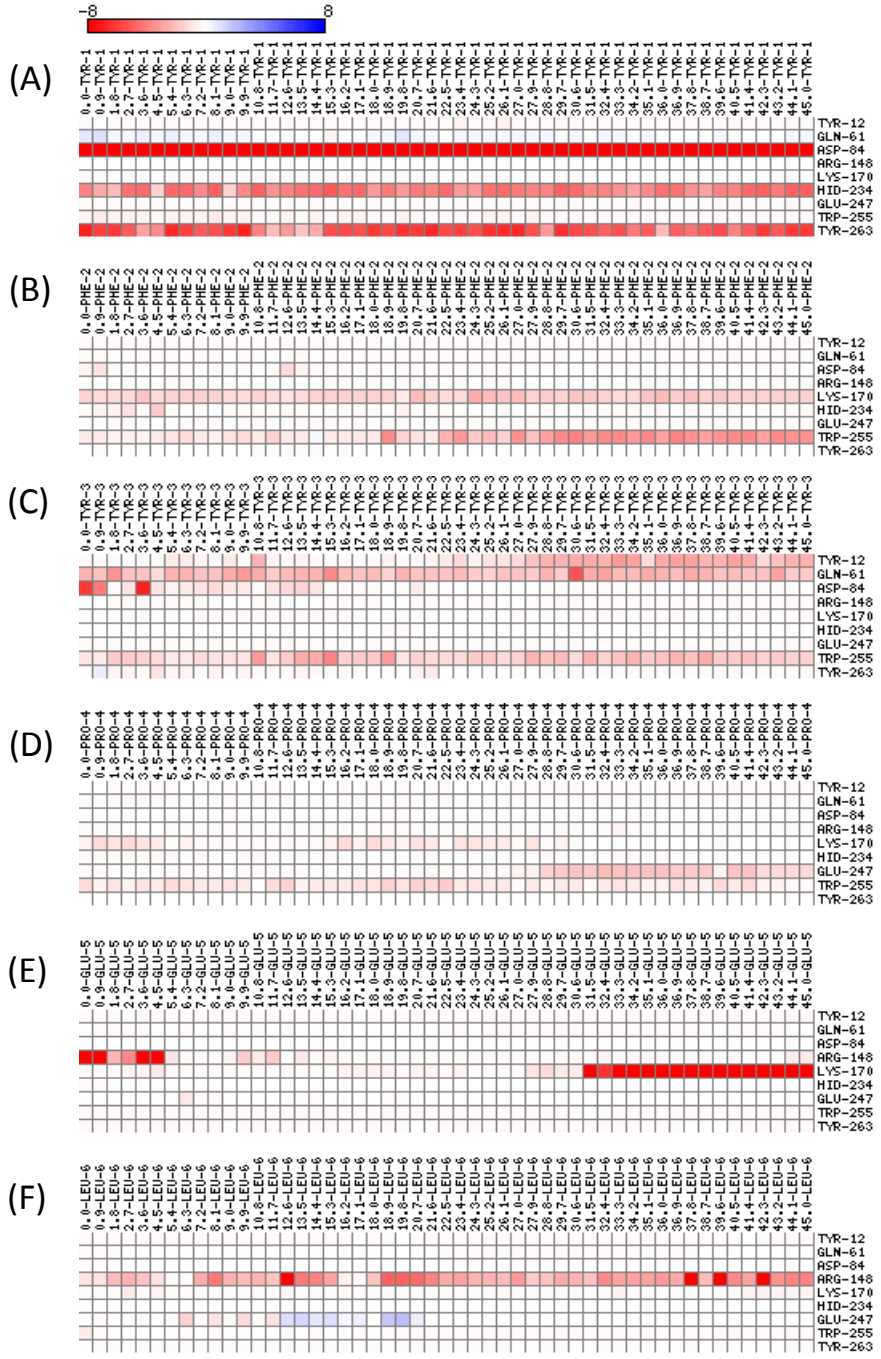
Supplementary Figure 1. Time course stability of α_{s1}-casein fragments ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147}, and ^{144}YFY^{146}. Peptides were added at 5.0×10^{-6} M to the organ bath and aliquots were withdrawn at time 0 ■ , 4 ▒ and 9 □ minutes. Relative area (expressed as % of initial peptide, considered as 100%) was determined by RP-HPLC-MS/MS from the extracted ion chromatogram of the molecular ion of ^{143}AYFYPEL^{149} *m/z* 902.4 (charge +1), ^{144}YFYPEL^{149} *m/z* 831.4 (charge +1), ^{144}YFYPE^{148} *m/z* 718.3 (charge +1), ^{144}YFYP^{147} *m/z* 589.3 (charge +1), and ^{144}YFY^{146} *m/z* 492.2 (charge +1).

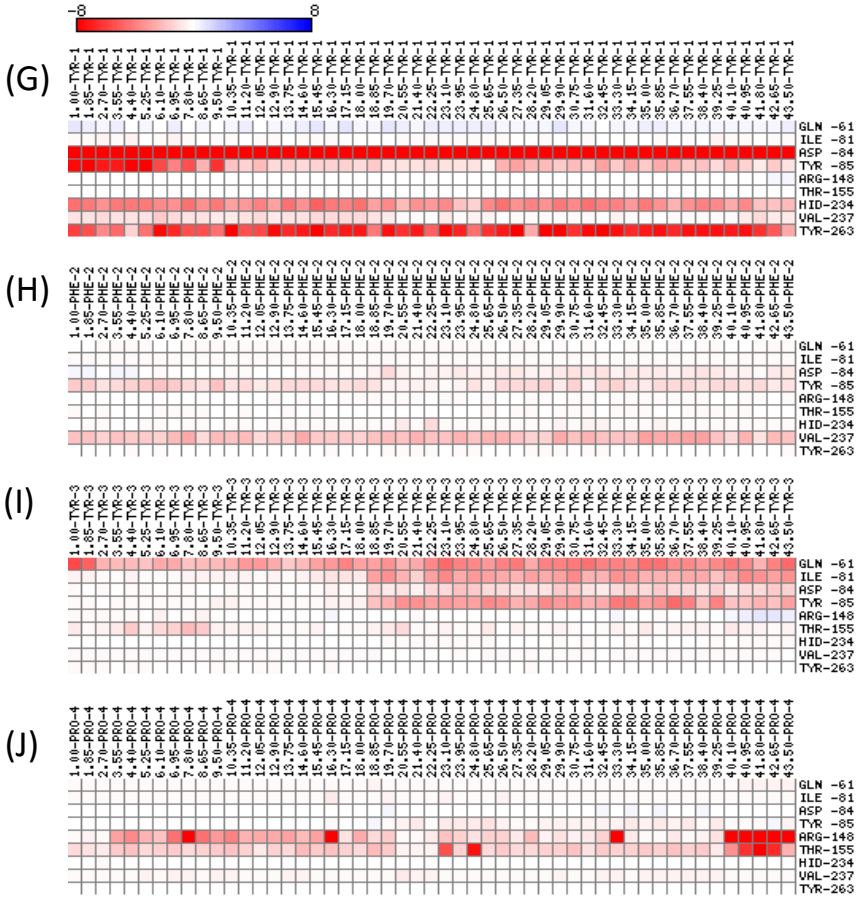
Supplementary Figure 2



Supplementary Figure 2. Per residue interactions of peptides (A) ^{144}YFYPEL^{149} and (B) ^{144}YFYP^{147} with the μ-opioid receptor. X axis shows the name and number of the μ-opioid receptor residues and Y axis the name and number of peptide residues. The color scale is based on the energy value from red (most favorable) to blue (less favorable).

Supplementary Figure 3





Supplementary Figure 3. Per residue interactions of peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} with the μ-opioid receptor. X axis shows the name of the peptide residue at each step of the molecular dynamic simulation. Y axis shows the name and number of the receptor residues interacting with the peptide. The color scale is based on the energy value from red (most favorable) to blue (less favorable). For each peptide ^{144}YFYPEL^{149} and ^{144}YFYP^{147}, respectively: N-terminal Tyr (A and G); Phe (B and H); Tyr at third position (C and I); Pro (D and J); Glu from ^{144}YFYPEL^{149} (E) and C-terminal Leu from ^{144}YFYPEL^{149} (F).

Supplementary References

Callum, J., Dickson, B. D., Madej, Å. A., Skjevik, R. M., Betz, K. T., Ian, R. G., & Ross C. W. (2014). Lipid14: The Amber Lipid Force Field. *The Journal of Chemical Theory and Computation, 10*, 865-879.

Case, D. A., Darden, T. A., Cheatham, T. E., Simmerling, C. L., Wang, J., Duke, R. E., Luo, R., Walker, R. C., Zhang, W., Merz, K. M., Roberts, B., Hayik, S., Roitberg, A., Seabra, G., Swails, J., Götz, A. W., Kolossváry, I., Wong, K. F., Paesani, F., Vanicek, J., Wolf, R. M., Liu, J., Wu, X., Brozell, S. R., Steinbrecher, T., Gohlke, H., Cai, Q., Ye, X., Wang, J., Hsieh M. -J., Cui, G., Roe, D. R., Mathews, D. H., Seetin, M. G., Salomon-Ferrer, R., Sagui, C., Babin, V., Luchko, T., Gusarov, S., Kovalenko, A., & Kollman, P. A. (2012). AMBER 12, University of California, San Francisco.

Darden, T., York, D., & Pedersen, L. (1993). Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. *The Journal of Chemical Physics, 98*, 10089−10092.

Duan, Y., Wu, C., Chowdhury, S., Lee, M. C., Xiong, G., Zhang, W., Yang, R., Cieplak, P., Luo, R., Lee, T., Caldwell, J., Wang, J., & Kollman, P. (2003). A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *Journal of Computational Chemistry, 24*, 1999−2012.

Jo, S., Kim, T., & Im, W. (2007). Automated builder and database of protein/membrane complexes for molecular dynamics simulations. *PLoS ONE, 2*, e880.

Jo, S., Kim, T., & Im, W. (2008). CHARMM-GUI: a web-based graphical user interface for CHARMM. *Journal of Computational Chemistry, 29*, 1859–1865.

Jo, S., Lim, J. B., & Im, W. (2009). CHARMM-GUI Membrane Builder for mixed bilayers and its application to yeast membranes. *Biophysical Journal*, 97, 50–58.

Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., & Klein, M. L. (1983). Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics, 79*, 926−935.

Pavlidis, P., & Noble, W. S. (2003). Matrix2png: A utility for visualizing matrix data. *Bioinformatics*, *19*, 295−296.

Ryckaert, J. -P., Ciccotti, G., & Berendsen, H. J. C. (1977). Numerical integration of the cartesian equations of motion of a system with constraints: Molecular dynamics of n-alkanes. *Journal of Computational Physics, 23,* 327−341.

Skjevik, Å. A., Madej, B. D., Walker, R. C., & Teigen, K. (2012). LIPID11: A modular framework for lipid simulations using Amber. *The Journal of Physical Chemistry B*, *116,* 11124−11136.

Still, W. C., Tempczyk, A., Hawley, R. C., & Hendrickson, T. (1990). Semianalytical treatment of solvation for molecular mechanics and dynamics. *Journal of the American Chemical Society, 112*, 6127−6129.

Short communication: Effect of a casein hydrolyzate on mucin secretion and gene expression in the zucker rat intestine

Samuel Fernández-Tomé*, Daniel Martínez-Maqueda*, María Tabernero†‡, Beatriz Miralles*, Carlota Largo†, Isidra Recio*[1]

*****Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

†Hospital La Paz, Health Research Institute (IdiPaz), Paseo de la Castellana, 261, 28046 Madrid, Spain

‡IMDEA-Food Institute, CEI (UAM+CSIC), Carretera de Canto Blanco, 8, 28049 Madrid, Spain

[1] Corresponding author: Isidra Recio

Nicolás Cabrera, 9. 28049 Madrid, Spain

Phone: +34 910017940

Fax: +34 910017905

e-mail: i.recio@csic.es

ABSTRACT

The effect of a peptic casein hydrolysate on the relative gene expression level of mucins *Muc2, Muc3* and *Muc4* in the intestine of obese rats was investigated by quantitative reverse transcription-PCR. In addition, secretion of fecal mucins at the 2nd and 8th week and of luminal mucins at the end of the experimental period (8 weeks) was also evaluated by a fluorimetric assay. This casein hydrolysate induced a significant increase in ileal and colonic mucin mRNA expression. Concretely, it was found an increased expression for the genes that codifies for the secreted *Muc2* and the membrane-bound *Muc3*. In agreement with this gene overexpression, it was found a significant rise of O-linked glycoproteins in feces after 2 and 8 weeks of administration of the casein hydrolyzate. Mucus material in the small intestine lumen of rats fed the casein hydrolyzate was higher than that found in control rats (124%), however the change did not reach statistical difference. This hydrolyzate had been previously shown a mucin stimulatory effect in human goblet cells (HT29-MTX) and this study confirms *in vivo* the potential effects on mucus gastrointestinal protection.

KEYWORDS: casein hydrolyzate, mucin, rat intestine, gene expression, mucin secretion

The mucus gel layer covering the gastrointestinal surface is a major component of physiological defense mechanisms. This protective capacity is mainly attributed to its principal component, mucins, high molecular weight glycoproteins secreted by goblet cells. Interestingly some dietary compounds can positively modulate the dynamics of mucus, proving thus potential to prevent the gastrointestinal barrier-related disorders (Bischoff et al., 2014). Obesity is associated with several metabolic and inflammatory pathologies. The impact of intestinal permeability in the inflammatory-obese disorders has been postulated, via an increase in the circulating levels of bacterial lipopolysaccharide (Cani et al., 2007). Interestingly, dietary administration of prebiotics has shown positive effects in intestinal integrity, reducing inflammation and improving metabolic conditions (Cani et al., 2009).

The specific effect of food proteins on intestinal mucus production was first demonstrated for milk casein and α-lactalbumin hydrolyzates by using an isolated *ex-vivo* preparation of rat jejunum (Claustre et al., 2002). Similarly, the opioid peptide β-casomorphin-7, β-casein f(60-66), also gave rise to a marked increase on mucin discharge in this tissue (Trompette et al., 2003). Later on, Zoghbi et al. (2006) found that this peptide induced mucin secretion and gene overexpression in rat (DHE) and human (HT29-MTX) goblet cells though activation of μ-opioid intestinal receptors. Likewise, the peptide β-casein fragment (94-123) increased mucin release and mRNA transcripts in HT29-MTX cells (Plaisancié et al., 2013), and similar results were shown for some fragments comprised within its 30-amino acid sequence (Plaisancié et al., 2015). However, just a few animal studies have been carried out to reveal *in vivo* evidence on mucus protection by dairy components.

Recently, our group found that a peptic casein hydrolyzate (CH) and the contained α_{s1}-casein peptides ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149} exerted stimulatory effect on mucin secretion in human HT29-MTX cells and on the expression of *MUC5AC*, the major secreted mucin gene in this cell line (Martínez-Maqueda et al., 2013). Therefore, the aim of the present study was to investigate the *in vivo* effect of this CH on the expression of various intestinal mucin genes. In rodent intestine the main mucins are the secreted *Muc2* confined to goblet cells, and the transmembrane *Muc3* and *Muc4* detectable in both goblet cells and enterocytes (Trompette et al., 2004). Zucker rats were used in order to evaluate the potential effect of the hydrolyzate to

improve intestinal integrity. In addition, the influence on mucin secretion in fecal and gut luminal contents was also evaluated. Based on previous animal studies, the relevance of dairy compounds on mucus gastrointestinal protection is briefly discussed.

The study protocol was approved by the Institutional Animal Ethics Committee of La Paz University Hospital (Madrid, Spain) and procedures were performed in accordance with the EU Directive 2010/63/EU and the Spanish law RD 53/2013 regarding the protection of experimental animals. Eighteen male 8-week-old zucker rats, weighing 426-521 g were purchased from Charles River Laboratories (Barcelona, Spain) and were randomly assigned to the control or the experimental group (n = 9 per group). Rats were placed in separate cages holding 3 animals per cage, and during 1 week of acclimation period they had access to water and commercial standard rodent diet (Scientific Animals Food & Engineering A04, Augy, France) *ad libitum*. After acclimation period, experimental group received a diet supplemented with 0.7% CH, providing approximately 0.4 g CH/kg of animal per day. Blood pressure, weight gain and food intake were monitored weekly.

Following the experimental feeding period (8 weeks), rats were anaesthetized using 1.5% isoflurane and subjected to complete intracardiac exsanguination. Plasma was separated out by centrifugation (10 min at 2500 *g*) in pre-treated EDTA tubes with complete peptidase inhibitor cocktail (Roche Applied Science, Madrid, Spain), and immediately frozen at – 20 ºC. Plasma biochemical analysis were performed in a COVAS Autoanalyzer (Roche Applied Science) and circulating levels of glucagon-like peptide-1, leptin, insulin and proinflamatory cytoquines (interleukin-6 and tumor necrosis factor-□) were determined by immunoassay in a Luminex LX200 analyzer using the Rat Adipokine multiplex kit (RADPK-81K, Milliplex® Map Kit, Rat Serum Adipokine Panel, Millipore, Billerica, MA, US), according to manufacturer´s instructions.

Duodenum, jejunum, ileum and colon samples were removed and immediately stored in RNAlater® (Applied Biosystems, Carlsbad, CA, USA), according to manufacturer's instructions. Total RNA from the gut sections (20 mg) was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany). The RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and

Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR amplification was performed using the ViiA™ 7 Real-Time PCR System in 384 wells microplates with TaqMan® Gene Expression Assays (FAM™ dye-labeled MGB probe), according to manufacturer's instructions (all from Applied Biosystems). For each sample, PCR amplification was carried out in a reaction volume of 10 µL with cDNA samples (1:5 dilution, 5.06 ng) in triplicate. No-template controls were also included. Based on previous experiments, *β-actin* (accession no. 116659) was used as reference gen. The relative expression levels of the target genes, *Muc2, Muc3* and *Muc4* (accession no. 24572, 687030 and 303877, respectively) were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data were analyzed with DataAssist 3.01 software (Applied Biosystems). Significant differences were determined by a two-tailed Mann-Whitney test, and *P* values were adjusted using Benjamini-Hochberg false discovery rate procedure.

Feces were collected from cages at weeks 2 and 8. Luminal contents of small intestine (jejunum to ileum) at the end of the experimental period (8 weeks) were collected by flushing with 20 mL of ice-cold PBS (sodium azide 0.02 *M*, pH 7.4) and the same volume of air. Feces and luminal contents were kept at – 20 ºC and freeze-dried. Mucin fraction from feces (Sprong et al., 2010) and luminal contents (Lien et al., 1996) were isolated as previously described, and measured using a fluorimetric assay (Crowther and Wetmore, 1987). Standard solution of commercial porcine stomach mucins (Sigma–Aldrich Química, Madrid, Spain) was used to calculate the total amount of fecal and luminal mucins. Data were analyzed using GraphPad Prism 6.01 software, and significant differences were determined by a two-tailed Mann-Whitney test.

Animals tolerated well the experimental conditions and gained weight during the feeding period, being similar the weight gained in both experimental groups. Plasma lipid profile, glucose, hormones and cytokines levels were not significantly different in treated rats, compared to control rats. Our results are in line with the safety data reported for this CH after acute and repeated dose (4 weeks) oral toxicity studies in rats (Anadón et al., 2010).

Figure 1 shows the modulatory effect of the CH on the relative gene expression level of mucins *Muc2, Muc3* and *Muc4* in the rat intestine, determined by quantitative reverse transcription-PCR. In duodenum tissue, the CH inhibited the expression of *Muc2* (0.70 ± 0.06-fold change) and *Muc4* (0.81 ± 0.04-fold change), while any statistical variation compared to control rats was found in the jejunum. However, the hydrolyzate induced an increase in ileal and colonic mucin mRNA expression. Transcription level of both the secreted *Muc2* and the transmembrane *Muc3* mucins were significantly raised in the ileum (1.31 ± 0.07- and 1.20± 0.06-fold change, respectively) and colon (1.51 ± 0.10- and 1.47 ± 0.06-fold change, respectively). Although the transmembrane *Muc4* mucin gene expression was also enhanced with respect to control rats in the colonic tissue, it was not statistically different. Interestingly, the CH revealed an increasing trend in the modulatory effect along the intestine location, i.e., mucins mRNA transcripts, mainly *Muc2*, tended to be below or within control levels at proximal sections (duodenum and jejunum) and over control levels at distal sections (ileum and colon). Mucus thickness in the rat intestine has been shown to vary depending on the gut section, being the colonic layer, and to a lower extent the ileal, up to four times thicker compared to other regions (Atuma et al., 2001). This situation might imply a worthy biological relevance for the CH regulatory effect on the intestinal mucus layer. Strong evidence about the protective implication of mucins at intestine, especially in colon, was demonstrated when spontaneous colitis (Van der Sluis et al., 2006) and colorectal cancer (Velcich et al., 2002) were developed in mice genetically deficient in the mucin *Muc2*. Moreover, Shigeshiro et al. (2012) reported that rats subjected to water immersion-stress were found to decrease their colonic *Muc2* gene relative expression and goblet cell population.

The mucus component in the fecal and small intestine luminal contents was determined and quantified by a fluorimetric assay of O-linked glycoproteins (mucins). As shown in Figure 2, compared to control rats, the CH induced a significant rise in the fecal mucin excretion after 2-(129%) and 8-weeks (147%) of experimental feeding. Sprong et al. (2010) showed the protective role played by a dietary cheese whey protein in the dextran sulfate sodium-induced model of rat colitis. Despite these authors did not find *Muc2* gene overexpression in colon, it was showed that the dairy protein ameliorated the colitis markers with an enhanced fecal mucin secretion.

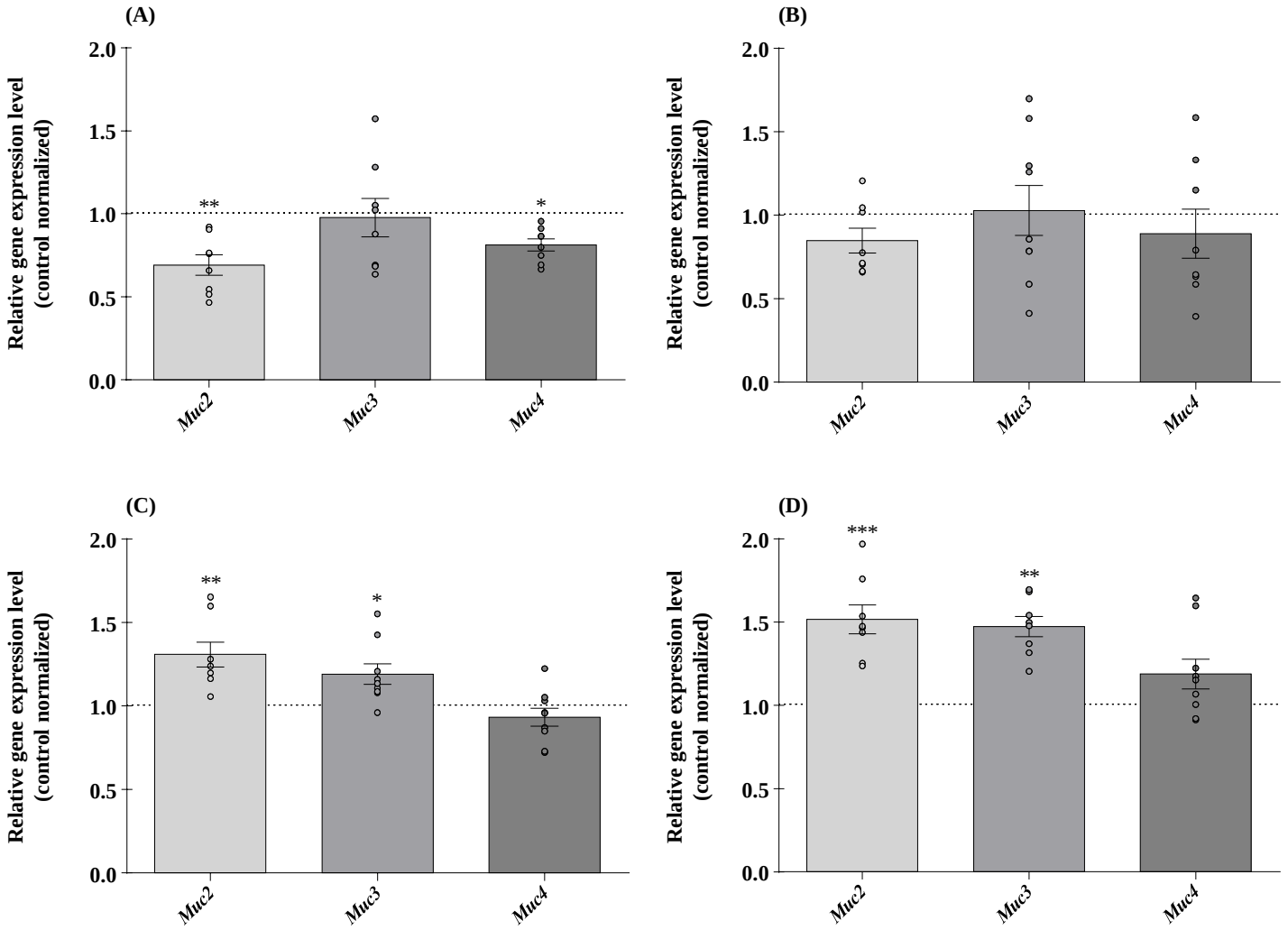


Figure 1. Effect of casein hydrolyzate on the mRNA expression of *Muc2, Muc3* and *Muc4* in the rat (A) duodenum, (B) jejunum, (C) ileum and (D) colon. Results (mean ± SEM, n = 9) were expressed as relative gene expression level of control (fold change value 1), using β-actin as reference gene. Significant differences versus control were determined by Mann-Whitney analysis of variance: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

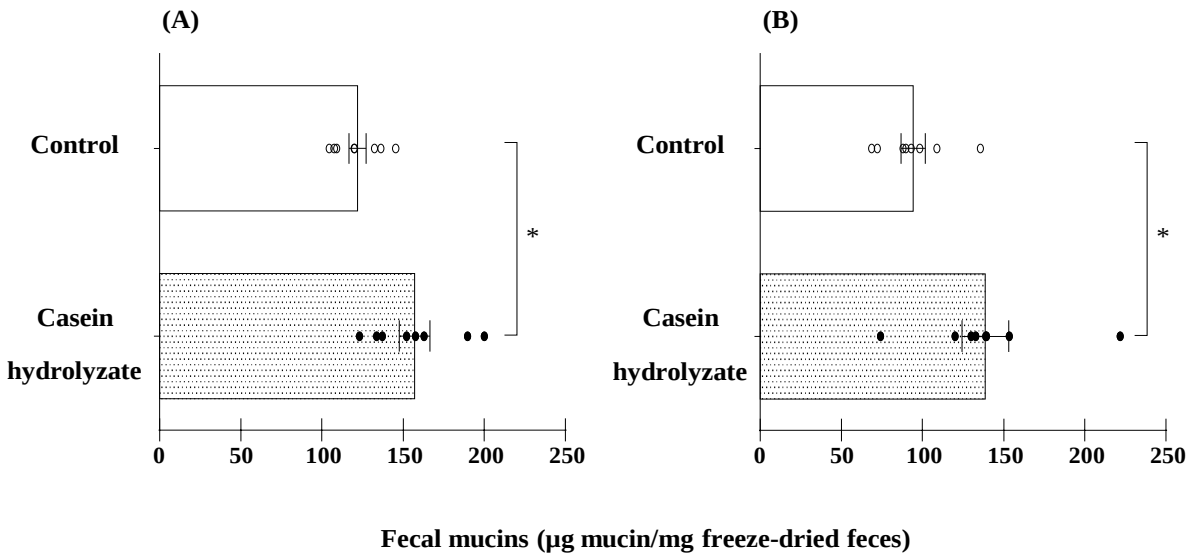


Figure 2. Effect of casein hydrolyzate on fecal mucin content after (A) 2 weeks and (B) 8 weeks of experimental feeding period. Results (mean ± SEM, *n* = 9) were represented as total amount of mucins (µg) per mg of freeze-dried feces. Significant differences versus control were determined by Mann-Whitney analysis of variance: $P < 0.05$ (*).

As illustrated in Figure 3, the CH also suggested an increased mucin synthesis at the gut luminal content. Mucus material in the small intestine lumen of rats fed the CH was higher than that found in control rats (124%), however the change did not reach statistical difference ($P = 0.15$). Nevertheless, this is the first study that evaluates the *in vivo* influence of dairy proteins in the mucus component of the gut lumen, while other food-compounds such as dietary fiber had already proved this protective modulation (Ito et al., 2009). In our study, the different results between mucus secretions at fecal and luminal contents might correspond to the variations shown in mucin gene expression levels between the small and large intestine sections. However, the regulatory mechanisms involved in mucus production and the intricate functionality of the gastrointestinal gel layer are still largely understood (Kim and Ho, 2010).

The results of our study might be explained by the previously demonstrated mucin stimulatory effect of this CH in human intestinal HT29-MTX cells (Martínez-Maqueda et al., 2013). It was found that this CH increased the expression of the secreted mucin *MUC5AC* (1.8-fold change) and the mucin-like glycoprotein production (210%). It was also proposed that the

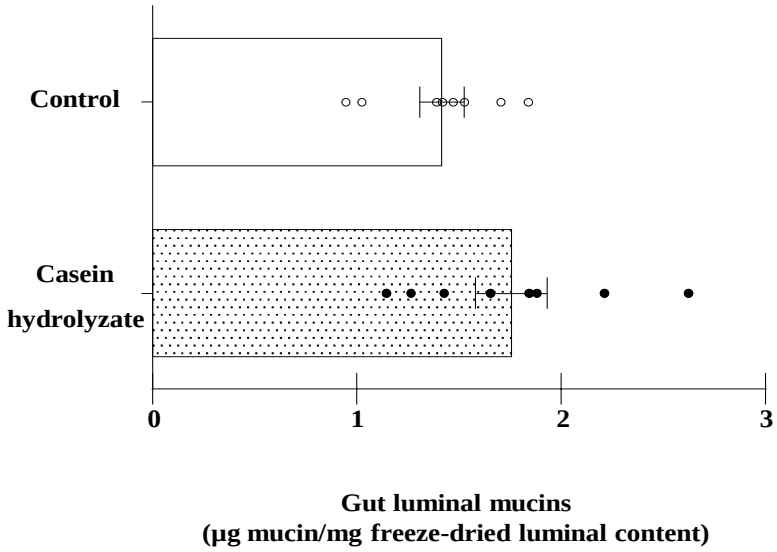


Figure 3. Effect of casein hydrolyzate on small intestine mucin luminal content. Results (mean ± SEM, *n* = 9) were represented as total amount of mucins (µg) per mg of freeze-dried luminal content. Significant differences versus control were determined by Mann-Whitney analysis of variance: $P < 0.05$ (*).

peptides α_{s1}-casein fragment ^{143}AYFYPEL^{149} and its derived-fragment ^{144}YFYPEL^{149}, at concentrations between 0.05 and 0.5 m*M*, were the main contributors to the activity. In the present study, the rat standard diet was supplemented with 0.4 g/kg/day of the hydrolyzate, which contained 4.1 mg of ^{143}AYFYPEL^{149} per g of hydrolysate, quantified by HPLC-tandem mass spectrometry. The concentration of peptide that can be reached with this dosage is within the range that proved activity in human intestinal goblet cells. However, it cannot be discarded the contribution of other peptides or components of the CH on the activity.

To date, only few *in vivo* studies have also pointed to the modulatory effect of dairy proteins, and hydrolyzates and peptides thereof, on the dynamics of the intestinal mucus layer. Interestingly, our findings are consistent with the study of Han et al. (2008) showing that pancreatin-hydrolyzed casein enhanced the ileal endogenous nitrogen flow and stimulated the transmembrane *Muc3* and *Muc4* mRNA expression in the rat small intestine and colon, respectively. Similarly, Plaisancié et al. (2013) found that the peptide β-casein fragment (94-123) supported the gut mucosa by inducing the expression of small intestine *Muc2* and *Muc4*, the number of goblet and Paneth cells and the antimicrobial factors *rat defensing 5* and *lysozyme*. Recently, oral administration of this peptide to rats has also shown protective benefits

against indomethacin-induced lesions and markers of enteritis, being these effects associated with increased recount of goblet cells replenished with mucin content and lower epithelial apoptosis (Bessette et al., 2016).

The emerging potential of natural compounds that can regulate the functionality of mucins and ultimately exert influence on health and disease has been recently stated (Macha et al., 2015). In the present study, the assayed CH induced the expression of mucin genes *Muc2* and *Muc3* in the distal segments of the rat intestine, i.e. the ileum and colon. Because of the physiological relevance of *Muc2*, coding at goblet cells for the main secreted mucin in rat intestine, and *Muc3*, coding for the membrane-located mucins at goblet as well as absorptive cells, a double-level of improved mucosal protection can be thus proposed. Besides, the CH enhanced the secretion of mucins by increasing the total mucins in the feces during the experimental period. However, no significant changes were shown on the mucin luminal content of rats fed the CH, although the average amount was higher than in control rats. Additional studies that further evaluate at a molecular level the CH effect on markers of gut health status would benefit from above findings. This study has allowed better knowledge of the promising role of dairy proteins as functional components that promote the mucus gel barrier and prevent the gastrointestinal-related diseases.

ACKNOWLEDGMENTS

This work has received financial support from project S2009/AGR-1469 (ALIBIRD) from the Comunidad de Madrid and project AGL2015-66886-R from the Spanish Ministry of Economy and Competitiveness (MINECO). S. F. –T. acknowledges MINECO for his FPI fellowship.

REFERENCES

Anadón, A., M. A. Martínez, I. Ares, E. Ramos, M. R. Martínez-Larrañaga, M. M. Contreras, M. Ramos, and I. Recio. 2010. Acute and repeated dose (4 weeks) oral toxicity studies of two antihypertensive peptides, RYLGY and AYFYPEL, that correspond to fragments (90-94) and (143-149) from α_{s1}-casein. Food Chem. Toxicol. 48: 1836-1845.

Atuma, C., V. Strugala, A. Allen, and L. Holm. 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state *in vivo*. Am. J. Physiol. Gastrointest. Liver Physiol. 280: G-922-G929.

Bessette, C., B. Benoit, S. Sekkal, J. Bruno, M. Estienne, J. Léonil, L. Ferrier, V. Théodorou, and P. Plaisancié. 2016. Protective effects of β-casofensin, a bioactive peptide from bovine β-casein, against indomethacin-induced intestinal lesions in rats. Mol. Nutr. Food Res. 60: 823-833.

Bischoff, S. C., G. Barbara, W. Buurman, T. Ockhuizen, J. –D. Schulzke, M. Serino, H. Tilg, A. Watson, and J. M. Wells. 2014. Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterol. 14: 189.

Cani, P. D., J. Amar, M. A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A. M. Neyrinck, F. Fava, K. M. Tuohy, C. Chabo, A. Waget, E. Delmée, B. Cousin, T. Sulpice, B. Chamontin, J. Ferrières, J. F. Tanti, G. R. Gibson, L. Casteilla, N. M. Delzenne, M. C. Alessi, and R. Burcelin. 2007. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56: 1761-1772.

Cani, P. D., S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. Neyrinck, D. M. Lambert, G. G. Muccioli, and N. M. Delzenne. 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58: 1091-1103.

Claustre, J., F. Toumi, A. Trompette, G. Jourdan, H. Guignard, J. A. Chayvialle, and P. Plaisancié. 2002. Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. Am. J. Physiol. Gastrointest. Liver Physiol. 283: G521-G528.

Crowther, R. S., and Wetmore, R. F. 1987. Fluorometric assay of O-linked glycoproteins by reaction with 2-cyanoacetamide. Anal. Biochem. 163: 170-174.

Han, K. –S., A. Deglaire, R. Sengupta, and P. J. Moughan. 2008. Hydrolyzed casein influences intestinal mucin gene expression in the rat. J. Agric. Food Chem. 56: 5572-5576.

Ito, H., M. Satsukawa, E. Arai, K. Sugiyama, K. Sonoyama, S. Kiriyama, and T. Morita. 2009. Soluble fiber viscosity affects both goblet cell number and small intestine mucin secretion in rats. J. Nutr. 139: 1640-1647.

Kim, Y. S., and S. B. Ho. 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr. Gastroenterol. Rep. 12: 319-330.

Lien, K. A., M. I. McBurney, B. I. Beyde, A. B. R. Thomson, and W. C. Sauer. 1996. Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. Am. J. Clin. Nutr. 63: 584-595.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408.

Macha, M. A., S. R. Krishn, R. Jahan, K. Banerjee, S. K. Batra, and M. Jain. 2015. Emerging potential of natural products for targeting mucins for therapy against inflammation and cancer. Cancer Treat. Rev. 41: 277-288.

Martínez-Maqueda, D., B. Miralles, E. Cruz-Huerta, and I. Recio. 2013. Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. Int. Dairy J. 32: 13-19.

Plaisancié, P., R. Boutrou, M. Estienne, G. Henry, J. Jardin, A. Paquet, and J. Léonil. 2015. β-Casein(94-123)-derived peptides differently modulate production of mucins in intestinal goblet cells. J. Dairy Res. 82: 36-46.

Plaisancié, P., J. Claustre, M. Estienne, G. Henry, R. Boutrou, A. Paquet, and J. Léonil. 2013. A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. J. Nutr. Biochem. 24: 213-221.

Shigeshiro, M., S. Tanabe, and T. Suzuki. 2012. Repeated exposure to water immersion stress reduces the Muc2 gene level in the rat colon via two distinct mechanisms. Brain Behav. Immun. 26: 1061-1065.

Sprong, R. C., A. J. Schonewille, and R. van der Meer. 2010. Dietary cheese whey protein protects rat against mild dextran sulfate sodium-induced colitis: role of mucin and microbiota. J. Dairy Sci. 93: 1364-1371.

Trompette, A., C. Blanchard, S. Zoghbi, J. Bara, J. Claustre, G. Jourdan, J. A. Chayvialle, and P. Plaisancié. 2004. The DHE cell line as a model for studying rat gastro-intestinal mucin expression: effects of dexamethasone. Eur. J. Cell Biol. 83: 347-358.

Trompette, A., J. Claustre, F. Caillon, G. Jourdan, J. A. Chayvialle, and P. Plaisancié. 2003. Milk bioactive peptides and β-casomorphins induce mucus release in rat jejunum. J. Nutr. 133: 3499-3503.

Van der Sluis, M., B. A. E. De Koning, A. C. J. M. De Bruijn, A. Velcich, J. P. P. Meijerink, J. B. Van Goudoever, H. A. Büller, J. Dekker, I. Van Seuningen, I. B. Renes, and A. W. C. Einerhand. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131: 117-129.

Velcich, A., W. Yang, J. Heyer, A. Fragale, C. Nicholas, S. Viani, R. Kucherlapati, M. Lipkin, K. Yang, and L. Augenlicht. 2002. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 295: 1726-1729.

Zoghbi, S., A. Trompette, J. Claustre, M. El Homsi, J. Garzón, G. Jourdan, J. –Y. Scoazee, and P. Plaisancié. 2006. β-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a μ-opioid pathway. Am. J. Physiol. Gastrointest. Liver Physiol. 290: G1105-G1113.

Estudio comparativo de procesos de simulación *in vitro* de la digestión gastrointestinal de proteínas lácteas (caseínas y proteínas de suero) con digeridos intestinales obtenidos en individuos sanos.

2.7. Publicación VII: **Comparison of the standardised *in vitro* digestion model (Infogest) with *in vivo* digestion data at peptide level.**

MANUSCRIPT

Comparison of the standardised *in vitro* digestion model (Infogest) with *in vivo* digestion data at peptide level

S. Fernández-Tomé[1], J. Sanchón[1], B. Miralles[1], B. Hernández-Ledesma[1], D. Tomé[2], C. Gaudichon[2], I. Recio[1*]

[1]Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain.

[2]AgroParisTech_UMR0914 Physiologie de la Nutrition et du Comportement Alimentaire, 16 rue Claude Bernard, 75005 Paris, France.

* Corresponding author: I. Recio

Nicolás Cabrera, 9. 28049 Madrid, Spain

Phone: +34 910017940

Fax: +34 910017905

e-mail: i.recio@csic.es

ABSTRACT

In this study, protein degradation and peptide formation during *in vitro* gastrointestinal digestion of casein and whey protein were compared with data obtained from human jejunal digests. *In vitro* digestion was performed by using a standardised static digestion method proposed in the context of the COST Action INFOGEST and jejunal digests after oral ingestion of the same substrates were collected by a nasogastric tube located at the proximal jejunum. By SDS-PAGE, no intact casein could be detected in any of the jejunal samples, nor in the *in vitro* samples taken during the intestinal phase. β-lactoglobulin was detected in the jejunal effluents taken 1 h after ingestion of whey protein, although with different intensity depending on volunteers. Similarly, β-lactoglobulin resisted the *in vitro* gastric phase and it was hydrolysed after 1 h into contact with the simulated pancreatic juice. *In vivo* and *in vitro* digests were analysed by HPLC-MS/MS under equal conditions. In addition, a selective precipitation step was performed to enrich and identify phosphorylated peptides. Comparable peptide profiles were observed in the *in vivo* and *in vitro* digests. Based on these peptide profiles, it was shown that the correlation coefficients found between *in vitro* digests and the jejunal effluents were within the range found for the different volunteers of the study. Similar resistant regions to digestion were identified after *in vitro* and *in vivo* digestion and the formation of certain peptides is discussed.

KEYWORDS: *in vitro*/*in vivo* digestion, peptidomic, mass spectrometry, milk protein digestion

1. INTRODUCTION

Digestion is an intricate process that food undergoes upon oral ingestion. Proteins are known to be extensively hydrolysed throughout the digestive tract by gastrointestinal enzymes and brush border membrane peptidases, leading to the release of numerous peptides and free amino acids. However, some specific regions of proteins have demonstrated a particular resistance against the digestive phenomena. Food protein-derived peptides present in the gut lumen might be biologically and metabolically relevant for different physiological functions, exerting not only beneficial properties (Rutherfurd-Markwick, 2012) but also deleterious effects, such as, those produced by epitopes involved in food allergies (Jiménez-Saiz et al., 2015). Because of the growing evidence on the health implications of dietary peptides, there has also been an interest to elucidate the factors that influence protein degradation and lead peptide release and stability in the gastrointestinal tract (Picariello et al., 2013; Sánchez-Rivera et al., 2014a).

Although animal and human digestion studies obviously provide the most meaningful results, they imply ethical, technical and financial issues. Thus, a large variety of *in vitro* methods simulating the digestion process have been applied to study the gastrointestinal behaviour of food and pharmaceuticals (Kaukonen et al., 2004; Versantvoort et al., 2005; Dupont et al., 2010; Martos et al., 2010; Kopf-Bolanz et al., 2012; Ménard et al., 2014). To overcome the wide variation between the individual parameters used on these methodologies (Guerra et al., 2012), in the context of the COST Action INFOGEST FA1005 network (Dupont et al., 2011), a static *in vitro* digestion method was proposed (Minekus et al., 2014). The consistency and validation of this model has been recently published (Egger et al., 2016). However, these authors emphasized the difficulty to fully mimic the kinetic behaviour of the complex *in vivo* digestion process by a static model, and prompted further experiments to directly compare the *in vitro* results towards *in vivo* data.

Milk proteins are one of the main sources of biologically active peptides (Hernández-Ledesma et al., 2014; Nongonierma & Fitzgerald, 2015). Moreover, these proteins represent a stable and complex food matrix commonly used as a suitable model in digestion experiments. Few *in vivo* studies have been performed on the presence of milk-derived peptides in the

gastrointestinal lumen of humans (Svedberg et al., 1985; Chabance et al., 1998; Boutrou et al., 2013) and animals (Bouzerzour et al., 2012; Barbé et al., 2014) after ingestion of dairy products. Despite these studies are essential to test the interaction between dietary proteins and the digestive tract and to shed light on the physiological-derived consequences (Foltz et al., 2010), the data are still scarce and a notable diversity has been recently stated (Boutrou et al., 2015).

Accordingly, the objective of this work was to compare the peptidome of samples obtained after gastrointestinal digestion of milk casein and whey protein by the harmonized INFOGEST *in vitro* digestion method, with *in vivo* digests collected from human jejunum. Nine different time combinations of the *in vitro* gastric and intestinal phases were carried out and evaluated against *in vivo* jejunal samples obtained 1, 2, 3 and 4 h after protein ingestion. Protein degradation was determined by SDS-PAGE, and the characterization at a peptide level of the digestion products was evaluated by HPLC-MS/MS. Moreover, an enrichment selective precipitation was performed on *in vivo* casein samples to study the caseinphosphopeptide (CPP) fraction.

2. MATERIALS AND METHODS

2.1 Human jejunal effluents

Human jejunal effluents were obtained as previously described (Boutrou et al., 2013). Briefly, individuals were fed with 30 g of milk protein powder, casein or whey, dissolved in 500 mL of water. Samples were obtained by a nasogastric tube that migrated to the proximal jejunum by aspiration of jejunal effluents at 1, 2, 3 and 4 hours after oral administration of the protein solution. Jejunal effluents were collected on ice and freeze-dried and kept at -80 °C until analysis. Nitrogen content in freeze-dried samples was determined by elemental analysis and further analyses were performed on protein basis. In this study, 9 jejunal samples were analysed: 5 of them obtained after feeding with casein and 4 after feeding with whey protein.

2.2 *In vitro* simulated gastrointestinal digestion

Casein and whey commercial milk protein powders were digested according to the *in vitro* harmonized protocol (Minekus et al., 2014). Briefly, freeze-dried casein or whey protein samples were dissolved in simulated saliva fluid at 30 mg of protein/mL without amylase because of the absence of starch in the samples. Then, the mix was diluted at a ratio of 50:50 (v/v) in simulated gastric fluid containing pepsin from porcine gastric mucosa (2000 U/mL of digesta, EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA). Samples were withdrawn at 10, 20, 30, 60 and 120 min during gastric digestion and the reaction was stopped by adjusting the pH at 7.0 with NaOH 1 M and snap freezing in liquid nitrogen. Intestinal phase was carried out by mixing the gastric phase with the same volume of simulated intestinal fluid containing pancreatin from porcine pancreas (100 U trypsin activity/mL of final mixture, Sigma-Aldrich) and bile extract porcine (10 mM in the final mixture, Sigma-Aldrich). All simulated fluids were tempered at 37 °C before use. Digestions were performed in duplicate by incubating at 37 °C in an orbital shaker at 150 rpm. Intestinal phases were started from 20 and 120 min of gastric phase digests, performed during 60 and 120 min and the digestion was stopped with Pefabloc® SC (5 mM, Sigma-Aldrich) and snap freezing. Samples were freeze-dried and kept at -20 °C until analysis. A digestion blank consisting of the mix of enzymes used in digestions at the same concentration without substrate protein was prepared. Enzyme activities and bile concentration were measured according to the assay described in the protocol (Minekus et al., 2014).

2.3 SDS-PAGE

Samples were dissolved at 1 mg of protein/mL in sample buffer that contains Tris-HCl (0.05 M, pH 6.8, Sigma-Aldrich), SDS (1.6%, w:v, Merck, Darmstadt, Germany), glycerol (8%, v:v, PanreacQuímica SAU, Castellar del Vallés, Barcelona, Spain), β-mercaptoethanol (2%, v:v, Sigma-Aldrich) and bromophenol blue indicator (0.002%, w:v, Merck), heated at 95 °C for 5 min and loaded on 12% Bis-Trispolyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA). Electrophoretic separations were run at 100 V for 5 min and then at 150 V, using the XT MES running buffer (Bio-Rad) in the criterion cell (Bio-Rad). A molecular weight marker (Precision Plus Protein™ Unstainend standard, Bio-Rad) was used on each gel. Gels were stained with Coomasie Blue (Instant blue, Expedeon, Swavesey, UK) and images were taken with a

Molecular Imager® VersaDoc™ MP 5000 system (Bio-Rad) and processed with Quantity One® 1-D analysis software (Bio-Rad).

2.4 Analysis by HPLC-tandem mass spectrometry

Freeze-dried samples were reconstituted in solvent A (water:formic acid, 100:0.1, v:v) and centrifuged at 13000 × *g*, 10 min, before injection of the supernatant. From *in vivo* casein samples, an enrichment selective precipitation of CPP was performed by adding calcium chloride (1%, w:v) and ethanol (50%, v:v) as described by Sánchez-Rivera et al. (2014b). Whey samples were treated for 60 min at 37 °C with 1,4-dithiothreitol 70 mM (Sigma-Aldrich) to reduce samples in order to improve the identification of disulfide-linked fragments (Martínez-Maqueda et al., 2013a).

Samples were analyzed by HPLC-MS/MS using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranea Sea_{18} column (150 x 2.1 mm, Teknokroma, Barcelona, Spain). HPLC system was connected to an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with an electrospray ionization source. The injection volume was 50 µL and the flow was set at 0.2 mL/min. Peptide elution was performed with a linear gradient from 0 to 45% of solvent B (acetonitrile:formic acid, 100:0.1, v:v) in 115 min. Same gradient and solvents were used for CPP, however the elution time was 60 min. Injections were analyzed in duplicate, being the spectra recorded over the mass/charge (*m/z*) range 100-1300 and 100-1700, selecting 500 and 1200, respectively, as the target mass. For CPP, spectra was recorded over the *m/z* range 100-2100 with a target mass of 1200. The results were processed by using Data Analysis (version 4.0, Bruker Daltonics). Homemade database of cow's casein and whey protein were used for the peptide sequencing in MASCOT v2.4 software (Matrix Science). Moreover, Biotools version 3.2 was used for the interpretation of the matched MS/MS spectra. No specific enzyme cleavage was used. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. Each identified peptide spectrum was revised manually, regardless of its *P* value.

3. RESULTS AND DISCUSSION

3.1. Protein degradation during *in vitro* and human gastrointestinal digestion

Protein patterns obtained by SDS-PAGE of the jejunal effluents collected at 1, 2, 3 and 4 h after oral administration of casein and whey protein are shown in Figure 1 (A and C, respectively). To obtain similar protein loadings in all lanes, samples were dissolved in the treatment buffer taking into account the protein content of each sample determined by elemental analysis. In all the jejunal digests from casein (Figure 1 A), there was a clear band with molecular weight (MW) 50 kDa, which likely corresponds to human pancreatic lipase, as previously described by other authors (Iizuka et al., 1991). In addition, there were several light bands with mobilities between 25 and 37 kDa, close to those of intact casein. However, no differences were observed at different sampling times or between volunteers and they did not react with antibodies against casein by immunoblotting (data not shown), thus, these bands could probably correspond to endogenous proteins. In a previous report, the nitrogen composition, endogenous and exogenous, in the upper jejunum of human volunteers after ingestion of casein or whey protein was investigated (Mahé et al., 1996). It was found that casein was slowly recovered in the jejunum and mainly in the form of degraded peptides which is consistent with our results where no intact protein is detected. Similarly, other *in vivo* studies in minipigs cannulated at duodenum have shown that after ingestion of non-heated liquid milk, intact casein can be detected during the first minutes of digestion, but they are rapidly digested in the next 20 min (Barbe et al., 2013).

When applying the standardised *in vitro* digestion conditions on the same casein substrate (Figure 1 B), a gradual degradation of the casein fraction with the digestion time was observed in the gastric phase. Electrophoretic bands corresponding to casein were observed up to 30 min peptic digestion but they were absent in the samples obtained in the gastric phase at 60 and 120 min. In the samples withdrawn during the intestinal phase an intense band corresponding to porcine pancreatic lipase could be observed ca. 50 kDa, as well as, other minor bands corresponding to other pancreatic enzymes between 20 and 35 kDa, which is compatible with the MW of the porcine pancreatic enzymes trypsin (23.3 kDa), chymotrypsin (25.5-29.10 kDa) or elastase (25.9 kDa), as previously shown (Ménard et al., 2014). The rapid

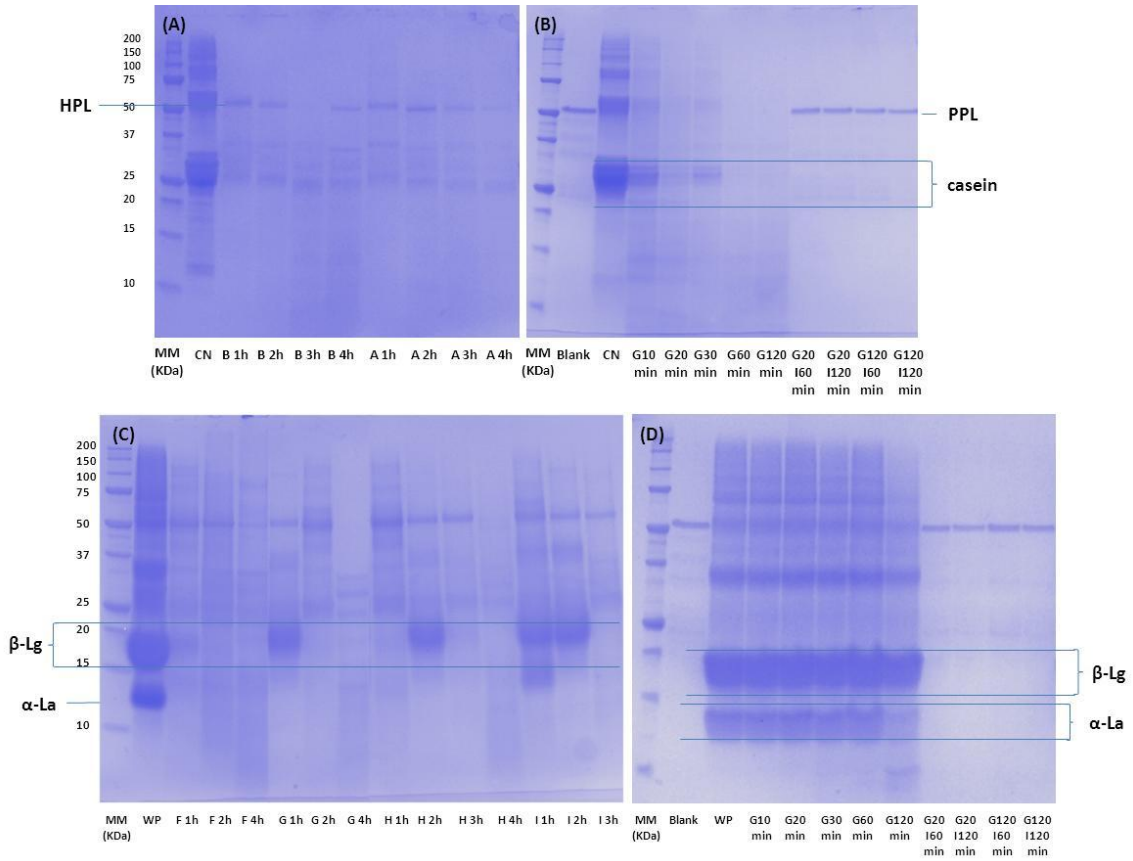


Figure 1. SDS-PAGE of jejunal digests (A and C) and *in vitro* simulated gastrointestinal digestion (B and D). A and B corresponded to casein and C and D to whey protein as substrate. Each lane in A and C corresponded to different subjects (referred to with capital letters from A to I) and times of jejunal sampling (1, 2, 3 and 4 h). Each lane in B and D correspond to different times (min) of gastric (G) and intestinal (I) digestion. MM, molecular weight marker; CN, casein; WP, whey protein; Blank, gastrointestinal digestion enzymes without milk protein; HPL, human pancreatic lipase; PPL, porcine pancreatic lipase; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

degradation of the casein fraction of milk during *in vitro* gastric digestion is in agreement with previous reports using this standardised method at different laboratories (Egger et al., 2016) where the casein bands were not detected at the end of the gastric phase (120 min) and by other authors using slightly different *in vitro* conditions (Picariello et al., 2010; Kopf-Bolanz et al., 2012; Ménard et al., 2014).

Figure 1C shows the SDS-PAGE analyses of the jejunal contents of different volunteers at different times when whey protein was orally administered. The electrophoretic band corresponding to β-lactoglobulin was visible in the samples taken at 1 h in all volunteers with

variations in intensity, and in some of them, this protein was also detected in the sample taken 2 h after oral administration of the whey protein (volunteers H and I). Again, this is in agreement with previous results on nitrogen content at jejunum where β-lactoglobulin could be recovered, mostly in the form of intact protein (Mahé et al., 1996). From Figure 1C, it can be seen that in volunteer I an important amount of α-lactalbumin could be found 1 hour after administration. As expected, during *in vitro* digestion of whey protein (Figure 1D) β-lactoglobulin was resistant to digestion by pepsin but it disappeared completely after 60 min of intestinal digestion regardless the duration of the gastric phase (20 or 120 min). The resistance of this protein to peptic digestion has been largely documented *in vivo* (Bouzerzour et al., 2012) and *in vitro* assays (Dupont et al., 2010; Kopf-Bolanz et al., 2012), as well as, the effect of heat treatment on the susceptibility of this protein to gastric digestion (Barbé et al., 2013; Sánchez-Rivera et al., 2015).

3.2. Peptidomic characterisation of *in vivo* and *in vitro* digests

Jejunal digests obtained at four different time points and *in vitro* digests withdrawn under gastric and intestinal conditions were analysed under equal conditions by HPLC-MS/MS. From the casein-jejunal samples, a total of 465 different peptides were unequivocally identified, most of them belonging to β-casein (176), followed by α_{s1}-casein peptides (131) and α_{s2}- and κ-casein-derived peptides (74 and 84, respectively). However, under the same analytical conditions, a total of 338 different peptides were identified from the *in vitro* digests (97 from β-casein; 78 from α_{s1}-casein; 97 from κ-casein; and 66 from α_{s2}-casein) although a higher number of time points were considered (4 vs 9). As an example, Table 1 shows the peptides derived from β-casein and α_{s1}-casein from a representative volunteer in the jejunal sample taken 2 h after the oral administration of casein, and peptides from these major two proteins obtained after *in vitro* digestion (20 min gastric phase+60 min intestinal phase). Under our analytical conditions, most of the identified peptides ranged 5 to 26 amino acid long, with masses between 500 and 3000 Da, although several phosphorylated peptides up to 30 amino acids were also identified (see Section 3.3).

Table 1: Identified peptides derived from β- and α_{s1}-casein in the jejunal digests of a representative volunteer 2 hour after oral administration of casein, and peptides from these two proteins identified in the *in vitro* simulated gastrointestinal digestion after 20 min of gastric phase and 60 min of intestinal phase (G20+I60 min).

JEJUNAL DIGESTS[a]					
β-casein-derived peptides			α_{s1}-casein-derived peptides		
Range		Sequence	Range		Sequence
1	5	RELEE	1	15	RPKHPIKHQGLPQEV
5	15	ELNVPGEIVES	11	15	LPQEV
6	14	LNVPGEIVE	12	21	PQEVLNENLL
45	57	LQDKIHPFAQTQS	24	31	FVAPFPEV
45	58	LQDKIHPFAQTQSL	24	32	FVAPFPEVF
59	70	VYPFPGPIPNSL	25	31	VAPFPEV
59	80	VYPFPGPIPNSLPQNIPPLTQT	25	33	VAPFPEVFG
60	69	YPFPGPIPNS	26	30	APFPE
69	80	SLPQNIPPLTQT	39	47	ELSKDIGSE
71	80	PQNIPPLTQT	57	63	IKQMEAE
81	89	PVVVPPFLQ	80	89	HIQKEDVPSE
81	91	PVVVPPFLQPE	112	119	VPNSAEER
81	92	PVVVPPFLQPEV	125	137	EGIHAQQKEPMIG
81	93	PVVVPPFLQPEVM	126	137	GIHAQQKEPMIG
85	92	PPFLQPEV	143	148	AYFYPE
85	93	PPFLQPEVM	143	149	AYFYPEL
115	119	PVEPF	157	164	DAYPSGAW
115	121	PVEPFTE	159	163	YPSGA
115	123	PVEPFTESQ	165	172	YYVPLGTQ
115	124	PVEPFTESQS	165	173	YYVPLGTQY
122	126	SQSLT	168	172	PLGTQ
128	132	TDVEN	174	178	TDAPS
128	139	TDVENLHLPLPL	180	190	SDIPNPIGSEN
128	140	TDVENLHLPLPLL	180	191	SDIPNPIGSENS
129	139	DVENLHLPLPL	180	193	SDIPNPIGSENSEK
130	139	VENLHLPLPL	180	196	SDIPNPIGSENSEKTTM
135	139	LPLPL	180	198	SDIPNPIGSENSEKTTMPL
157	163	FPPQSVL	182	186	IPNPI
158	162	PPQSV	185	196	PIGSENSEKTTM
164	168	SLSQS	194	198	TTMPL
164	182	SLSQSKVLPVPQKAVPYPQ			
164	189	SLSQSKVLPVPQKAVPYPQRDMPIQA			
171	175	LPVPQ			
172	189	PVPQKAVPYPQRDMPIQA			
174	183	PQKAVPYPQR			
177	189	AVPYPQRDMPIQA			
185	189	MPIQA			
192	209	LYQEPVLGPVRGPFPIIV			
193	199	YQEPVLG			
193	207	YQEPVLGPVRGPFPI			
193	208	YQEPVLGPVRGPFPII			
193	209	YQEPVLGPVRGPFPIIV			
196	201	PVLGPV			
196	205	PVLGPVRGPF			
199	209	GPVRGPFPIIV			
200	208	PVRGPFPII			
200	209	PVRGPFPIIV			
203	207	GPFPI			
205	209	FPIIV			

***IN VITRO* DIGESTION G20 +I60 min[b]**					
β-casein-derived peptides			α_{s1}-casein-derived peptides		
Range		Sequence	Range		Sequence
1	5	RELEE	8	13	HQGLPQ
1	6	RELEEL	9	13	QGLPQ
6	14	LNVPGEIVE	17	28	NENLLRFFVAPF
7	14	NVPGEIVE	24	28	FVAPF
9	14	PGEIVE	24	30	FVAPFPE
39	45	QQTEDEL	24	31	FVAPFPEV
57	66	SLVYPFPGPI	25	31	VAPFPEV
58	66	LVYPFPGPI	25	33	VAPFPEVFG
59	66	VYPFPGPI	27	31	PFPEV
59	68	VYPFPGPIPN	29	33	PEVFG
60	66	YPFPGPI	35	41	EKVNELS
61	68	PFPGPIPN	37	41	VNELS
62	66	FPGPI	53	57	AMEDI
69	76	SLPQNIPP	53	58	AMEDIK
69	82	SLPQNIPPLTQTPV	70	74	EIVPN
73	77	NIPPL	84	90	EDVPSER
73	82	NIPPLTQTPV	102	107	KKYKVP
75	82	PPLTQTPV	106	114	VPQLEIVPN
83	87	VVPPF	134	138	PMIGV
83	91	VVPPFLQPE	136	140	IGVNQ
83	92	VVPPFLQPEV	139	143	NQELA
85	91	PPFLQPE	155	161	QLDAYPS
85	92	PPFLQPEV	157	161	DAYPS
92	99	VMGVSKVK	174	178	TDAPS
92	100	VMGVSKVKE	174	186	TDAPSFSDIPNPI
100	105	EAMAPK	182	186	IPNPI
101	105	AMAPK	194	198	TTMPL
108	113	EMPFPK			
114	119	YPVEPF			
115	119	PVEPF			
125	129	LTLTD			
126	132	TLTDVEN			
128	132	TDVEN			
134	138	HLPLP			
144	154	MHQPHQPLPPT			
147	154	PHQPLPPT			
150	154	PLPPT			
156	162	MFPPQSV			
157	161	FPPQS			
164	168	SLSQS			
177	182	AVPYPQ			
193	201	YQEPVLGPV			
194	201	QEPVLGPV			
196	201	PVLGPV			
203	207	GPFPI			

[a] Only data for one volunteer is shown; [b] Only data from one duplicate is shown.

It is also worthy to mention that certain regions, especially those rich in proline residues, were resistant to gastrointestinal digestion and reached jejunum. For instance, the opioid peptide β-casomorphin-7 (β-casein ^{60}YPFPGPI^{66}) and various peptides containing the same

were found at jejunum after consumption of casein. These peptides had been previously quantified in jejunal contents and it was estimated that the amount considering β-casomorphin-7 and its precursors could reach a concentration ca. 17 μmol/L (Boutrou et al., 2013). However, the *in vitro* gastric digestion samples contained several precursors of β-casomorphin-7 but in any of the samples the active form starting with tyrosine at the N-terminus was found. It has been described that pepsin cleaves the ^{58}Leu-Val^{59} peptide bond at β-casein and leucine aminopeptidase removes the Val^{59}, rendering the tyrosine residue at the N-terminus (Jinsmaa and Yoshikawa, 1999). In our study, the enzymatic action of the pancreatic extract employed in the *in vitro* simulation was needed to release the peptide β-casomorphin-7. Other β-casein regions especially resistant and found in jejunum and *in vitro* digests contained the clusters ^{74}IPPLT^{78}, ^{83}VVPP^{86}, ^{134}HLPLP^{138}, ^{150}PLPP^{153}, ^{157}FPP^{159}, ^{172}PVP^{174}, ^{204}PFPI^{207}. In the case of α_{s1}-casein, for example the bioactive peptide ^{143}AYFYPEL^{149} was found in both the *in vitro* and *in vivo* digests. This α_{s1}-casein peptide had been previously identified in *in vivo* gastric human digests after milk ingestion (Chabance et al., 1998), and it is known for its proved antihypertensive effects in spontaneously hypertensive rats (Contreras et al., 2009), intestinal mucin stimulatory properties in human goblet HT29-MTX cells (Martínez-Maqueda et al., 2013b), and opioid activity in the guinea pig ileum assay (Fernández-Tomé et al., 2016). A more detailed comparison between peptides found *in vitro* and *in vivo* digests is shown in Section 3.4.

In order to compare the peptide profile obtained from the analysis of the jejunal digests and *in vitro* digests, a heat map was built (Figure 2). This graph represents the appearance frequency of each amino acid identified as part of different peptides for a given protein. This graph gives qualitative information about the protein coverage and about those protein regions where peptides were identified, although it does not provide quantitative information or relative abundancies. Each line in the heat map corresponds to a human volunteer (Figures 2A and 2C) or a different duplicate in the *in vitro* assays (Figures 2B and 2D). In the *in vivo* digests, there is a common pattern at the different time points and for the different volunteers, although certain inter-individual variability can be inferred. For instance, there are regions, such as β-casein 157-161 where peptides were commonly found but this region was absent in some individuals. Comparing the *in vivo* and *in vitro* peptide profiles for β-casein, it can be seen that jejunal digests at any time point are comparable to the intestinal phase of the *in vitro* simulated

Figure 2. Heat map built with the frequency of appearance of each amino acid on the β- and α_{s1}-casein protein chain. A and C corresponds to profiles obtained from human jejunal digests obtained at 1, 2, 3 and 4 h after oral administration of casein and B and D corresponds to profiles obtained from simulated *in vitro* digestion of the same substrate. The green colour represents low frequency and red high frequency, being the maximum 24, 9, 18 and 8 in A, B, C, and D, respectively. G, gastric; I, intestinal.

gastrointestinal digestion. The β-casein N-terminal region and the region 57-99 is highly represented in both cases, but the peptides in the central region of the protein (from residue 115 to 175) were more abundant in the jejunal digests.

For α_{s1}-casein (Figures 2C and 2D), the peptide profile was much more complex in the *in vivo* digests that in the *in vitro* ones. While in the simulated *in vitro* digestions peptides were mainly found in the N- and C-terminal regions, in the jejunal digests, peptides were found along the whole protein chain. Thus, many peptides belonging to the central domain of the α_{s1}-casein protein chain could be identified in the jejunal digests which were absent in the *in vitro* digests.

The frequency of appearance of each amino acid for β-casein was used to compare the *in vitro* and the *in vivo* digestion. Table 2 shows the Spearman correlation coefficients obtained for each time point. As expected from the peptide profile, the average correlation coefficient was higher when comparing the human jejunal digests with the intestinal phase from the *in vitro* digests (average 0.59 ± 0.09) than with the gastric phase (average 0.16 ± 0.12). In general, the correlation coefficients obtained between the *in vivo* data and the *in vitro* intestinal phase ranged from 0.39 to 0.67. All the *in vitro* intestinal time points correlated better with the human jejunal samples taken at 1 h, since they varied from 0.60 to 0.67. The correlation matrix for β-casein-derived peptides comparing the profile obtained from the 5 different subjects was also built. The coefficients varied from 0.51 to 0.77, which are within the range obtained for the *in vivo*/*in vitro* comparison.

Figure 3 shows the peptide pattern of the two major proteins, β-lactoglobulin (Figure 3A and 3B) and α-lactalbumin (Figure 3C and 3C), obtained when whey protein was used as substrate. In this case, β-lactoglobulin-derived peptides were only found in the intestinal phase during *in vitro* gastrointestinal simulation, and only few α-lactalbumin peptides were identified at the end of the gastric phase, in agreement with the results obtained by SDS-PAGE. The resistance of β-lactoglobulin to the action of pepsin is well documented (Miranda and Pelissier, 1983). Other *in vitro* digestion studies have reported the release of certain peptides sequences from β-lactoglobulin during gastric digestion (Benedé et al., 2014; Egger et al., 2016), but it is known that the susceptibility of this protein to peptic hydrolysis may vary with the denaturation degree of β-lactoglobulin by heating or other treatments (Peram et al., 2013; Singh et al., 2014;

Table 2: Spearman correlation coefficients calculated for β-casein peptide profiles found in jejunal digests and *in vitro* simulated gastrointestinal digests when casein was used as substrate.

	IN VIVO			
IN VITRO	jejunum 1h	jejunum 2h	jejunum 3h	jejunum 4h
G10 min	-0.09	-0.02	-0.13	-0.08
G20 min	-0.03	0.00	-0.06	-0.04
G30 min	0.06	0.09	0.01	0.01
G60 min	0.12	0.18	0.13	0.08
G120 min	0.24	0.40	0.27	0.24
G20+I60 min	0.67	0.61	0.64	0.54
G20+I120 min	0.60	0.42	0.47	0.39
G120+I60 min	0.62	0.45	0.50	0.41
G120+I120 min	0.62	0.49	0.54	0.44

Sánchez-Rivera et al., 2015). During the *in vitro* intestinal phase, 46 different β-lactoglobulin sequences were released mainly from regions comprised between 42 and 68 and 107 and 135 with few peptides from the N- and C- terminal regions of the protein. The peptidic profile obtained from jejunal digests was more complex than the *in vitro* one, with 112 different peptides along the whole protein chain. A considerable number of β-lactoglobulin peptides found in jejunum belonged to regions which were also overrepresented in the intestinal *in vitro* profile (40-60; 120-140). It has to be noted that the ability of peptides to be ionized can influence the final appearance of these peptidic profiles, i.e., peptides easily ionized are highly detected and may be overrepresented in these heat maps. Previous studies have also shown a higher abundancy of β-lactoglobulin peptides from region 40-60 in human jejunal contents after ingestion of whey protein (Boutrou et al., 2013), in piglet jejunum after administration of infant formula (Bouzerzour et al., 2012) and after the incubation of β-lactoglobulin with human gastrointestinal fluids (Benedé et al., 2014).

In the case of the α-lactalbumin protein, both peptidic profiles, *in vitro* and *in vivo* were coincident in several regions: 40-52, 63-68, 80-90 (Figure 3C). Interestingly, peptides were

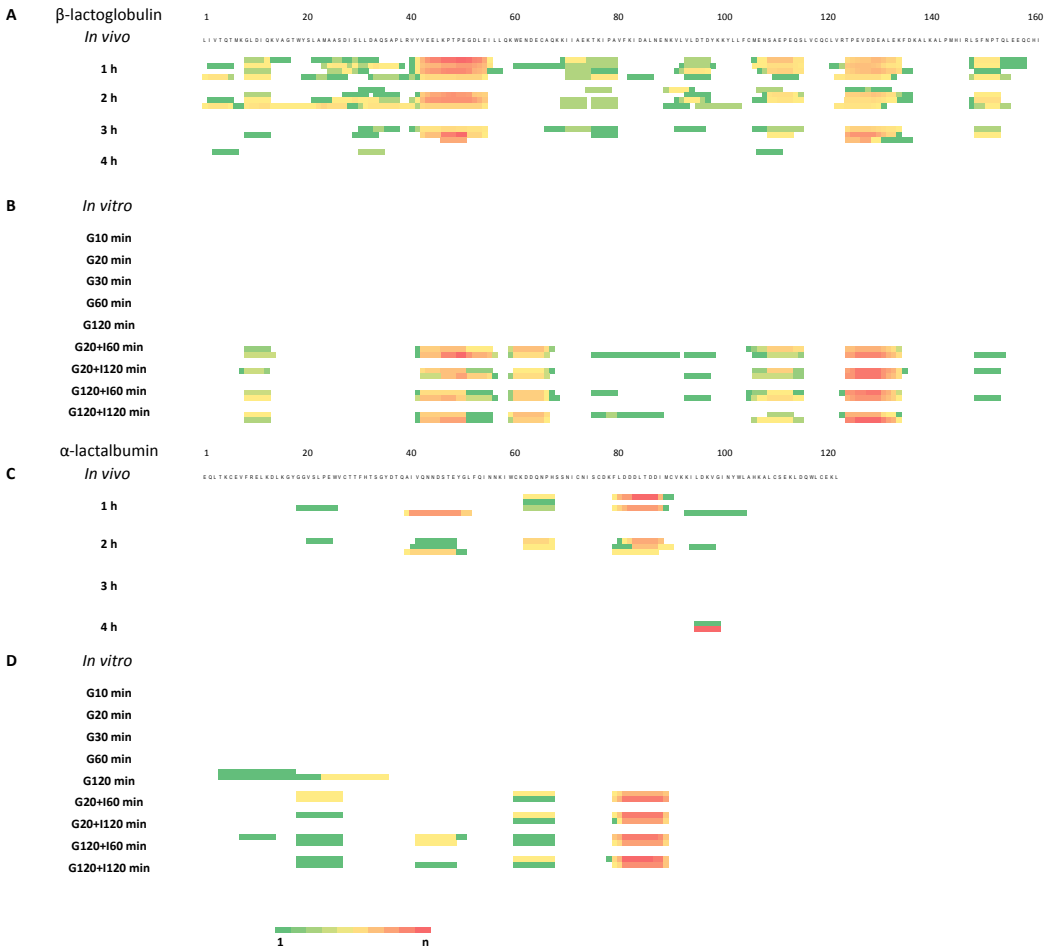


Figure 3. Heat map built with the frequency of appearance of each amino acid on the β-lactoglobulin and α-lactalbumin protein chain. A and C corresponds to profiles obtained from human jejunal digests obtained at 1, 2, 3 and 4 h after oral administration of whey proteins and B and D corresponds to profiles obtained from simulated *in vitro* digestion of the same substrate. The green colour represents low frequency and red high frequency, being the maximum 32, 12, 8 and 9 in A, B, C, and D, respectively. G, gastric; I, intestinal.

found in jejunal digests at samples withdrawn during the first two hours, but only few α-lactalbumin peptides were identified at 3 and 4 h. In this sense, casein-derived peptides tended to maintain their presence along the digestion times evaluated (Figure 2), while the number of whey-derived peptides started to decrease after 3 h of ingestion. Similarly, Boutrou et al. (2013) also found these differences between casein and whey protein, regarding the kinetics of dietary nitrogen flux and the detection and size distribution of peptides in the jejunum after human ingestion.

A correlation matrix for the *in vitro*/*in vivo* comparison of β-lactoglobulin-derived peptides was also calculated (Table 3). In this case, the *in vitro* gastric phase was not included since no peptides were released from this protein by the action of pepsin and neither jejunal samples at 4 h due to the low number of identified peptides. The average correlation coefficient was 0.64 ± 0.05 with correlation coefficients ranging between 0.55 to 0.71. Again, these values were comprised within the range found when assessing inter-individual variability after whey protein consumption (from 0.28 to 0.77). The highest correlation coefficients were found for the human jejunal samples obtained after 1 h with any of the *in vitro* samples from the intestinal phase. This higher correlation could be due to the higher number of peptides found *in vivo* samples at short times and because they might be less affected by the absorption process.

Table 3: Spearman correlation coefficients calculated for β-lactoglobulin peptide profiles found in jejunal digests and in vitro simulated gastrointestinal digests when whey protein was used as substrate.

	IN VIVO		
IN VITRO	jejunum 1h	jejunum 2h	jejunum 3h
G20+I60 min	0.69	0.65	0.66
G20+I120 min	0.71	0.64	0.58
G120+I60 min	0.71	0.62	0.67
G120+I120 min	0.66	0.55	0.64

3.3. Identification of *in vivo* phosphorylated peptides

In vivo digests were subjected to a selective precipitation procedure with $CaCl_2$ in order to identify phosphorylated regions. Various phosphorylated fragments belonging to the region β-casein 1-24 were identified in all jejunal digests, but especially, in the samples taken at 1 and 2 h where phosphorylated peptides were found in all volunteers (Figure 4). However, after the same enrichment procedure, no peptides belonging to the phosphorylated N-terminal region of β-casein were found. Similar results were obtained for α_{s1}-casein (Figure 5), for which several fragments from the region 42-52, in addition to other peptides phosphorylated at serine cluster 64-68 or Ser^{115}, were found. Some of the peptides identified in jejunum are similar to fragments previously identified in ileum of rats fed with CPP (Brommage et al., 1991; Hirayama et al., 1992) or in duodenal digests from mini-pigs fed with different dairy matrices (Barbé et al., 2014).

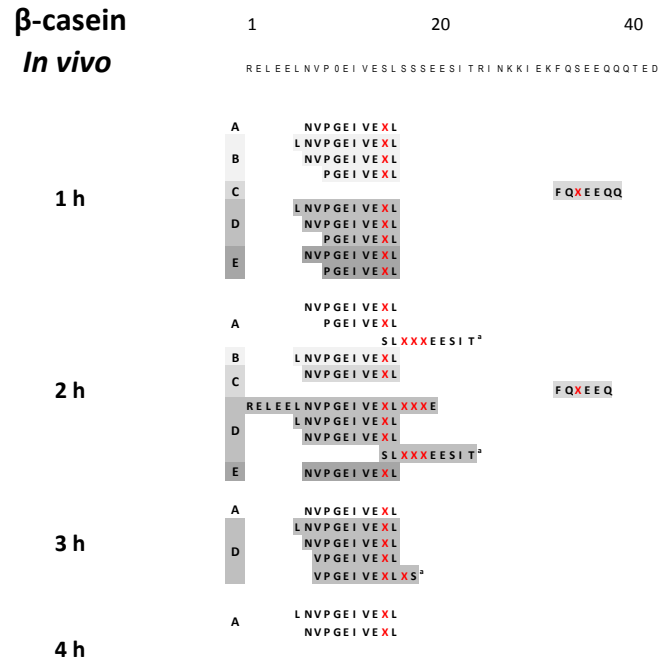


Figure 4: Phosphorylated peptides derived from β-casein identified in jejunal digests. Each grey colour denotes a different subject. In those peptides marked with an asterisk, the position of the phosphorylated residues may vary.

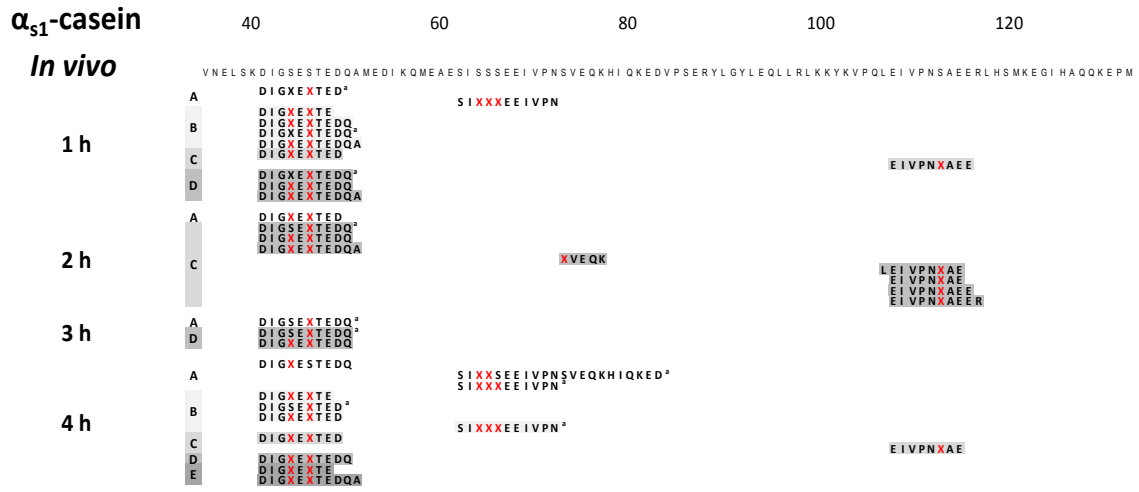


Figure 5: Phosphorylated peptides derived from α_{s1}-casein identified in jejunal digests. Each grey colour denotes a different subject. In those peptides marked with an asterisk, the position of the phosphorylated residues may vary.

3.4. Analysis of peptide homology *in vivo* vs *in vitro*

In vitro and *in vivo* identified peptides resulting from the HPLC-MS/MS analyses of digests were assessed for homology of their sequences by Venn diagrams (Figure 6). A total of 465 peptides were obtained from human jejunal effluents fed with casein and 338 were found by the *in vitro* protocol, being 101 of these sequences (14.4%) common between both digestions (Figure 6A). β-casein and α_{s1}-casein were compared separately but the percentages of homology found were similar than that in whole casein. Around 23% of β-casein and 16% of α_{s1}-casein peptides were identical between *in vitro* and *in vivo* results (Figure 6C and 6E). In the case of whey protein, 179 and 117 peptides were identified *in vivo* and *in vitro,* respectively. The percentage of homology between both groups was 13.0%, sharing 34 sequences (Figure 6B). As illustrated in Figure 6D and 6F, comparable patterns were demonstrated for the two major whey proteins. 112 peptides were observed in the *in vivo* β-lactoglobulin jejunal digests, 27 of them matched with a total of 46 peptides found *in vitro* (Figure 6D). From α-lactalbumim, 25 sequences were recognized *in vivo* and 14 in the *in vitro* digests, sharing 5 of them (Figure 6F). Therefore, the percentage of identical sequences between *in vitro* samples and *in vivo* jejunal

effluents showed a certain similarity for most abundant milk proteins, ranging from 14.7% (α-lactalbumin) to 23% (β-casein).

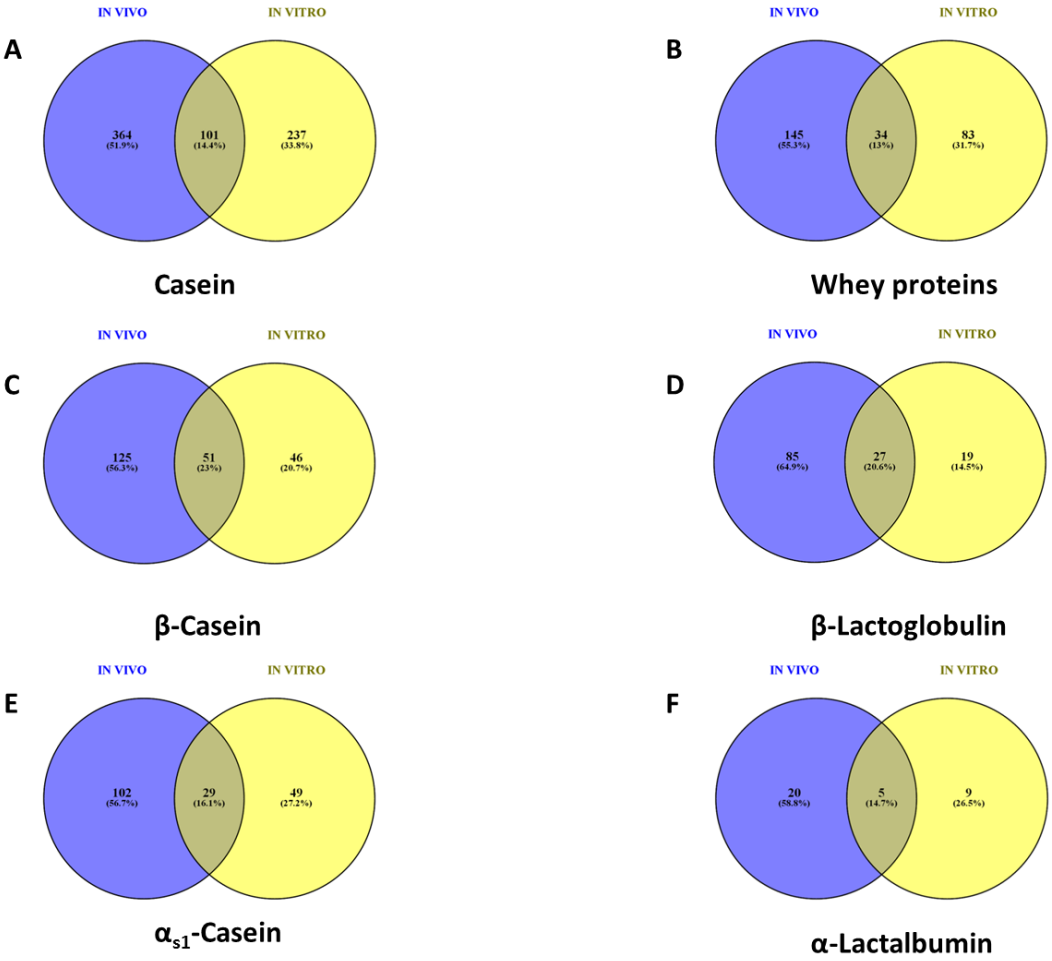


Figure 6: Venn diagrams with the number of identical and different peptides found in human jejunal digests and *in vitro* simulated gastrointestinal digestion of casein (A) and whey protein (B) and specified for β-casein (C), α_{s1}-casein (E), β-lactoglobulin (D) and α-lactalbumin (F).

Validation of *in vitro* methods with results obtained from *in vivo* studies has been urgently stated. The static *in vitro* protocol of Minekus et al. (2014) was built by consensus of different research groups, considering the physiologically *in vivo* data published in the literature. It is presented in a robust and simply mode and it has recently shown consistency in inter-laboratory trials (Egger et al., 2016). However, these authors indicated the limitations of static *in vitro* models as simplified methods of the intricate *in vivo* digestion process. Several parameters

such as chewing, gastric emptying, continuous changes in pH, secretion flow rates and feedback systems that regulate the progress of digestion, among others, have been described as limiting factors for these protocols (Guerra et al., 2012). In the present work, an *in vitro*/*in vivo* comparison at peptide level was attempted. Although some differences were found, the *in vitro* protocol resembles the *in vivo* intestinal digestion regarding protein degradation and peptide release. Finally, it was found shown that the correlation found for the *in vivo/in vitro* comparison was similar to the inter-individual variability, i.e. the correlation coefficients found between *in vitro* digests and the jejunal effluents were within the range found for the different volunteers of the study. Therefore, these results illustrate that the proposed *in vitro* digestion protocol constitutes a good approximation of the physiological gastrointestinal digestion of milk proteins.

ACKNOWLEDGEMENTS

This work has received financial support from project AGL2015-66886-R from the Spanish Ministry of Economy and Competitiveness (MINECO). S. F.-T. and B. H.-L. acknowledge MINECO for their FPI fellowship and “Ramón y Cajal” post-doctoral contract, respectively. J. S. is the recipient of a “CSIC-CM- FSE, Iniciativa de Empleo Juvenil" contract.

REFERENCES

Barbé, F., Le Feunteun, S., Remond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., & Dupont, D. (2014). Tracking the *in vivo* release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63*, 147-156.

Barbé, F., Ménard, O., Le Gouar, Y., Buffiere, C., Famelart, M.H., Laroche, B., Le Feunteun, S., Dupont, D., & Remond, D. (2013). The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. *Food Chemistry, 136*, 1203-1212.

Benedé, S., Lopez-Exposito, I., Gimenez, G., Grishina, G., Bardina, L., Sampson, H.A., Lopez-Fandino, R., & Molina, E. (2014). Mapping of IgE epitopes in *in vitro* gastroduodenal digests of beta-lactoglobulin produced with human and simulated fluids. *Food Research International, 62*, 1127-1133.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tome, D., & Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition, 97*, 1314-1323.

Boutrou, R., Henry, G., & Sánchez-Rivera, L. (2015). On the trail of milk bioactive peptides in human and animal intestinal tracts during digestion: a review. *Dairy Science & Technology, 95*, 815-829.

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huerou-Luron, I., & Dupont, D. (2012). *In vivo* digestion of infant formula in piglets: protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition, 108*, 2105-2114.

Brommage, R., Juillerat, M.A., & Jost, R. (1991). Influence of caseinphosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Lait, 71*, 173-180.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollès, P., & Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie, 80*, 155-165.

Contreras, M. M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal*, *19,* 566-573.

Dupont, D., Bordoni, A., Brodkorb, A., Capozzi, F., Velickovic, T. C., Corredig, M., Cotter, P. D., De Noni, I., Gaudichon, C., Golding, M., Lea, T., Le Huërou-Luron, I., Mackie, A., Madsen, C., De Meulenaer, B., Nys, Y., Pihlanto, A., Recio, I., Rémond, D., Requena, T., Souchon, I., Swiatecka, D., Turgeon, S., Vegarud, G., Vreeburg, R., Weitschies, W., & Wickham, M. (2011).

An international network for improving health properties of food by sharing our knowledge on the digestive process. *Food Digestion, 2*, 23-25.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Leonil, J., Faulks, R. M., Wickham, M. S. J., Mills, E. N. C., & Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant *in vitro* digestion models. *Molecular Nutrition & Food Research, 54*, 767-780.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunçao, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M. J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., Martins, C., Meyneir, A., Miralles, B., Murray, B. S., Pihlanto, A., Picariello, G., Santos, C. N., Simsek, S., Recio, I., Rigby, N., Rioux, L. –E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E. K., Vegarud, G. E., Vergères, G., & Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International, 88*, 217-225.

Fernández-Tomé, S., Martínez-Maqueda, D., Girón, R., Goicoechea, C., Miralles, B., & Recio, I. (2016). Novel peptides derived from α_{s1}-casein with opioid activity and mucin stimulatory effect on HT29-MTX. *Journal of Functional Foods, 25*, 466-476.

Foltz, M., van der Pijl, P.C., & Duchateau, G.S. (2010). Current *in vitro* testing of bioactive peptides is not valuable. *Journal of Nutrition*, *140,* 117-118.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology, 30*, 591-600.

Hernández-Ledesma, B., García-Nebot, M.J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). Dairy protein hydrolysates: Peptides for health benefits. *International Dairy Journal, 38*, 82-100.

Hirayama, M., Toyota, K., Hidaka, H., & Naito, H. (1992). Phosphopeptides in rat intestinal digests after ingesting casein phosphopeptides. *Bioscience, Biotechnology and Biochemistry, 56*, 1128-1129.

Iizuka, K., Higurashi, H., Fujimoto, J., Hayashi, Y., Yamamoto, K., & Hiura, H. (1991). Purification of human pancreatic lipase and the influence of bicarbonate on lipase activity. *Annals of Clinical Biochemistry, 28*, 373-378.

Jiménez-Saiz, R., Benedé, S., Molina, E., López-Expósito I. (2015). Effect of processing technologies on the allergenicity of food products. *Critical Reviews in Food Science and Nutrition, 55*, 1902-1917.

Jinsmaa, Y., & Yoshikawa, M. (1999). Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. *Peptides, 20*, 957-962.

Kaukonen, A.M., Boyd, B.J., Charman, W.N., & Porter, C.J., (2004). Drug solubilization during *in vitro* digestion of suspension formulations of poorly water-soluble drugs in triglyceride lipids. *Pharmaceutical Research, 21*, 254-260.

Kopf-Bolanz, K.A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *Journal of Nutrition, 142*, 245-250.

Mahe, S., Roos, N., Benamouzig, R., Davin, L., Luengo, C., Gagnon, L., Gausserges, N., Rautureau, J., & Tomé, D. (1996). Gastrojejunal kinetics and the digestion of N-15 beta-lactoglobulin and casein in humans: The influence of the nature and quantity of the protein. *American Journal of Clinical Nutrition, 63*, 546-552.

Martínez-Maqueda, D., Miralles, B., Ramos, M., & Recio, I. (2013a). Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells. *Food Research International, 54*, 1287-1291.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., & Recio, I. (2013b). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *International Dairy Journal, 32*, 13-19.

Martos, G., Contreras, P., Molina, E., & López-Fandiño, R. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *Journal of Agricultural and Food Chemistry, 58*, 5640-5648.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039-1045.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food & Function, 5*, 1113-1124.

Miranda, G., & Pelissier, J.P. (1983). Kinetic-studies of *in vivo* digestion of bovine unheated skim-milk proteins in the rat stomach. *Journal of Dairy Research, 50*, 27-36.

Nongonierma, A.B., & FitzGerald, R.J. (2015). The scientific evidence for the role of milk protein-derived bioactive peptides in humans: a review. *Journal of Functional Foods, 17*, 640-656.

Peram, M.R., Loveday, S.M., Ye, A., & Singh, H. (2013). *In vitro* gastric digestion of heat-induced aggregates of beta-lactoglobulin. *Journal of Dairy Science, 96*, 63-74.

Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S., & Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 878*, 295-308.

Picariello, G., Mamone, G., Nitride, C., Addeo, F., & Ferranti, P. (2013). Protein digestomics: integrated platforms to study food-protein digestion and derived functional and active peptides. *Trends in Analytical Chemistry, 52*, 120-134.

Rutherfurd-Markwick, K. J. (2012). Food proteins as a source of bioactive peptides with diverse functions. *British Journal of Nutrition, 108*, S149-S157.

Sánchez-Rivera, L., Diezhandino, I., Gómez-Ruiz, J.A., Fresno, J.M., Miralles, B., & Recio, I. (2014b). Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. *Electrophoresis, 35*, 1627-1636.

Sánchez-Rivera, L., Martínez-Maqueda, D., Cruz-Huerta, E., Miralles, B., & Recio, I. (2014a). Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food Research International, 63*, 170-181.

Sánchez-Rivera, L., Ménard, O., Recio, I., & Dupont, D. (2015). Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder. *Food Research International, 77*, 132-139.

Singh, T.K., Oiseth, S.K., Lundin, L., & Day, L. (2014). Influence of heat and shear induced protein aggregation on the in vitro digestion rate of whey proteins. *Food & Function, 5*, 2686-2698.

Svedberg, J., de Haas, J., Leimenstoll, G., Paul, F., & Teschemacher, H. (1985). Demonstration of β-casomorphin immunoreactive materials in *in vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides, 6*, 825-830.

Versantvoort, C.H., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J., & Sips, A.J. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology, 43*, 31-40.

3. DISCUSIÓN

El tracto digestivo es considerado como el órgano endocrino más extenso del organismo. Se encuentra en contacto continuo y directo con una gran cantidad de compuestos derivados de la digestión de los alimentos. Esto ha llevado a que en los últimos años haya crecido el interés científico por el efecto de los péptidos bioactivos en el tracto digestivo, bien por su actividad moduladora sobre las células y los receptores de este sistema del organismo (Shimizu y Hachimura, 2011), como por la influencia directa ejercida por los procesos digestivos sobre la funcionalidad biológica de dichos péptidos (Sánchez-Rivera y col., 2014a). Los trabajos experimentales incluidos en esta Tesis Doctoral revelan nuevos datos acerca de la digestión y las propiedades beneficiosas relacionadas con la salud digestiva del péptido lunasina, de proteínas lácteas y de péptidos derivados. En concreto, se ha demostrado el papel protector que tiene el inhibidor de proteasas IBB1 sobre la digestión *in vitro* del péptido lunasina simulando las condiciones fisiológicas y se han identificado los péptidos liberados tras la acción de las enzimas gástricas y pancreáticas sobre ambos polipéptidos. En base a estos resultados, se ha evaluado el transporte intestinal *in vitro* del péptido lunasina y de cuatro fragmentos liberados durante su digestión que comprenden la secuencia completa del péptido. Además, se demuestra el potencial efecto de los digeridos gastrointestinales de la lunasina y de algunos de los péptidos contenidos en dichos digeridos sobre la viabilidad de células tumorales del tracto gastrointestinal. Posteriormente, se profundizó en los mecanismos protectores relacionados con la actividad quimiopreventiva del péptido lunasina. Para ello, se ha evaluado el efecto antiproliferativo de este péptido en células nativas y células madre de cáncer colorrectal, así como su capacidad de protección frente al estrés oxidativo inducido químicamente en células hepáticas. Por otro lado, se ha evaluado el mecanismo modulador de péptidos lácteos sobre la expresión relativa de genes que codifican para mucinas en células caliciformes intestinales, identificándose en este estudio nuevas secuencias con actividad opioide. Además, se determinó el efecto *in vivo* sobre el mucus intestinal de un hidrolizado de proteínas lácteas que contiene

dichos péptidos. Este efecto estaba mediado por la estimulación de la expresión génica y de la secreción fecal y luminal de mucinas intestinales. Por último, se ha llevado a cabo un estudio comparativo de la digestión gastrointestinal de proteínas lácteas entre un protocolo *in vitro* recientemente consensuado a nivel internacional y muestras obtenidas del contenido intestinal del yeyuno de individuos humanos tras la ingestión de caseínas y proteínas de suero.

3.1. Digestión, biodisponibilidad y mecanismos quimiopreventivos del péptido lunasina en modelos celulares

La digestión gastrointestinal de las proteínas alimentarias supone tanto una barrera física y enzimática como el origen de múltiples modificaciones en la estructura y secuencia de los péptidos derivados. Estos procesos pueden provocar la liberación de nuevos fragmentos con mayor, menor o nula actividad comparada con la de sus precursores. Dada esta importancia, en los últimos años, se ha incrementado notablemente el número de trabajos que consideran aspectos propios de la digestión y la biodisponibilidad de los péptidos bioactivos a la hora de establecer una relación causa-efecto con la salud y su potencial aplicación como ingredientes funcionales (Miner-Williams y col., 2014; Nongonierma y FitzGerald, 2016).

Algunos estudios basados en ensayos inmunológicos habían sugerido que el péptido lunasina resistía la acción proteolítica de las enzimas digestivas debido a la actividad de los inhibidores de proteasas presentes de forma natural en las proteínas vegetales, como el BBI y el KTI (Jeong y col., 2007a; b; Park y col., 2007; Hernández-Ledesma y col., 2009a). Por ello, se planteó, por primera vez, el estudio mediante cromatografía de líquidos de alta eficacia acoplada a espectrometría de masas en tándem (HPLC-MS/MS) de la digestión *in vitro* del péptido lunasina simulando las condiciones fisiológicas en ausencia y presencia de la principal isoforma del BBI, el IBB1, reconocida por su actividad inhibitoria de tripsina y quimiotripsina. Se comprobó

la funcionalidad del isoinhibidor IBB1 mediante el uso de su forma activa y químicamente inactivada (Clemente y col., 2010). Se observó que dicha inactivación reducía su actividad inhibitoria de las enzimas tripsina y quimiotripsina en más de un 95%, lo que permitía determinar la influencia del estado del IBB1 (activo *versus* inactivo) sobre la potencial protección frente a la digestión de la lunasina. Además, el análisis por HPLC-MS/MS corroboró que un 2% del IBB1 total permaneció todavía activo en la preparación químicamente inactivada. En comparación con los trabajos anteriores, este estudio permitía avanzar en la investigación del péptido lunasina mediante su cuantificación a nivel gástrico e intestinal y la identificación de los péptidos liberados durante el proceso digestivo.

Se confirmó, por un lado, el efecto protector que ejerce el IBB1 sobre la degradación que sufre el péptido lunasina durante el proceso digestivo. En ausencia del IBB1, la pepsina hidrolizó la lunasina, permaneciendo un 2,6% del péptido inicial intacto. Sin embargo, en presencia del IBB1 activo en ratios lunasina:IBB1 1:1 y 1:2 (peso:peso), el porcentaje de lunasina intacto tras la acción de la enzima gástrica fue significativamente mayor, del 34,3 y 35,1%, respectivamente, demostrando que está protección no dependía del ratio empleado. Estos resultados indicaban que la protección ejercida frente a la digestión gástrica estaba mediada por factores independientes al mecanismo enzimático. De acuerdo con este planteamiento, Hajela y col. (1999) habían descrito previamente la mínima actividad inhibitoria que presenta el BBI frente a la pepsina. Esta hipótesis se reforzó cuando la digestión se llevó a cabo en presencia del IBB1 en un estado inactivo (lunasina:IBB1, 1:2), obteniéndose resultados estadísticamente similares (28,4%). Por ello, se sugirió que la simple presencia del isoinhibidor y su mayor tamaño con respecto a la lunasina, independientemente de su actividad, podría ser responsable del efecto preventivo frente al ataque de la enzima gástrica. Por el contrario, a nivel intestinal, el IBB1 ejerció un efecto protector dosis-dependiente mediado por su actividad inhibitoria de

tripsina y quimiotripsina. En ausencia del IBB1, la lunasina se degradó prácticamente en su totalidad (0,1%), pero su resistencia intestinal aumentó significativamente con la presencia de IBB1 a ambos ratios 1:1 y 1:2, permaneciendo intacta un 1,8 y 5,3% del péptido inicial, respectivamente. Sin embargo, el isoinhibidor en un mismo ratio de lunasina:IBB1 (1:2) pero inactivado químicamente tuvo un efecto significativamente menor. En este caso, el porcentaje de lunasina intacta al final del proceso digestivo fue del 1,5%, similar al observado en presencia del IBB1 a una relación 1:1. Paralelamente, se confirmó la extraordinaria resistencia que presenta el isoinhibidor a su paso por el tracto gastrointestinal (Clemente y col., 2011). Esta resistencia se debe a la particular estabilidad de su estructura mantenida por una red compacta de siete puentes disulfuro (Clemente y Domoney, 2006; Trivedi y col., 2009). En nuestro estudio, la inactivación química del IBB1 inducida mediante reducción-alquilación, además de reducir en un 95% su actividad inhibitoria de proteasas intestinales, aumentó notablemente su sensibilidad a las enzimas digestivas, liberándose un total de 19 y 24 fragmentos durante su digestión a nivel gástrico e intestinal, respectivamente. En el caso del análisis del péptido lunasina, el mayor número de fragmentos derivados se identificó en el digerido obtenido a partir de lunasina en ausencia del IBB1 (20 y 18 del total de 22 y 33 secuencias liberadas a nivel gástrico e intestinal, respectivamente). Además, en el caso de esta simulación, se formaron 5 fragmentos durante la fase gástrica y 15 durante la intestinal, que no fueron identificadas en los digeridos obtenidos a partir de mezclas de lunasina con el IBB1. Esto indicó el papel protector que ejercía el isoinhibidor, no sólo en la digestión de la lunasina, sino también y, predominantemente, en la región central entre los aminoácidos 11 y 28, donde se originaron la mayoría de estas nuevas secuencias. Sin embargo, en presencia del IBB1 activo a ambos ratios lunasina:IBB1, el perfil peptídico de las digestiones gástricas y gastrointestinales fue bastante similar. El número de fragmentos liberados en estos digeridos varió ligeramente, entre 13 y 16, originándose

a partir de cuatro regiones concretas, 1-10, 11-18, 19-28 y 29-43, que mostraron una destacada estabilidad durante toda la simulación digestiva.

En base a estos resultados, se propuso evaluar la biodisponibilidad del péptido lunasina en el modelo de células Caco-2 en monocapa mediante HPLC-MS/MS. Además, se planteó el mismo estudio con cuatro fragmentos identificados que habían demostrado una marcada resistencia a las enzimas gastrointestinales y que integran la secuencia completa del péptido lunasina: ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{19}LTPCEKHIME^{28} y ^{29}KIQGRGDDDDDDDDD^{43}. En primer lugar, en el estudio del compartimento apical, se observó que el péptido lunasina y los fragmentos ^{11}RKQLQGVN^{18} y ^{19}LTPCEKHIME^{28} se hidrolizaban en contacto con las enzimas del epitelio intestinal en un 42,8, 20,3 y 21,3%, respectivamente, mientras que los fragmentos ^{1}SKWQHQQDSC^{10} y ^{29}KIQGRGDDDDDDDDD^{43} permanecieron prácticamente intactos. Resulta interesante destacar como en el ensayo con lunasina, se comprobó que la acción de las enzimas epiteliales fue predominantemente dirigida hacia la región central de su secuencia, como sucede con las proteasas pepsina, tripsina y quimiotripsina en ausencia del IBB1. En el caso de los otro cuatro péptidos analizados, la mayoría de los fragmentos liberados se formaron a consecuencia de cortes desde el extremo N-terminal, sugiriéndose la acción de la enzima dipeptidil peptidasa-IV, de acuerdo con los resultados publicados en varios estudios similares (Howell y col., 1992; Quirós y col., 2008; Ding y col., 2015). En el estudio del compartimento basolateral, se comprobó la biodisponibilidad de la lunasina y del péptido derivado ^{11}RKQLQGVN^{18}, con coeficientes de transporte, expresados como permeabilidad aparente, de 3,3 x 10^{-7} y 2,5 x 10^{-7} cm/s, respectivamente. Estos valores son similares a los descritos para otros péptidos bioactivos como los fragmentos ^{90}RYLGY^{94} y ^{143}AYFYPEL^{149} de la α_{s1}-CN (Contreras y col., 2012), y el péptido recombinante VLPVP (Lei y col., 2008). Ninguno de los otros fragmentos evaluados, ^{1}SKWQHQQDSC^{10}, ^{19}LTPCEKHIME^{28} y ^{29}KIQGRGDDDDDDDDD^{43} de la lunasina, ni

el resto de secuencias formadas en el compartimento apical fue capaz de atravesar la monocapa de células Caco-2. Por ello, se sugirió que la secuencia ^{11}RKQLQGVN^{18} podría ser potencialmente responsable de la absorción intestinal mostrada para el péptido lunasina. En este sentido, también merece la pena indicar que el IBB1 ejerció un papel determinante en la protección de esta región frente a la acción de las proteasas intestinales sobre los enlaces ^{11}R-K^{12}, ^{12}K-Q^{13}, ^{13}Q-L^{14}, ^{14}L-Q^{15}, ^{15}Q-G^{16} y ^{16}G-V^{17} (Publicación I). Este hecho sería fisiológicamente muy relevante si se confirma mediante más estudios la hipótesis planteada sobre el papel que cumple esta región en la biodisponibilidad de la lunasina. Además, se demostró que tanto el péptido lunasina como el fragmento ^{11}RKQLQGVN^{18} eran absorbidos a través de la barrera intestinal mediante un mecanismo de difusión paracelular. Esta vía es una ruta favorable para la absorción de moléculas solubles y evita la acción de las peptidasas intracelulares (Salamat-Miller y Johnston, 2005). De manera similar, también se ha identificado esta vía en el transporte intestinal de varios péptidos (Segura-Campos y col., 2011).

La FDA recomendó el consumo de 25 g de proteínas de soja al día para reducir el riesgo de desarrollar enfermedades cardiovasculares (FDA, 1999). Según el trabajo de de Mejia y col. (2010), estos 25 g supondrían una ingesta total diaria de lunasina de 0,94 g. Bajo las condiciones experimentales de nuestros estudios, en presencia de IBB1 activo (lunasina:IBB1 1:2), 49,82 mg de lunasina (5,3% de la inicial ingerida) llegarían a nivel intestinal tras el proceso gastrointestinal. Un porcentaje del 42,8% sería hidrolizado por las peptidasas del borde del epitelio intestinal, dando lugar a 28,50 mg en el lumen intestinal, que podrían actuar a nivel local sobre las células y los receptores digestivos. De esta cantidad, un 0,88% sería biodisponible con una permeabilidad aparente de $3,3 \times 10^{-7}$ cm/s. Por lo tanto, 0,25 mg de lunasina totales podrían pasar al torrente sanguíneo en forma intacta para ejercer efectos sistémicos. En este sentido, Dia y col. (2009) determinaron la presencia de 0,21 mg de lunasina en

el plasma de individuos adultos a los 60 min de la ingesta de 50 g de una dieta basada en proteínas de soja. A pesar de las diferencias experimentales entre este estudio *in vivo* y la evaluación llevada a cabo en nuestros trabajos, los resultados obtenidos se acercaron razonablemente.

El péptido lunasina había sido ampliamente estudiado por sus propiedades quimiopreventivas demostradas en ensayos *in vitro*, cultivos celulares y modelos animales (Hernández-Ledesma y Hsieh, 2015). Inicialmente, se decidió evaluar el efecto de los digeridos gastrointestinales de las mezclas de lunasina, con o sin IBB1, frente al crecimiento de dos líneas de células de adenocarcinoma colorrectal, HT-29 y Caco-2. En ausencia del IBB1, los digeridos de lunasina redujeron la viabilidad de las células HT-29 con un valor de IC_{50} de 0,29 mg/mL. Este efecto sería atribuible a los fragmentos derivados de la digestión de la lunasina, ya que la presencia de este péptido en el digerido fue residual (0,1%). Por el contrario, este digerido no mostró efecto en células Caco-2. La presencia del IBB1 en las digestiones aumentó los efectos observados. Este hecho podría deberse a la propia actividad quimiopreventiva previamente descrita para el IBB1 (Clemente y col., 2010), así como a la mayor presencia de lunasina y de péptidos derivados protegidos de la acción de las enzimas digestivas en presencia del isoinhibidor. Los valores IC_{50} para los distintos digeridos fueron menores en las células HT-29 (0,16-0,23 mg/mL) que en las Caco-2, observándose además se observó un claro efecto dosis-dependiente.

Partiendo de estos resultados, se planteó evaluar el efecto individual del péptido lunasina y de algunos fragmentos derivados identificados en sus digeridos gástricos y gastrointestinales, ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{17}VNLTPCEKHIME^{28}, ^{19}LTPCEKHIME^{28}, ^{29}KIQGRGDDDDDDD^{41}, ^{29}KIQGRGDDDDDDDDD^{43} y ^{34}GDDDDDDDDD^{43}, sobre la viabilidad de la línea celular de adenocarcinoma gástrico AGS y de las dos líneas de adenocarcinoma colorrectal usadas previamente (HT-29 y Caco-2). De acuerdo con los resultados encontrados en

el trabajo anterior, las células HT-29 resultaron ser las más susceptibles a la acción del péptido lunasina, que mostró efectos dosis- y tiempo-dependientes con una máxima actividad inhibitoria tras 72 h de tratamiento con la concentración de 200 µM (inhibición de la viabilidad celular en un 23,8% con respecto al control). Sin embargo, esta actividad fue notablemente menor a la descrita en estas células para la lunasina aislada a partir de harinas de soja a una concentración de 100 µM (62,8%) (Dia y de Mejia, 2010). Por ello, se decidió evaluar la pureza del péptido lunasina sintético (usado en nuestros trabajos). Mediante purificación por HPLC, se determinó que el péptido sintético presentaba tan solo un 10% de impurezas. Por lo que se sugirió que las diferencias estructurales entre el péptido sintético y el aislado desde la fuente proteica natural podrían ser, principalmente, las responsables de la menor actividad demostrada en el presente estudio. De una forma similar, los siete fragmentos derivados de la lunasina mostraron un mayor efecto en la línea celular HT-29, en comparación con los experimentos en células AGS y Caco-2. Esta situación fue particularmente destacable para el fragmento ^{1}SKWQHQQDSC^{10} que inhibió la viabilidad de las células HT-29 en un porcentaje máximo del 25,8% (200 µM, 72 h). En este sentido, Chang y col. (2014) recientemente encontraron que el fragmento ^{1}SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQG^{32} tenía un efecto inmunomodulante equiparable a la secuencia completa de la lunasina mediante la activación de células "natural killer"; mientras que Hernández-Ledesma y col. (2011) vieron que la región N-terminal, ^{1}SKWQHQQDSCRKQLQGVNLTPC^{22}, era menos activa que la C-terminal, ^{23}EKHIMEKIQGRGDDDDDDDDD^{43}, y el péptido lunasina completo en la inhibición del crecimiento de células de cáncer de mama MDA-MB-231, con valores de IC_{50} de 323,0, 138,0 y 181,0 µM respectivamente. Por lo tanto, nuestro trabajo ha sido el primer estudio que ha propuesto una actividad quimiopreventiva para la región N-terminal, concretamente para el fragmento ^{1}SKWQHQQDSC^{10} de la lunasina. Sin embargo, con los resultados del presente estudio no se pudo justificar la actividad observada para los digeridos de lunasina:IBB1 (Publicación I), por lo que el efecto

inhibitorio de otros componentes del digerido, del IBB1, o un efecto sinérgico entre los péptidos identificados podría haber contribuido a los efectos previamente demostrados.

El siguiente paso consistió en profundizar en el conocimiento de los mecanismos implicados en la actividad quimiopreventiva del péptido lunasina. La reciente teoría de las células madre cancerosas sugiere que muchos tipos de cáncer, entre ellos, predominantemente el cáncer colorrectal, se inician y mantienen por una pequeña población de células tumorogénicas capaces de auto-renovarse y diferenciarse en múltiples tipos celulares de forma continuada, desarrollando nuevos tumores a partir de los originales (Anderson y col., 2011). Además, estas células pueden ser responsables de las capacidades de recidiva, metástasis y resistencia a los tratamientos anticancerígenos que se presentan frecuentemente en esta enfermedad (Clevers, 2011). Esta situación ha dado lugar a la búsqueda de nuevos agentes farmacológicos, nutricionales y fitoquímicos con capacidad preventiva frente a esta subpoblación tumorogénica (Kim y col., 2012; Oh y col., 2016). Partiendo de este planteamiento, se evaluó el efecto citotóxico del péptido lunasina en la línea celular de carcinoma colorrectal HCT-116, cultivada bajo distintas condiciones que permitieron ensayar la actividad tanto en células nativas (parentales) como en células madre (esferas) tumorales (Publicación III). La lunasina fue capaz de inhibir la viabilidad de las células nativas tumorales a las 72 h de tratamiento con un valor de IC_{50} de 107,5 µM, mientras que su capacidad preventiva frente a la formación de esferas tumorales tras 10 días se dio a un valor de IC_{50} de 161,0 µM. Así, las células madre tumorales HCT-116 mostraron una menor sensibilidad a la lunasina, al igual que se había descrito en células de adenocarcinoma colorrectal SW620 para el ácido graso docosahexaenoico (Yang y col., 2013). Sin embargo, McConnell y col. (2015) encontraron que el efecto inhibitorio de la lunasina en células de cáncer de pulmón no microcítico aumentó notablemente cuando las células se cultivaron en un ambiente de

crecimiento no-adherente (propio de las células madre tumorales), en comparación con las condiciones adherentes. En esta misma línea, estos resultados también han sido recientemente demostrados en ensayos con células madre y nativas de melanoma de piel (Shidal y col., 2016). De estas observaciones y de nuestros trabajos, se puede confirmar que el efecto antiproliferativo del péptido lunasina depende de las condiciones de cultivo y de la línea tumoral celular evaluada.

Algunos de los mecanismos implicados en la supervivencia y proliferación de las células tumorales se relacionan con su elevado umbral de apoptosis y su replicación incontrolada durante el progreso del ciclo celular (Hanahan y Weinberg, 2011). Mediante el ensayo de anexina V-yoduro de propidio por citometría de flujo, se observó que la lunasina ejercía un efecto inductor de la apoptosis de hasta 1,8 y 2,0 veces, con respecto al control, a la máxima dosis empleada (80 µM) en las células nativas y madre HCT-116, respectivamente. Mediante Western-Blot se comprobó que este efecto desencadenante del proceso apoptótico estaba mediado por la activación de la vía de señalización caspasa-3, la cual se acompañaba de una disminución de la señal proteica PARP, relacionada con la viabilidad celular y la reparación del ADN. En este sentido, Dia y de Mejia (2010; 2011a) habían demostrado que el péptido lunasina participaba en la activación de la vía apoptótica mitocondrial de las células de cáncer colorrectal HT-29 y KM12L4, mediante un aumento de las señales Bax, citocromo c, caspasa-3, -2 y -9 y el cluster nuclear y una disminución de la señal Bcl-2. Paralelamente, en nuestro trabajo, ambas poblaciones tumorales nativas y madre se tiñeron con yoduro de propidio para determinar por citometría de flujo la cantidad de ADN presente y evaluar la fase del ciclo celular en la que se encontraban. Los resultados mostraron un moderado incremento concentración-dependiente en el porcentaje de células tratadas que se encontraban en la fase G1. Así, la dosis de 80 µM aumentó significativamente este porcentaje tanto en las células nativas (control: 66,5% *versus* lunasina: 72,0%) como células madre tumorales (control: 69,4% *versus*

lunasina: 74,0%). Este hecho demostraba la capacidad preventiva del péptido al bloquear la progresión del ciclo celular en dicha fase. Además, en el caso de las células nativas HCT-116, se observó un incremento de la población celular en fase sub-G0/G1, lo que sugiere degradación del ADN potencialmente debida a la muerte celular inducida por el efecto pro-apóptotico anteriormente descrito. Asociado con este efecto regulatorio sobre el ciclo celular, se demostró que el péptido lunasina era capaz de inducir levemente la expresión proteica del inhibidor de la quinasa dependiente de ciclina 1a, p21, que regula el avance en el ciclo celular de la fase G1 hacia la fase S, como también había sido descrito previamente en las células HT-29 y KM12L4 (Dia y de Mejia 2010; 2011a). En conjunto, estos resultados permitieron avanzar en el conocimiento de los mecanismos antiproliferativos ejercidos por el péptido lunasina frente al cáncer colorrectal al inhibir, tanto la proliferación de las células cancerígenas nativas como la expansión de la subpoblación de células madre tumorales.

El estrés oxidativo ha sido descrito como agente causal implicado en el inicio y la progresión de los procesos tumorales, mediante inducción de las mutaciones del ADN, inestabilidad genómica, daño a las moléculas celulares y aumento de la proliferación celular (Reuter y col., 2010; Sosa y col., 2013). Además, el exceso en los niveles de ROS ha sido vinculado con la patogénesis de múltiples enfermedades crónicas y degenerativas, como la inflamación, las enfermedades cardiovasculares y neurodegenerativas, la diabetes y el envejecimiento (Ramos, 2008; Wadley y col., 2013). En concreto, el hígado es un órgano diana del daño inducido por ROS, donde estas especies reactivas participan en patologías como la hepatitis vírica y alcohólica, la esteatosis no alcohólica, la fibrosis hepática o el hepatocarcinoma (Cichoz-Lach y Michalak, 2014). En varios estudios *in vivo* se había aislado el péptido lunasina en una forma intacta y bioactiva a partir del hígado de ratas alimentadas con dietas enriquecidas en dicho péptido (Jeong et al., 2007a; 2007c; 2009; 2010). Por ello, se propuso evaluar el efecto de la lunasina sobre los mecanismos celulares hepáticos de

defensa antioxidante (Publicación IV). En virtud de dichas consideraciones, se seleccionó la línea celular HepG2, como modelo aceptado en estudios bioquímicos, farmacológicos y nutricionales que evalúan la funcionalidad de las células del hígado en cultivo (Goya y col., 2009). El *t*-BOOH se utilizó como agente químico inductor del estrés oxidativo en esta línea celular (Alía y col., 2005). Se ha descrito que para determinar la actividad antioxidante celular de un compuesto es necesario evaluar sus efectos sobre la viabilidad celular, los niveles intracelulares de ROS, la respuesta de los mecanismos de defensa enzimáticos y no enzimáticos, y los marcadores de daño oxidativo celular (Valko y col., 2006). En los resultados del efecto directo del péptido lunasina (0,5 – 10 µM, 20 h) sobre las células HepG2 no estresadas, se encontró que la lunasina no afectaba a la viabilidad celular, además de disminuir la producción basal de ROS (máximo efecto a 5 µM: 63,6% con respecto al control), y aumentar el contenido en GSH (máximo efecto a 10 µM: 125,6% con respecto al control). Estos efectos situarían a la célula en un estado más favorable a la hora de afrontar una situación de estrés oxidativo (Goya y col., 2009). Se observó que el agente químico *t*-BOOH (400 µM, 3 h) inducía muerte celular en un 67,5%, mediante un aumento de las ROS (190,0% con respecto al control) y un descenso del GSH citosólico (42,2% con respecto al control). Sin embargo, el pretratamiento con el péptido lunasina durante 20 h fue capaz de proteger a las células frente al *t*-BOOH, recuperando por completo su viabilidad y mejorando parcialmente la generación de ROS (5 µM: 122,0%) y el contenido de GSH (0,5 µM: 66,5%), pero sin mostrar una tendencia dosis-dependiente. El GSH es el principal mecanismo intracelular de defensa no enzimático, estando presente en el hígado en altas concentraciones como agente detoxificante de la producción en exceso de ROS y mitigador de la peroxidación celular (Mari y col., 2009). Las enzimas GPx, en su relación con el GSH, y CAT, independiente de este mecanismo, son fundamentales para controlar el equilibrio redox celular. En este trabajo se demostró como el péptido lunasina disminuía la actividad de ambas enzimas CAT (máximo efecto a 5 µM: 102,1%) y, de una forma dosis-dependiente en

el caso de la GPx (máximo efecto a 10 µM: 127,9%), con respecto al marcado aumento inducido durante el daño oxidativo con *t*-BOOH (CAT: 256,8% y GPx: 235,3%). La oxidación de los grupos sulfuro de las proteínas y la consiguiente formación de grupos carbonilos, biomarcadores de daño oxidativo, puede conllevar la pérdida de la funcionalidad de enzimas, receptores y múltiples proteínas implicadas en la señalización celular (Wang y col., 2016). El agente químico *t*-BOOH provocó una elevación notable en la formación de grupos carbonilo en las células HepG2 (290,3% con respecto al control). Sin embargo, el péptido lunasina consiguió disminuirlos al nivel basal. Además, el estrés oxidativo también es capaz de inducir daño celular alterando vías de señalización sensibles al estado redox (Singh y Czaja, 2008) y al contenido intracelular de GSH (Franco y Cidlowski, 2012), como las desencadenantes de la apoptosis. Utilizando la caspasa-3 como biomarcador, se observó como el péptido lunasina (1 – 10 µM) evitaba la activación de la caspasa-3 inducida por el *t*-BOOH (185,7% con respecto al control). Por lo tanto, en este trabajo se identificó, por primera vez para el péptido lunasina, un notable efecto potenciador de los mecanismos de defensa antioxidantes de las células HepG2 que podría contribuir a la actividad quimiopreventiva previamente demostrada.

Las peptidasas presentes en el cultivo celular podrían degradar la lunasina, liberándose formas inactivas o bien generándose nuevas secuencias con mayor actividad a la descrita para el péptido precursor. Por lo tanto, resultó interesante evaluar, mediante HPLC-MS/MS, la estabilidad del péptido lunasina en las líneas celulares empleadas (Publicación II y IV). El análisis de los sobrenadantes de las células de cáncer gástrico (AGS) y colorrectal (HT-29 y Caco-2), reveló una degradación moderada del péptido lunasina durante las 72 h de tratamiento en contacto con las células AGS (27,0%) y HT-29 (32,0%). Este descenso en el porcentaje de lunasina se vio acompañado de la formación de varios fragmentos derivados de su extremo C-terminal: ^{23}EKHIMEKIQGRGDDDDDDDDD^{43},

^{25}HIMEKIQGRGDDDDDDDDD^{43}, ^{29}KIQGRGDDDDDDDDD^{43} y ^{32}GRGDDDDDDDDD^{43}. Por el contrario, en el caso de las células Caco-2, la señal del péptido lunasina se mantuvo al mismo nivel durante las 72 h, por lo que la pérdida del efecto inhibitorio observado a este tiempo de tratamiento no se pudo atribuir ni a la degradación de la lunasina ni a la formación de fragmentos derivados con menor actividad. En el caso de las células hepáticas HepG2 tratadas con dicho péptido, se observó una marcada diferencia con respecto al trabajo anterior, ya que el contenido en lunasina descendió completamente con el tiempo de incubación quedando menos de un 1,0% de lunasina intacta a las 20 h. Además se identificaron varios fragmentos derivados: ^{25}HIMEKIQGRGDDDDDDDDD^{43}, ^{26}IMEKIQGRGDDDDDDDDD^{43}, ^{29}KIQGRGDDDDDDDDD^{43}, ^{30}IQGRGDDDDDDDDD^{43} y ^{32}GRGDDDDDDDDD^{43}. Merece la pena mencionar que los fragmentos identificados en ambos trabajos provienen de la misma región C-terminal de la lunasina, por lo que se podría sugerir que las líneas celulares AGS, HT-29 y HepG2 comparten mecanismos enzimáticos frente a la incubación con dicho péptido, aunque las células hepáticas indujeron un descenso en su contenido mucho mayor. Además, estos fragmentos pertenecen al dominio C-terminal descrito, hasta el momento, como la región activa de la lunasina (Hernández-Ledesma y col., 2009c) y contienen el motivo ^{33}RGD^{35} relacionado con la interacción del péptido con los receptores e integrinas celulares (Galvez y col., 2001; Dia y de Mejia, 2011a). En este sentido, la reducción del contenido en lunasina en el sobrenadante del medio celular podría también deberse a la internalización celular del péptido, cómo ha sido descrito en varios trabajos (Galvez y col., 2001; Dia y de Mejia, 2011a; Galvez y col., 2011; Cam y col., 2013).

3.2. Efectos de péptidos lácteos sobre la mucosa intestinal. Estudio peptidómico de digeridos humanos en comparación con digeridos *in vitro*

El epitelio gastrointestinal se encuentra protegido por una capa de mucus, cuya producción puede estar modulada por diferentes componentes alimentarios (Macha y col., 2015). Entre ellos, el péptido β-CM-7, derivado de la β-CN, había demostrado influencia en la producción de mucinas mediada por los receptores opioides del subtipo μ en yeyuno aislado de rata (Claustre y col., 2002; Trompette y col., 2003) y en células caliciformes intestinales humanas y de rata (Zoghbi y col., 2006). Recientemente, Martínez-Maqueda y col. (2013a) demostraron que los péptidos derivados de la α_{s1}-CN ^{143}AYFYPEL^{149} e ^{144}YFYPEL^{149} inducían un aumento en la secreción y expresión génica de la mucina 5AC en células HT29-MTX. Debido a que estas dos secuencias presentaban una estructura favorable para interaccionar con los receptores opioides localizados en el tracto digestivo (Meisel, 1997), se decidió evaluar la actividad opioide de estos péptidos, y de los fragmentos derivados ^{144}YFYPE^{148}, ^{144}YFYP^{147} e ^{144}YFY^{146} (Publicación V). En los primeros ensayos con íleon aislado de cobaya, usando una curva dosis-acumulativa, se observó efecto opioide para los péptidos ^{144}YFYPEL^{149} e ^{144}YFYPE^{148} a la dosis de $5{,}5 \times 10^{-7}$ M. Sin embargo, el efecto tendía a disminuir durante el tiempo del ensayo, lo que planteó la hipótesis de que los péptidos pudieran estar degradándose por las peptidasas propias de la preparación, como se había descrito previamente para péptidos opioides endógenos (McKnight y col., 1983). Este hecho se confirmó mediante HPLC-MS/MS y al repetir los ensayos en presencia de inhibidores de proteasas (Aoki y col., 1984). En estas condiciones, se describió, por primera vez, una moderada actividad opioide dosis-dependiente para ^{144}YFYPE^{148} (máximo efecto a $5{,}0 \times 10^{-6}$ M: 31,6% de inhibición) seguida de ^{144}YFYPEL^{149} (26,2%), ^{144}YFY^{146} (25,5%) y ^{143}AYFYPEL^{149} (22,2%). Estos efectos en el tejido de íleon de cobaya fueron revertidos por el antagonista opioide naloxona, pero disminuyeron notablemente en la preparación de deferente de ratón, lo que sugería

selectividad de los péptidos por el subtipo μ-opioide, tal como se había descrito para las β-CMs (Teschemacher, 2003). Para explicar la falta de actividad del péptido ^{144}YFYP^{147}, en este trabajo se llevó a cabo, por primera vez, una modelización de la interacción molecular entre un péptido opioide alimentario (^{144}YFYPEL^{149}, activo, *versus* ^{144}YFYP^{147}, inactivo) y el receptor μ-opioide. Las simulaciones con ambos péptidos mostraron una variación marcada de energía en las posiciones del receptor 145-150, 195-200, 240-250 y la región C-terminal. Sin embargo, la flexibilidad de la región 195-200, íntimamente relacionada con la actividad del receptor (Waldhoer y col., 2004), se vio comprometida en el caso del péptido ^{144}YFYP^{147}. Además, las interacciones entre los residuos de los péptidos y los del receptor opioide se distribuyeron de forma diferente en ambos casos, tanto por el tipo de unión establecida como por la intensidad de las mismas. El residuo prolina N-terminal del péptido ^{144}YFYP^{147} fue determinante en la interacción con el receptor, lo que no se observó en la simulación con ^{144}YFYPEL^{149}, resultando en un claro cambio en la disposición y orientación de ambos péptidos con respecto al lugar de unión activo del receptor opioide.

Paralelamente, se planteó determinar, mediante reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa (qRT-PCR), la expresión relativa de MUC5AC en células HT29-MTX bajo la acción de estos péptidos lácteos. Considerando los efectos opioides descritos, cabría esperar que además de ^{143}AYFYPEL^{149} e ^{144}YFYPEL^{149} (Martínez-Maqueda y col., 2013a), la secuencia ^{144}YFYPE^{148} pudiera mostrar un efecto estimulatorio sobre la expresión de mucinas. Aunque se observó un incremento por encima del nivel basal de las células control, este aumento no fue significativo, como ocurrió en el caso de los péptidos ^{144}YFYP^{147} e ^{144}YFY^{146}. Por ello, se estableció que el péptido ^{144}YFYPEL^{149} constituye el fragmento mínimo con actividad estimulante sobre la expresión génica de MUC5AC. Nuestros resultados muestran una aparente falta de correspondencia entre la actividad opioide

en íleon de cobaya y la sobreexpresión de MUC5AC en células HT29-MTX. En este sentido, algunos estudios con otros péptidos alimentarios han conseguido caracterizar bien esta relación (Zoghbi y col., 2006; Martínez-Maqueda y col., 2013b). Sin embargo, en otros trabajos se han obtenido resultados contradictorios entre la interacción opioide y la producción de mucinas (Martínez-Maqueda y col., 2012; Plaisancié y col., 2013; 2015). Por todo ello, se sugiere que el efecto de las proteínas alimentarias y sus péptidos derivados sobre la capa mucosa puede estar mediado por más de un mecanismo, además del descrito a nivel de los receptores opioides.

Partiendo de estos resultados, y del estudio de Martínez-Maqueda y col. (2013a) en el que se demostró el efecto estimulatorio de los péptidos ^{143}AYFYPEL^{149} e ^{144}YFYPEL^{149}, y de un hidrolizado de caseínas con pepsina que los contiene, sobre el fortalecimiento de la mucosa intestinal, se planteó evaluar *in vivo* el efecto de este hidrolizado lácteo (Publicación VI). Para ello, el hidrolizado se incorporó a la dieta de ratas Zucker durante 8 semanas (0,43 g de hidrolizado/kg/día) y se estudió la expresión génica correspondiente a las tres principales mucinas del intestino de rata (Muc2, Muc3 y Muc4) y la secreción de contenido mucoso en el lumen digestivo y en las heces. Mediante qRT-PCR, se observó que las ratas que habían consumido el hidrolizado de caseínas presentaban un aumento significativo en la expresión relativa de los genes que codifican para la mucina secretada Muc2 y la mucina de membrana Muc3 en el íleon (1,31 y 1,20 veces la expresión basal, respectivamente) y el colon (1,51 y 1,47, respectivamente). Plaisancié y col. (2013) demostraron que la administración intragástrica en ratas del péptido β-CN f(94-123) (0,01-100 μM) incrementaba los niveles de ARNm de los genes Muc2 en el yeyuno (1,39 – 1,52) y Muc4 en el duodeno (1,56), yeyuno (1,60 – 1,61) e íleon (1,57), con respecto al control, aunque no se describió una actividad dosis-dependiente. A diferencia de nuestros resultados, en este trabajo no se encontraron efectos a nivel de la mucosa del colon, que cobran especial importancia debido al papel protector que ejerce

principalmente la mucina Muc2 en este tejido. Se ha descrito que ratones genéticamente deficientes en la mucina Muc2 pueden desarrollar de forma espontánea colitis (Van der Sluis y col., 2006) y cáncer colorrectal (Velcich y col., 2002). Por ello, el hidrolizado de caseínas utilizado en nuestro estudio podría demostrar un efecto particularmente beneficioso a nivel de la mucosa colónica mediante el incremento en la expresión de genes correspondientes a las principales mucinas intestinales. Por otro lado, este hidrolizado también demostró efectos regulatorios en la secreción de mucinas. En comparación con las ratas control, el contenido mucoso fecal a las 2 y 8 semanas de tratamiento se incrementó en un 129 y 147%, respectivamente. Estos resultados son similares a los descritos por Sprong y col. (2010) para proteínas de suero lácteo en un modelo animal de colitis inducida. Sin embargo, aunque la cantidad de mucinas en el contenido luminal de las ratas alimentadas con el hidrolizado de caseínas también fue mayor al de las ratas control (124%), las diferencias encontradas no fueron estadísticamente significativas.

El potencial papel regulatorio que pueden ejercer los componentes alimentarios en contacto continuo con la barrera mucosa gastrointestinal había sido propuesto en la bibliografía (Moughan y col., 2007; Bischoff y col., 2014). El hidrolizado de caseínas empleado en el presente trabajo había mostrado previamente un efecto estimulante, con respecto a las células HT29-MTX control, de la secreción de mucinas (210%) y de la expresión génica de MUC5AC (1,80) (Martínez-Maqueda y col. 2013a). Estos efectos se atribuyeron, principalmente, a los péptidos ^{143}AYFYPEL^{149} e ^{144}YFYPEL^{149}, secuencias que han demostrado actividad opioide en esta memoria (Publicación V). Mediante HPLC-MS/MS se cuantificó la presencia del péptido ^{143}AYFYPEL^{149} en el hidrolizado de caseínas (4,1 mg de péptido/g de hidrolizado). Considerando el volumen del contenido gastrointestinal de la rata, esta determinación sería fisiológicamente compatible con el rango de concentraciones (0,05-0,50 mM) que mostró actividad en las células HT29-MTX (Martínez-Maqueda y col. 2013a). Además,

tal como describieron estos autores, otros componentes y péptidos del hidrolizado podrían contribuir en los efectos descritos.

Una gran parte de los trabajos experimentales incluidos en esta Tesis Doctoral han contribuido al conocimiento existente acerca de la influencia directa ejercida por el proceso digestivo sobre la actividad biológica de las proteínas alimentarias y los péptidos derivados (Picariello y col., 2013b; Sánchez-Rivera y col., 2014a). Impulsados por este objetivo, se ha publicado en la bibliografía una amplia variedad de metodologías para reproducir *in vitro* las condiciones fisiológicas de la digestión gastrointestinal (Guerra y col., 2012). En el ámbito de la Acción COST FA 1005 INFOGEST, Minekus y col. (2014) propusieron un protocolo consensuado a nivel internacional entre distintos grupos de investigación acerca de las condiciones estándar a aplicar en estos ensayos de simulación *in vitro* de la digestión gastrointestinal. Por ello, se planteó un estudio comparativo de los resultados obtenidos tras la digestión de caseínas y proteínas de suero lácteo mediante dicho protocolo y la digestión *in vivo* a nivel del yeyuno de individuos sanos (Publicación VII). Este estudio ha permitido avanzar hacia la validación de las metodologías *in vitro* con datos obtenidos *in vivo*, lo cual constituye una prioridad reconocida a nivel internacional.

La degradación que sufrieron las proteínas lácteas durante el proceso digestivo se evaluó mediante electroforesis en gel de poliacrilamida (SDS-PAGE). Tal como se había encontrado en otras simulaciones *in vitro* (Dupont y col., 2010; Picariello y col., 2010; Kopf-Bolanz y col., 2012; Ménard y col., 2014), las caseínas se degradaron rápidamente durante la fase gástrica, no detectándose a partir de los 30 minutos con este protocolo, mientras que las proteínas de suero β-Lg y α-La resistieron la actividad de la pepsina, pero se hidrolizaron completamente tras 60 minutos de fase intestinal. En el caso de las muestras obtenidas *in vivo*, no se detectó la presencia de caseínas intactas a nivel del yeyuno, pero sí de β-Lg en algunos individuos, con intensidad

variable, 1 y 2 h tras la ingesta de las proteínas de suero. Estos resultados están de acuerdo con lo descrito por Mahé y col. (1996), donde se describe que tras la ingesta de las proteínas lácteas, mientras las caseínas se degradan completamente, las proteínas de suero resisten parcialmente llegando en una forma intacta al yeyuno humano.

El perfil peptídico de los digeridos obtenidos *in vitro/in vivo* se analizó mediante HPLC-MS/MS, y se elaboraron unos mapas térmicos para facilitar la comparación a nivel cualitativo. En general, se observó que la diversidad del peptidoma *in vivo* era mayor a la encontrada *in vitro*, probablemente debido a la mayor complejidad de las condiciones propias de la digestión y a la variabilidad entre los distintos individuos. Por otro lado, los resultados encontrados fueron distintos según la proteína láctea analizada. Los perfiles peptídicos *in vivo* e intestinales *in vitro* de la β-CN fueron comparables en todos los tiempos evaluados, predominantemente en las regiones N- y C-terminales. Sin embargo, los péptidos procedentes de la región central de la proteína, y de una manera más marcada en el caso de la α_{s1}-CN, se identificaron en mayor medida en las muestras de la digestión *in vivo*. Los péptidos derivados de la digestión *in vitro* de la β-Lg se detectaron únicamente en la fase intestinal, de acuerdo a lo observado por SDS-PAGE. En este caso, la mayoría de los péptidos encontrados pertenecían a la región central, en especial entre los aminoácidos 42-68 y 107-135, mientras que en la digestión *in vivo* se identificó una mayor variabilidad de fragmentos que cubrieron la secuencia de la proteína prácticamente en su totalidad. Sin embargo, los perfiles peptídicos *in vitro/in vivo* de la α-La fueron bastante parecidos, coincidiendo en las regiones 40-52, 63-68 y 80-90. Merece la pena destacar que en este trabajo, al igual que describieron Boutrou y col. (2013), los péptidos obtenidos *in vivo* tras la ingesta de caseínas tendieron a mantener su presencia en el yeyuno durante las 4 h del estudio, mientras que el número de los péptidos derivados de proteínas de suero disminuyó notablemente después de las 3 h.

Además, la frecuencia de aparición de cada aminoácido de las proteínas mayoritarias β-CN y β-Lg se usó para correlacionar los resultados encontrados en los digeridos *in vitro* e *in vivo*. Al comparar los digeridos intestinales *in vitro* y los digeridos *in vivo*, los coeficientes de correlación de Spearman variaron entre 0,39 y 0,67 en el caso de la β-CN, y entre 0,55 y 0,71 para la β-Lg. Resulta interesante destacar que estos coeficientes son del mismo orden a los encontrados entre los distintos individuos del estudio: 0,51-0,77 (β-CN) y 0,28-0,77 (β-Lg). Por lo tanto, la comparación llevada a cabo entre los resultados *in vitro* e *in vivo* podría ser equiparable a la propia variación inter-individual.

Ciertas regiones proteicas, como aquellas ricas en aminoácidos hidrofóbicos y residuos de prolina, habían demostrado una especial resistencia al ataque de las enzimas digestivas (Hausch y col., 2002). Por ejemplo, el péptido opioide β-CM-7 (β-CN ^{60}YPFPGPI^{66}) así como varios fragmentos que contenían las secuencias de la β-CN ^{74}IPPLT^{78}, ^{83}VVPP^{86}, ^{134}HLPLP^{138}, ^{150}PLPP^{153}, ^{157}FPP^{159}, ^{172}PVP^{174} y ^{204}PFPI^{207}, se detectaron tanto en los digeridos *in vitro* como en los digeridos obtenidos en el yeyuno humano. Asimismo, en ambos digeridos, se identificó el péptido derivado de la α_{s1}-CN ^{143}AYFYPEL^{149}, precursor de secuencias con efecto opioide y estimulante de la expresión génica de mucinas intestinales (Publicación V). Este péptido también había sido identificado en el estómago de humanos que consumieron leche (Chabance y col., 1998). Asimismo, los CPPs han sido descritos como regiones con particular resistencia al proceso digestivo (Vegarud y col., 2000; García-Nebot y col., 2010). En el presente estudio se llevó a cabo un procedimiento de precipitación selectiva sobre los digeridos *in vivo* de caseínas para favorecer la identificación de regiones fosforiladas. Así, se detectó en el yeyuno humano la presencia de varios fragmentos fosforilados como aquellos pertenecientes a la región 1-24 de la β-CN, y 42-52, 64-68 y 110-119 de la α_{s1}-CN. Resulta interesante mencionar que algunas secuencias como α_{s1}-CN f(43-52)2P, identificada durante las 4 h de la digestión *in vivo* en varios

individuos, han sido recientemente detectadas en el plasma de humanos tras la ingesta de queso (Caira y col., 2016), lo que reforzaría la importancia de su presencia en el tracto digestivo.

Con el fin de comparar más en profundidad los resultados *in vivo/in vitro* se cuantificó el número de péptidos comunes entre los distintos digeridos. En el caso de las caseínas, se identificaron un total de 465 péptidos después de la ingesta *in vivo* (β-CN: 176, α_{s1}-CN: 131, κ-CN: 84 y α_{s2}-CN: 74) y 338 tras la simulación *in vitro* (β-CN: 97, α_{s1}-CN: 78, κ-CN: 97 y α_{s2}-CN: 66). De todos ellos, 101 secuencias (14,4%) fueron idénticas entre ambas digestiones. El mayor porcentaje de péptidos comunes se encontró en la proteína β-CN (23,0% del total de los péptidos identificados). Una situación similar se encontró para las proteínas de suero con 34 secuencias comunes (13,0%) entre las 179 y 117 encontradas *in vivo* e *in vitro*, respectivamente.

La metodología empleada en este trabajo se había presentado como una herramienta simple y eficaz para simular *in vitro* la digestión gastrointestinal (Minekus y col., 2014), y su reproducibilidad ha sido demostrada recientemente mediante ensayos inter-laboratorio (Egger y col., 2016). Con el presente estudio comparativo se ha conseguido avanzar hacia su correlación y validación con resultados obtenidos *in vivo*. Aunque todavía presenta las limitaciones propias de los métodos estáticos de simulación gastrointestinal en comparación al conjunto de complejos procesos involucrados en el fenómeno de la digestión, los resultados del presente trabajo muestran una buena aproximación del método *in vitro* hacia la digestión fisiológica de las proteínas lácteas.

4. CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. Bowman-Birk isoinhibitor 1 exerts a protective effect on lunasin degradation under conditions that simulate gastrointestinal digestion. The regions ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{19}LTPCEKHIME^{28} and ^{29}KIQGRGDDDDDDDDD^{43} from lunasin are particularly resistant to the action of digestive enzymes.

2. It has been demonstrated the absorption of peptide lunasin and the derived fragment ^{11}RKQLQGVN^{18} through Caco-2 cell monolayers, being the paracellular pathway the main mechanism involved in the transport of these peptides.

3. Lunasin and some derived fragments identified in its gastric and gastrointestinal digests have preventive effects against the growth of gastric cancer AGS and colorectal cancer HT-29 and Caco-2 cells. The fragment ^{1}SKWQHQQDSC^{10} is the main responsible for the proved inhibitory activity on cellular viability, especially in HT-29 cells.

4. Lunasin exerts a chemopreventive effect in colorectal cancer HCT-116 cells, both, the bulk tumor population and the derived cancer stem cells. This effect is mediated by inhibiting cell proliferation, inducing apoptosis and arresting cell cycle at G1 phase.

5. In liver HepG2 cells, peptide lunasin plays a protective role against chemical-induced oxidative stress. This capacity is modulated through stimulation of antioxidant defense cellular systems, and reduction of apoptosis and oxidative damage to proteins.

6. Milk α_{s1}-casein peptides ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} and, to a lower extent, ^{144}YFY^{146} and ^{143}AYFYPEL^{149} show opioid activity in guinea pig ileum preparations. By molecular dynamics simulations, it is demonstrated that the C-terminal proline residue from peptide ^{144}YFYP^{147} affects to its interaction with the μ-opioid receptor active binding site, decreasing activity.

7. The peptic casein hydrolyzate that contains the previously identified opioid peptides promotes the rat intestinal mucus barrier. This hydrolyzate stimulates gene expression of ileal and colonic mucins, Muc2 and Muc3, and induces the secretion of fecal mucus material.

8. Protein degradation and peptidome of human jejunal digests from casein and whey protein are comparable to those found after *in vitro* simulated gastrointestinal digestion following a harmonized protocol. Spearman correlation coefficients between *in vivo* and *in vitro* digests are within the range found for the different volunteers of the study, suggesting that the *in vitro* method constitutes a good approximation of the physiological digestion of milk proteins.

CONCLUSIONES

1. El isoinhibidor-1 de Bowman Birk ejerce un efecto protector frente a la degradación del péptido lunasina en condiciones que simulan la digestión gastrointestinal. Las regiones de la lunasina ^{1}SKWQHQQDSC^{10}, ^{11}RKQLQGVN^{18}, ^{19}LTPCEKHIME^{28} y ^{29}KIQGRGDDDDDDDDD^{43} son especialmente resistentes a la acción de las enzimas digestivas.

2. Se ha demostrado la absorción del péptido lunasina y el fragmento derivado ^{11}RKQLQGVN^{18} en un modelo de células Caco-2 en monocapa, siendo la vía paracelular el principal mecanismo de transporte para estos péptidos.

3. El péptido lunasina y algunos fragmentos derivados identificados en sus digeridos gástricos y gastrointestinales ejercen efectos preventivos frente al crecimiento de células de cáncer gástrico AGS y colorrectal HT-29 y Caco-2. La región ^{1}SKWQHQQDSC^{10} es la principal responsable de la actividad inhibitoria sobre la viabilidad celular, especialmente en células HT-29.

4. El péptido lunasina ejerce un efecto quimiopreventivo en células de cáncer colorrectal HCT-116, tanto en la población nativa como en la correspondiente a células madre tumorales. Este efecto está mediado por una acción antiproliferativa, inductora de la apoptosis y bloqueante del ciclo celular en la fase G1.

5. En células hepáticas HepG2, el péptido lunasina protege frente al estrés oxidativo inducido químicamente. Esta protección está modulada por la estimulación de los sistemas celulares de defensa antioxidante, y la disminución de la apoptosis celular y del daño oxidativo a proteínas.

6. Los péptidos lácteos derivados de la α_{s1}-caseína ^{144}YFYPEL^{149} e ^{144}YFYPE^{148} y, en menor medida, ^{144}YFY^{146} y ^{143}AYFYPEL^{149} presentan actividad opioide en íleon aislado de cobaya. Mediante modelización dinámica, se demuestra que la presencia de prolina en el extremo carboxi-terminal del péptido ^{144}YFYP^{147} afecta negativamente a su interacción con el centro activo del receptor μ-opioide, disminuyendo su actividad.

7. El hidrolizado de caseínas con pepsina que contiene los péptidos opioides previamente identificados fortalece la capa mucosa del intestino en un modelo de rata. Este hidrolizado estimula la expresión génica de las mucinas Muc2 y Muc3 en íleon y colon, y produce un aumento del contenido mucoso fecal.

8. La degradación proteica y el peptidoma de los digeridos de caseínas y proteínas de suero obtenidos en yeyuno humano son comparables a los encontrados tras aplicar un protocolo de digestión gastrointestinal *in vitro* consensuado a nivel internacional. Los coeficientes de correlación de Spearman obtenidos tras la comparación de la digestión *in vitro* e *in vivo* son del mismo orden a los encontrados entre los distintos individuos del estudio, demostrando que la metodología *in vitro* es una buena aproximación a la digestión fisiológica de proteínas lácteas.

5. BIBLIOGRAFÍA

Alía, M., Ramos, S., Mateos, R., Bravo, L., y Goya, L. (2005). Response of the antioxidant defence system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology, 19*, 119-128.

Alzoghaibi, M. A. (2013). Concepts of oxidative stress and antioxidant defense in Crohn´s disease. *World Journal of Gastroenterology, 19*, 6540-6547.

Amigo-Benavent, M., Clemente, A., Caira, S., Stiuso, P., Ferranti, P., y del Castillo, M. D. (2014). Use of phytochemomics to evaluate the bioavailability and bioactivity of antioxidant peptides of soybean β-conglycinin. *Electrophoresis, 35*, 1582-1589.

Amin, A. R., Kucuk, O., Khuri, F. R., y Shin, D. M. (2009). Perspectives for cancer pervention with natural compounds. *Journal of Clinical Oncology, 27*, 2712-2725.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung, B., y Aggarwal, B. B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research, 25*, 2097-2116.

Anderson, E. C., Hessman, C., Levin, T. G., Monroe, M. M., y Wong, M. H. (2011). The role of colorectal cancer stem cells in metastatic disease and therapeutic response. *Cancers, 3*, 319-339.

Antunes, F., Andrade, F., Ferreira, D., Nielsen, H. M., y Sarmento, B. (2013). Models to predict intestinal absorption of therapeutic peptides and proteins. *Current Drug Metabolism, 14*, 4-20.

Aoki, K., Kajiwara, M., y Oka, T. (1984). The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive "enkephalinase" in the potency of enkephalins in the guinea-pig ileum. *Japan Journal of Pharmacology, 36*, 59-65.

Arbogast, S., Smith, J., Matuszczak, Y., Hardin, B. J., Moylan, J. S., Smith, J. D., Ware, J., Kennedy, A. R., y Reid, M. B. (2007). Bowman-Birk inhibitor concentrate prevents atrophy, weakness, and oxidative stress in soleus muscle of hindlimb-unloaded mice. *The Journal of Applied Physiology, 102*, 956-964.

Barnes, P. J., y Adcock, I. M. (2009). Glucocorticoid resistance in inflammatory diseases. *Lancet, 373*, 1915-1917.

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., y Dupont, D. (2014). Tracking the *in vivo* release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63*, 147-156.

Bendtsen, L. Q., Lorenzen, J. K., Bendsen, N. T., Rasmussen, C., y Astrup, A. (2013). Effect of dairy proteins on appetite, energy expenditure, body weight, and composition: a review of the evidence from controlled clinical trials. *Advances in Nutrition, 4*, 418-438.

Benedé, S., López-Expósito, I., Giménez, G., Grishina, G., Bardina, L., Sampson, H. A., López-Fandiño, R., y Molina, E. (2014a). Mapping of IgE epitopes in *in vitro* gastroduodenal digests of β-lactoglobulin produced with human and simulated fluids. *Food Research International, 62*, 1127-1133.

Benedé, S., López-Expósito, I., Giménez, G., Grishina, G., Bardina, L., Sampson, H. A., Molina, E., y López-Fandiño, R. (2014b). *In vitro* digestibility of bovine β-casein with simulated and human oral and gastrointestinal fluids. Identification and IgE-reactivity of the resultant peptides. *Food Chemistry, 143*, 514-521.

Bessette, C., Benoit, B., Sekkal, S., Bruno, J., Estienne, M., Léonil, J., Ferrier, L., Théodorou, V., y Plaisancié, P. (2016). Protective effects of β-casofensin, a bioactive peptide from bovine β-casein, against indomethacin-induced intestinal lesions in rats. *Molecular Nutrition & Food Research, 60*, 823-833.

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., y Crowe, S. E. (2014). Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews, 94,* 329-354.

Bhutia, S. K., y Maiti, T. K. (2008). Targeting tumors with peptides from natural sources. *Trends in Biotechnology, 26*, 210-217.

Bimonte, S., Barbieri, A., Leongito, M., Piccirillo, M., Giudice, A., Pivonello, C., De Angelis, C., Granata, V., Palaia, R., e Izzo, F. (2016). Curcumin anticancer studies in pancreatic cancer. *Nutrients, 8,* 433.

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J. –D., Serino, M., Tilg, H., Watson, A., y Wells, J. M. (2014). Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology, 14*, 189.

Blanco-Míguez, A., Gutiérrez-Jácome, A., Pérez-Pérez, M., Pérez-Rodríguez, G., Catalán-García, S., Fdez-Riverola, F., Lourenço, A., y Sánchez, B. (2016). From amino acid sequence to bioactivity: The biomedical potential of antitumor peptides. *Protein Science, 25*, 1084-1095.

Boirie, Y., Dangin, M., Gachon, P., Vasson, M. –P., Maubois, J. –L., y Beaufrère, B. (1997). Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 14930-14935.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tomé, D., y Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition, 97*, 1314-1323.

Boutrou, R., Henry, G., y Sánchez-Rivera, L. (2015). On the trail of milk bioactive peptides in human and animal intestinal tracts during digestion: A review. *Dairy Science & Technology, 95*, 815-829.

Brantl, V., Gramsch, C., Lottspeich, F., Mertz, R., Jaeger, K. H., y Herz, A. (1986). Novel opioid peptides derived from hemoglobin: hemorphins. *European Journal of Pharmacology, 125*, 309-310.

Brantl, V., Teschemacher, H., Henschen, A., y Lottspeich, F. (1979). Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie, 360*, 1211-1216.

Brantl, V., Teschemacher, H., Bläsig, J., Henschen, A., y Lottspeich, F. (1981). Opioid activities of β-casomorphins. *Life Sciences, 28*, 1903-1909.

Brinkmann, C. R., Thiel, S., y Otzen, D. E. (2013). Protein-fatty acid complexes: biochemistry, biophysics and function. *FEBS Journal, 280*, 1733-1749.

Brommage, R., Juillerat, M. A., y Jost, R. (1991). Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Lait, 71*, 173-180.

Caira, S., Pinto, G., Vitaglione, P., Dal Piaz, F., Ferranti, P., y Addeo, F. (2016). Identification of casein peptides in plasma of subjects after a cheese-enriched diet. *Food Research International, 84*, 108-112.

Cam, A., y de Mejia, E. G. (2012). RGD-peptide lunasin inhibits Akt-mediated NF-κB activation in human macrophages though interaction with the αVβ3 integrin. *Molecular Nutrition & Food Research, 56*, 1569-1581.

Cam, A., Sivaguru, M., y de Mejia, E. G. (2013). Endocytic mechanism of internalization of dietary peptide lunasin into macrophages in inflammatory condition associated with cardiovascular disease. *PLoS ONE, 8*, e72115.

Capriotti, A. L., Caruso, G., Cavaliere, C., Samperi, R., Ventura, S., Chiozzi, R. Z., y Laganà, A. (2015). Identification of potential bioactive peptides generated by simulated gastrointestinal digestion of soybean seed and soy milk proteins. *Journal of Food Composition and Analysis, 44*, 205-213.

Capriotti, A. L., Cavaliere, C., Piovesana, S., Samperi, R., y Laganà, A. (2016). Recent trends in the analysis of bioactive peptides in milk and dairy products. *Analytical & Bioanalytical Chemistry, 408,* 2677-2685.

Carbonaro, M., Maselli, P., y Nucara, A. (2015). Structural aspects of legume proteins and nutraceutical properties. *Food Research International, 76*, 19-30.

Carli, A. P., Vieira, P. M. A., Santos Silva, K. T., Guerra de Sá Cota, R., Martins Carneiro, C., Castro-Borges, W., y Guerra de Andrade, M. H. (2012). Bowman-Birk inhibitors, proteasome peptidase activities and colorectal pre neoplasias induced by 1,2-dimethylhydrazine in *Swiss* mice. *Food and Chemical Toxicology, 50*, 1405-1412.

Carocho, M., y Ferreira, I. (2013). A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and futute perspectives. *Food and Chemical Toxicology, 51*, 15-25.

Celec, P., Hodosy, J., Pálffy, R., Gardlík, R., Halcak, L., y Ostatníková, D. (2013). The short-term effects of soybean intake on oxidative and carbonyl stress in men and women. *Molecules, 18*, 5190-5200.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollès, P., y Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie, 80*, 155-165.

Chakrabarti, S., Jahandideh, F., y Wu, J. (2014). Food-derived bioactive peptides on inflammation and oxidative stress. *BioMed Research International, 2014*, *608979*.

Chang, H. –C., Lewis, D., Tung, C. –Y., Han, L., Henriquez, S. M. P., Voiles, L., Lupov, I. P., Pelloso, D., Sinn, A. L., Pollok, K. E., de Lumen, B. O., Li, F., Blum, J. S., Srivastava, S., y Robertson, M. J. (2014). Soypeptide lunasin in cytokine immunotherapy for lymphoma. *Cancer Immunology, Immunotherapy, 63*, 283-295.

Chang, K. –J., Lillian, A., Hazum, E., Cuatrecasas, P., y Chang, J. –K. (1981). Morphiceptin (NH_4-Tyr-Pro-Phe-Pro-$CONH_2$): A potent and specific agonist for morphine (μ) receptors. *Science, 212*, 75-77.

Chiba, H., Tani, F., y Yoshikawa, M. (1989). Opioid antagonist peptides derived from κ-casein. *Journal of Dairy Research, 56*, 363-366.

Chitapanarux, T., Tienboon, P., Pojchamarnwiputh, S., y Leelarungrayub, D. (2009). Open-labeled pilot study of cystein-rich whey protein isolate supplementation for nonalcoholic steatohepatitis patients. *Journal of Gastroenterology and Hepatology, 24*, 1045-1050.

Cichoz-Lach, H., y Michalak, A. (2014). Oxidative stress as a crucial factor in liver diseases. *World Journal of Gastroenterology, 20,* 8082-8091.

Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., y Plaisancié, P. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 283*, G521-G528.

Clemente, A., y Arqués, M. D. (2014). Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents. *World Journal of Gastroenterology, 20*, 10305-10315.

Clemente, A., y Domoney, C. (2006). Biological significance of polymorphism in legume protease inhibitors from the Bowman-Birk family. *Current Protein & Peptide Science, 7*, 201-216.

Clemente, A., Moreno, F. J., Marín-Manzano, M. C., Jiménez, E., y Domoney, C. (2010). The cytotoxic effect of Bowman-Birk isoinhibitors, IBB1 and IBBD2, from soybean (*Glycine max*) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases. *Molecular Nutrition & Food Research, 54*, 396-405.

Clemente, A., Sonnante, G., y Domoney, C. (2011). Bowman-Birk inhibitors from legumes and human gastrointestinal health: current status and perspectives. *Current Protein & Peptide Science, 12*, 358-373.

Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nature Medicine, 17*, 313-319.

Contreras, M. M., Sancho, A. I., Recio, I., y Mills, C. (2012). Absorption of casein antihypertensive peptides through an *in vitro* model of intestinal epithelium. *Food Digestion, 3*, 16-24.

Dalgleish, D. G. (1993). Bovine milk protein properties and the manufacturing quality of milk. *Livestock Production Science, 35,* 75-93.

Daniel, H., Vohwinkel, M., y Rehner, G. (1990). Effect of casein and β-casomorphins on gastrointestinal motility in rats. *Journal of Nutrition, 120*, 252-257.

Davis, C. D., y Milner, J. A. (2007). Molecular targets for nutritional preemption of cancer. *Current Cancer Drug Targets, 7*, 410-415.

Defilippi, C., Gomez, E., Charlin, V., y Silva, C. (1994). Inhibition of small intestinal motility by casein: a role of beta casomorphins? *Nutrition, 11*, 751-754.

de Kok, T. M., van Breda, S. G., y Manson, M. M. (2008). Mechanisms of combined action of different chemopreventive dietary compounds. *European Journal of Nutrition, 47*, 51-59.

de Mejia, E. G., y Dia, V. P. (2010). The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Reviews, 29*, 511-528.

de Mejia, E. G., Wang, W., y Dia, V. P. (2010). Lunasin, with an arginine-glycine-aspartic acid motif, causes apoptosis to L1210 leukemia cells by activation of caspase-3. *Molecular Nutrition & Food Research, 54*, 406-414.

De Noni, I., y Cattaneo, S. (2010). Occurrence of β-casomorphins 5 and 7 in commercial dairy products and their digests following *in vitro* simulated gastro-intestinal digestion. *Food Chemistry, 119*, 560-566.

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., Vanacore, D., Chianese, L., Addeo, F., y Ferranti, P. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258.

Dia, V. P., Bringe, N. A., y de Mejia, E. G. (2014). Peptides in pepsin-pancreatin hydrolysates from commercially available soy products that inhibit lipopolysaccharide-induced inflammation in macrophages. *Food Chemistry, 152*, 423-431.

Dia, V. P., y de Mejia, E. G. (2010). Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. *Cancer Letters, 295*, 44-53.

Dia, V. P., y de Mejia, E. G. (2011a). Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. *Molecular Nutrition & Food Research, 55*, 623-634.

Dia, V. P., y de Mejia, E. G. (2011b). Lunasin potentiates the effect of oxaliplatin preventing outgrowth of colon cancer metastasis, binds to $\alpha_5\beta_1$ integrin and suppresses FAK/ERK/NF-κB signaling. *Cancer Letters, 313*, 167-180.

Dia, V. P., y de Mejia, E. G. (2013). Potential of lunasin-orally administered in comparison to intraperitoneal injection to inhibit colon cancer metastasis *in vivo*. *Journal of Cancer Therapy, 4*, 34-43.

Dia, V. P., Torres, S., de Lumen, B. O., Erdman, J. W., y de Mejia, E. G. (2009). Presence of lunasin in plasma of men after soy protein consumption. *Journal of Agricultural and Food Chemistry, 57*, 1260-1266.

Ding, L., Wang, L. Y., Zhang, Y., y Liu, J. B. (2015). Transport of antihypertensive peptide RVPSL, ovotransferrin 328-332, in human intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry, 63*, 8143-8150.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., Wickham, M. S. J., Mills, E. N. C., y Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant *in vitro* digestion models. *Molecular Nutrition & Food Research, 54*, 767-780.

Dziuba, J., Minkiewicz, P., Darewicz, M., y Dziuba, B. (2010). Milk proteins. In: *Handbook of Dairy Food Analysis.* L. M. L. Nollet, F. Toldrá, Eds. CRC Press, Boca Raton, FL, USA, pp. 79-109.

Ebaid, H., Salem, A., Sayed, A., y Metwalli, A. (2011). Whey protein enhances normal inflammatory responses during cutaneous wound healing in diabetic rats. *Lipids in Health and Disease, 10*, 235.

EFSA Panel on Dietetic Products, Nutrition and Allergies (2010a). Scientific Opinion on the substantiation of health claims related to protein and increase in satiety leading to a reduction in energy intake (ID 414, 616, 730), contribution to the maintenance or achievement of a normal body weight (ID 414, 616, 730), maintenance of normal bone (ID 416) and growth or maintenance of muscle mass (ID 415, 417, 593, 594, 595, 715) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal, 8*, 1811.

EFSA Panel on Dietetic Products, Nutrition and Allergies (2010b). Scientific Opinion on the substantiation of health claims related to wheat bran fibre and increase in faecal bulk (ID 3006), reduction in intestinal transit time (ID 828, 839, 3067, 4699) and contribution to the maintenance or achievement of a normal body weight (ID 829) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal, 8*, 1817.

EFSA Panel on Dietetic Products, Nutrition and Allergies (2010c). Scientific Opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal, 8*, 1763.

EFSA Scientific Report prepared by a DATEX Working Group (2009). Review of the potential health impact of β-casomorphins and related peptides. *EFSA Scientific Report, 231*, 1-107.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunçao, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M. J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., Martins, C., Meyneir, A., Miralles, B., Murray, B. S., Pihlanto, A., Picariello, G., Santos, C. N., Simsek, S., Recio, I., Rigby, N., Rioux, L. –E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E. K., Vegarud, G. E., Vergères, G., y Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International, 88*, 217-225.

Eliassen, L. T., Berge, G., Leknessund, A., Wikman, M., Lindin, I., Lokke, C., Ponthan, F., Johnsen, J. I., Sveinbjornsson, B., Kogner, P., Flaegstad, T., y Rekdal, O. (2006). The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells *in vitro* and inhibits xenograft growth *in vivo. International Journal of Cancer, 119*, 493-500.

Eliassen, L. T., Berge, G., Sveinbjornsson, B., Svendsen, J. S., Vorland, L. H., y Rekdal, O. (2002). Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Research, 22*, 2703-2710.

Fang, B., Zhang, M., Tian, M., Jiang, L., Guo, H. Y., y Ren, F. Z. (2014). Bovine lactoferrin binds oleic acid to form an anti-tumor complex similar to HAMLET. *Biochimica et Biophysica Acta, 1841*, 535-543.

Fang, E. F., Wong, J. H., y Ng, T. B. (2010). Thermostable Kunitz trypsin inhibitor with cytokine inducing, antitumor and HIV-1 reverse transcriptase inhibitory activities from Korean large black soybeans. *Journal of Bioscience and Bioengineering, 109*, 211-217.

Farrell, H. M. Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., y Swaisgood, H. E. (2004). Nomenclature of the proteins of cows´ milk – Sixth revision. *Journal of Dairy Science, 87*, 1641-1674.

FDA Talk Paper (1999). FDA approves new health claim for soy protein and coronary heart disease. FDA, United States Department of Health and Human Services. Washington, DC: United States Government Printing Office.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D., y Bray, F. (2015). Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer, 136*, E359-E386.

Filizola, M., y Devi, L. A. (2012). Structural biology: How opioid drugs bind to receptor. *Nature, 485*, 314-317.

Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T. M., y Kloek, J. (2007). Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *Journal of Nutrition, 137*, 953-958.

Foltz, M., van der Pijl, P. C., y Duchateau, G. S. (2010). Current *in vitro* testing of bioactive peptides is not valuable. *Journal of Nutrition, 140*, 117-118.

Fosset, S., Fromentin, G., Gietzen, D. W., Dubarry, M., Huneau, J. F., Antoine, J. M., Lang, V., Mathieu-Casseron, F., y Tomé, D. (2002). Peptide fragments released from Phe-caseinomacropeptide *in vivo* in the rat. *Peptides, 23*, 1773-1781.

Franco, R., y Cidlowski, J. A. (2012). Glutathione efflux and cell death. *Antioxidants & Redox Signaling, 17*, 1694-1713.

Freiburghaus, C., Janicke, B., Lindmark-Mànsson, H., Oredsson, S. M., y Paulsson, M. A. (2009). Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. *Journal of Dairy Science, 92*, 2477-2484.

Froetschel, M. (1996). Bioactive peptides in digesta that regulate gastrointestinal function and intake. *Journal of Animal Science, 74*, 2500-2508.

Fukudome, S., y Yoshikawa, M. (1992). Opioid peptides derived from wheat gluten: their isolation and characterization. *FEBS Letters, 296*, 107-111.

Fukudome, S., y Yoshikawa, M. (1993). Gluten exorphin C: a novel opioid peptide derived from wheat gluten. *FEBS Letters, 316*, 17-19.

Gad, A. S., Khadrawy, Y. A., El-Nekeety, A. A., Mohamed, S. R., Hassan, N. S., y Abdel-Wahhab, M. A. (2011). Antioxidant activity and hepatoprotective effects of whey protein and *Spirulina* in rats. *Nutrition, 27*, 582-589.

Galvez, A. F., Chen, N., Macasieb, J., y de Lumen, B. O. (2001). Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. *Cancer Research, 61*, 7473-7478.

Galvez, A. F., Huang, L., Magbanua, M. M. J., Dawson, K., y Rodriguez, R. L. (2011). Differential expression of thrombospondin (THBS1) in tumorigenic and nontumorigenic prostate epitelial cells in response to a chromatin-binding soy peptide. *Nutrition and Cancer, 63*, 623-636.

García-Nebot, M. J., Alegría, A., Barberá, R., Contreras, M. M., y Recio, I. (2010). Milk *versus* caseinophosphopeptides added to fruit beverages: Resistance and release from simulated gastrointestinal digestion. *Peptides, 31*, 555-561.

García-Nebot, M. J., Cilla, A., Alegría, A., y Barberá, R. (2011). Caseinophosphopeptides exert partial and site-specific cytoprotection against H_2O_2-induced oxidative stress in Caco-2 cells. *Food Chemistry, 129*, 1495-1503.

García-Nebot, M. J., Recio, I., y Hernández-Ledesma, B. (2014). Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. *Food and Chemical Toxicology, 65*, 155-161.

Garg, S., Nurgali, K., y Mishra, V. K. (2016). Food proteins as source of opioid peptides-A review. *Current Medicinal Chemistry, 23*, 893-910.

Gill, H. S., Doull, F., Rutherfurd, K. J., y Cross, M. L. (2000). Immunoregulatory peptides in bovine milk. *British Journal of Nutrition, 84*, S111-S117.

Gomes, L. S., Senna, R., Sandim, V., Silva-Neto, M. A. C., Perales, J. E. A., Zingali, R. B., Soares, M. R., y Fialho, E. (2014). Four conventional soybean [*Glycine max* (L.) Merrill] seed exhibit different protein profiles as revealed by proteomic analysis. *Journal of Agricultural and Food Chemistry, 62*, 1283-1293.

Goya, L., Martín, M. Á., Ramos, S., Mateos, R., y Bravo, L. (2009). A cell culture model for the assessment of the chemopreventive potential of dietary compounds. *Current Nutrition & Food Science, 5*, 56-64.

Goya, L., Martín, M. Á., Sarriá, B., Ramos, S., Mateos, R., y Bravo, L. (2016). Effect of cocoa and its flavonoids on biomarkers of inflammation: Studies of cell culture, animals and humans. *Nutrients, 8*, 212.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., y Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology, 30*, 591-600.

Habib, H. M., Ibrahim, W. H., Schneider-Stock, R., y Hassan, H. M. (2013). Camel milk lactoferrin reduces the proliferation of colorectal cancer cells and exerts antioxidant and DNA damage inhibitory activities. *Food Chemistry, 141*, 148-152.

Hajela, N., Pande, A. H., Sharma, S., Rao, D. N., y Hajela, K. (1999). Studies on a doubleheaded protease inhibitor from *Phaseolus mungo*. *Journal of Plant Biochemistry and Biotechnology, 8*, 57-60.

Half, E., y Arber, N. (2013). Chemoprevention of gastrointestinal neoplasia. *Current Gastroenterology Reports, 15*, 320.

Hanahan, D., y Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*, 646-674.

Hausch, F., Shan, L., Santiago, N. A., Gray, G. M., y Khosla, C. (2002). Intestinal digestive resistance of immunodominant gliadin peptides. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 283*, 996-1003.

Hazum, E., Sabatka, J. J., Chang, K. –J., Brent, D. A., Findlay, J., y Cuatrecasas, P. (1981). Morphine in cow and human milk: Could dietary morphine constitute a ligand for specific morphine (μ) receptors? *Science, 213,* 1010-1012.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., y Recio, I. (2014). Dairy protein hydrolysates: peptides for health benefits. *International Dairy Journal, 38*, 82-100.

Hernández-Ledesma, B., y Hsieh, C. –C. (2015). Chemopreventive role of food-derived proteins and peptides: a review. *Critical Reviews in Food Science and Nutrition,* en prensa. Doi 10.1080/10408398.2015.1057632.

Hernández-Ledesma, B., Hsieh, C. –C., y de Lumen, B. O. (2009a). Lunasin and Bowman-Birk protease inhibitor (BBI) in US comercial soy foods. *Food Chemistry, 115*, 574-580.

Hernández-Ledesma, B., Hsieh, C. –C., y de Lumen, B. O. (2009b). Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications, 390*, 803-808.

Hernández-Ledesma, B., Hsieh, C. –C., y de Lumen, B. O. (2009c). Lunasin, a novel seed peptide for cancer prevention. *Peptides, 30*, 426-430.

Hernández-Ledesma, B., Hsieh, C. –C., y de Lumen, B. O. (2011). Relationship between lunasin´s sequence and its inhibitory activity of histones H3 and H4 acetylation. *Molecular Nutrition & Food Research, 55*, 989-998.

Hernández-Ledesma, B., Quirós, A., Amigo, L., y Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal, 17*, 42-49.

Hill, J. E., y Breidenbach, R. W. (1974). Proteins of soybean seeds. *Plant Physiology, 53*, 742-746.

Ho, V. S. M., y Ng, T. B. (2008). A Bowman-Birk trypsin inhibitor with antiproliferative activity from Hokkaido large black soybeans. *Journal of Peptide Science, 14*, 278-282.

Hoskin, D. W., y Ramamoorthy, A. (2008). Studies on anticancer activities of antimicrobial peptides. *Biochimica et Biophysica Acta, 1778*, 357-375.

Howell, S., Kenny, A. J., y Turner, A. J. (1992). A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. *Biochemical Journal, 284*, 595-601.

Hsieh, C. –C., Hernández-Ledesma, B., Jeong, H. J., Park, J. H., y de Lumen, B. O. (2010). Complementary roles in cancer prevention: Protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS ONE, 5*, e8890.

Huang, D., Ou, B., y Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841-1856.

Hubatsch, I., Ragnarsson, E. G. E., y Artursson, P. (2007). Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols, 2*, 2111-2119.

Hughes, J., Smith, T. –W., Kosterlitz, H. –W., Fothergill, L. A., Morgan, B. –A., y Morris, H. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature, 258*, 577-580.

Hur, S. J., Lim, B. O., Decker, E. A., y McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry, 125*, 1-12.

Hwang, J. –T., Ahn, C. W., Kim, H. J., Lee, K. A., Park, O. J., y Kwon, D. Y. (2011). Black soybean peptide mixture purified from *Rhynchosia volubilis* exerts antioxidant activity against H_2O_2- induced cytotoxicity and improves thrombosis. *Journal of Medicinal Plants Research, 5*, 6477-6483.

Iigo, M., Shimamura, M., Matsuda, E., Fujita, K., Nomoto, H., Satoh, J., Kojima, S., Alexander, D. B., Moore, M. A., y Tsuda, H. (2004). Orally administered bovine lactoferrin induces caspase-1 and interleukin-18 in the mouse intestinal mucosa: a posible explanation for inhibition of carcinogenesis and metastasis. *Cytokine, 25*, 36-44.

Iwan, M., Jarmolowska, B., Bielikowicz, K., Kostyra, E., Kostyra, H., y Kaczmarski, M. (2008). Transport of μ-opioid receptor agonists and antagonist peptides across Caco-2 monolayer. *Peptides, 29*, 1042-1047.

Janecka, A., Fichna, J., y Janecki, T. (2004). Opioid receptors and their ligands. *Current Topics in Medicinal Chemistry, 4*, 1-17.

Jarmalowska, B., Teodorowicz, M., Fiedorowicz, E., Sienkiewicz-Szlapka, E., Matysiewicz, M., y Kostyra, E. (2013). Glucose and calcium ions may modulate the efficiency of bovine β-casomorphin-7 permeability through a monolayer of Caco-2 cells. *Peptides, 49*, 59-67.

Jeong, H. J., Jeong, J. B., Kim, D. S., Park, J. H., Lee, J. B., Kweon, D. –H., Chung, G. Y., Seo, E. W., y de Lumen, B. O. (2007a). The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. *Cancer Letters, 255*, 42-48.

Jeong, J. B., Jeong, H. J., Park, J. H., Lee, S. H., Lee, J. R., Lee, K. L., Chung, G. Y., Choi, J. D., y de Lumen, B. O. (2007b). Cancer-preventive peptide lunasin from *Solanum nigrum* L. inhibits acetylation of core histone H3 and H4 and phosphorylation of retinoblastoma protein (Rb). *Journal of Agricultural and Food Chemistry, 55*, 10707-10713.

Jeong, H. J., Jeong, J. B., Kim, D. S., y de Lumen, B. O. (2007c). Inhibition of core hystone acetylation by the cancer preventive peptide lunasin. *Journal of Agricultural and Food Chemistry, 55*, 632-637.

Jeong, H. J., Jeong, J. B., Hsieh, C. –C., Hernández-Ledesma, B., y de Lumen, B. O. (2010). Lunasin is prevalent in barley and is bioavailable and bioactive in *in vivo* and *in vitro* studies. *Nutrition and Cancer, 62*, 1113-1119.

Jeong, H. J., Lee, J. R., Jeong, J. B., Park, J. H., Cheong, Y. K., y de Lumen, B. O. (2009). The cancer preventive seed peptide lunasin from rye is bioavailable and bioactive. *Nutrition and Cancer, 61*, 680-686.

Jiménez-Saiz, R., Benedé, S., Molina, E., y López-Expósito I. (2015). Effect of processing technologies on the allergenicity of food products. *Critical Reviews in Food Science and Nutrition, 55*, 1902-1917.

Jin, M. –M., Zhang, L., Yu, H. –X., Meng, J., Sun, Z., y Lu, R. –R. (2013). Protective effect of whey protein hydrolysates on H_2O_2-induced PC12 cells oxidative stress *via* a mitochondria-mediated pathway. *Food Chemistry, 141*, 847-852.

Jin, Y., Yu, Y., Qi, Y., Wang, F., Yan, J., y Zou, H. (2016). Peptide profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. *Journal of Proteomics, 141*, 24-46.

Jinsmaa, Y., y Yoshikawa, M. (1999). Enzymatic release of neocasomorphin and β-casomorphin from bovine β-casein. *Peptides, 20*, 957-962.

Kampa, M., Loukas, S., Hatzoglou, A., Martin, P., Martin, P. W., y Castanas, E. (1996). Identification of a novel opioid peptide (Tyr-Val-Pro-Phe-Pro) derived from human α_{s1} casein (α_{s1}-casomorphin, and α_{s1}-casomorphin amide). *Biochemical Journal, 319*, 903-908.

Kasai, T., Iwasaki, R., Tanaka, M., y Kiriyama, S. (1995). Caseinphosphopeptides (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. *Bioscience, Biotechnology, and Biochemistry, 59*, 26-30.

Kennedy, A. R., Billings, P. C., Wan, X. S., y Newberne, P. M. (2002). Effects of Bowman-Birk inhibitor on rat colon carcinogenesis. *Nutrition and Cancer, 43*, 174-186.

Khoogar, R., Kim, B. C., Morris, J., y Wargovich, M. J. (2016). Chemoprevention in gastrointestinal physiology and disease. Targeting the progression of cancer with natural products: a focus on gastrointestinal cancer. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 310*, G629-G644.

Kim, Y. S., Farrar, W., Colburn, N. H., y Milner, J. A. (2012). Cancer stem cells: potential target for bioactive food components. *Journal of Nutritional Biochemistry, 23*, 691-698.

Koch, G., Wiedemann, K., y Teschemacher, H. (1985). Opioid activities of human β-casomorphins. *Naunyn-Schmiedeberg´s Archives of Pharmacology, 331*, 351-354.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., y Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *Journal of Nutrition, 142*, 245-250.

Kovacs-Nolan, J., Zhang, H., Ibuki, M., Nakamori, T., Yoshiura, K., Turner, P. V., Matsui, T., y Mine, Y. (2012). The PepT1-transportable soy tripeptide VPY reduces intestinal inflammation. *Biochimica et Biophysica Acta, 1820*, 1753-1763.

Kreider, R. B., Iosia, M., Cooke, M., Hudson, G., Rasmussenl, C., Chen, H., Mollstedt, O., y Tsai, M. H. (2011). Bioactive properties and clinical safety of a novel milk protein peptide. *Nutrition Journal, 10*, 99.

Kuhara, T., Iigo, M., Itoh, T., Ushida, Y., Sekine, N., Terada, N., Okamura, H., y Tsuda, H. (2000). Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. *Nutrition and Cancer, 38*, 192-199.

Ledoux, N., Mahé, S., Dubarry, M., Bourras, M., Benamouzig, R., y Tomé, D. (1999). Intraluminal immunoreactive caseinomacropeptide after milk protein ingestion in humans. *Nahrung/Food, 43*, 196-200.

Lei, L., Sun, H., Liu, D., Liu, L., y Li, S. (2008). Transport of Val-Leu-Pro-Val-Pro in human intestinal epithelial (Caco-2) cell monolayers. *Journal of Agricultural and Food Chemistry, 56*, 3582-3586.

Lewandowska, H., Kalinowska, M., Lewandowski, W., Stepkowski, T. M., y Brzoska, K. (2016). The role of natural polyphenols in cell signaling and cytoprotection against cancer development. *Journal of Nutritional Biochemistry, 32*, 1-19.

Li, J., Ye, L., Cook, D. R., Wang, X., Liu, J., Kolson, D. L., Persidsky, Y., y Ho, W. –Z. (2011). Soybean-derived Bowman-Birk inhibitor inhibits neurotoxicity of LPS-activated macrophages. *Journal of Neuroinflammation, 8*, 15.

Lichtenstein, G. R., Deren, J. J., Katz, S., Lewis, J. D., Kennedy, A. R., y Ware, J. H. (2008). Bowman-Birk inhibitor concentrate: a novel therapeutic agent for patients with active ulcerative colitis. *Digestive Diseases and Sciences, 53*, 175-180.

Lin, P., Ye, X., y Ng, T. (2008). Purification of melibiose-binding lectins from two cultivars of Chinese black soybeans. *Acta Biochimica et Biophysica Sinica, 40*, 1029-1038.

Lo, W. M. Y., y Li-Chan, E. C. Y. (2005). Angiotensin I converting enzyme inhibitory peptides from *in vitro* pepsin-pancreatin digestion of soy protein. *Journal of Agricultural and Food Chemistry, 53*, 3369-3376.

Lollo, P. C. B., Amaya-Farfan, J., Faria, I. C., Salgado, J. V. V., Chacon-Mikahil, M. P. T., Cruz, A. G., Oliveira, C. A. F., Montagner, P. C., y Arruda, M. (2014). Hydrolysed whey protein reduces muscle damage markers in Brazilian elite soccer players compared with whey protein and maltodextrin. A twelve-week in-championship intervention. *International Dairy Journal, 34*, 19-24.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., y Klee, W. A. (1983). Opioid activities and structures of α-casein-derived exorphins. *Biochemistry, 22*, 4567-4573.

Macha, M. A., Krishn, S. R., Jahan, R., Banerjee, K., Batra, S. K., y Jain, M. (2015). Emerging potential of natural products for targeting mucins for therapy against inflammation and cancer. *Cancer Treatment Reviews, 41,* 277-288.

Mader, J. S., Salsman, J., Conrad, D. M., y Hoskin, D. W. (2005). Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. *Molecular Cancer Therapeutics, 4*, 612-624.

Mader, J. S., Smyth, D., Marshall, J., y Hoskin, D. W. (2006). Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor$_{165}$-induced angiogenesis by competing for heparin-like binding sites on endothelial cells. *The American Journal of Pathology, 169*, 1753-1766.

Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E., y Malcata, F. X. (2007). Bovine whey proteins – Overview on their main biological properties. *Food Research International, 40*, 1197-1211.

Mahé, S., Messing, B., Thuillier, F., y Tomé, D. (1991). Digestion of bovine milk proteins in patients with a high jejunostomy. *American Journal of Clinical Nutrition, 54*, 534-538.

Mahé, S., Roos, N., Benamouzig, R., Davin, L., Luengo, C., Gagnon, L., Gausserges, N., Rautureau, J., y Tomé, D. (1996). Gastrojejunal kinetics and the digestion of [N-15]beta-lactoglobulin and casein in humans: the influence of the nature and quantity of the protein. *American Journal of Clinical Nutrition, 63*, 546-552.

Mari, M., Morales, A., Colell, A., García-Ruiz, C., y Fernández-Checa, J. C. (2009). Mitochondrial glutathione, a key survival antioxidant. *Antioxidants & Redox Signaling, 11*, 2685-2700.

Martínez-Augustin, O., Rivero-Gutiérrez, B., Mascaraque, C., y Sánchez de Medina, F. (2014). Food derived bioactive peptides and intestinal barrier function. *International Journal of Molecular Sciences, 15*, 22857-22873.

Martínez-Maqueda, D., Miralles, B., de Pascual-Teresa, S., Reverón, I., Muñoz, R., y Recio, I. (2012). Food-derived peptides stimulate mucin secretion and gene expression in intestinal cells. *Journal of Agricultural and Food Chemistry, 60*, 8600-8605.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., y Recio, I. (2013a). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *International Dairy Journal, 32*, 13-19.

Martínez-Maqueda, D., Miralles, B., Ramos, M., y Recio, I. (2013b). Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells. *Food Research International, 54,* 1287-1291.

McConnell, E. J., Devapatla, B., Yaddanapudi, K., y Davis, K. R. (2015). The soybean-derived peptide lunasin inhibits non-small cell lung cancer cell proliferation by suppressing phosphorylation of the retinoblastoma protein. *Oncotarget, 6*, 4649-4662.

Mcknight, A. T., Corbett, A. D., y Kosterlitz, H. W. (1983). Increase in potencies of opioid peptides after peptidase inhibition. *European Journal of Pharmacology*, *86*, 393-402.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers*, *43*, 119-128.

Meisel, H., y Frister, H. (1989). Chemical characterization of bioactive peptides from *in vivo* digests of casein. *Journal of Dairy Research, 56*, 343-349.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., y Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039-1045.

Miguel, M., Recio, I., Ramos, M., Delgado, M. A., y Aleixandre, M. A. (2006). Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats. *Journal of Dairy Science, 89,* 3352–3359.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., y Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food & Function, 5*, 1113-1124.

Miner-Williams, W. M., Stevens, B. R., y Moughan, P. J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutrition Research Reviews, 27*, 308-329.

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., y Malik, B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & Redox Signaling, 20,* 1126-1167.

Moughan, P. J., Fuller, M. F., Han, K. –S., Kies, A. K., y Miner-Williams, W. (2007). Food-derived bioactive peptides influence gut function. *International Journal of Sport Nutrition and Exercise Metabolism, 17*, S5-S22.

Moura, F. A., Queiroz de Andrade, K., Farias dos Santos, J. C., Pimentel Araújo, O. R., y Fonseca Goulart, J. C. (2015). Antioxidant therapy for treatment of inflammatory bowel disease: Does it work? *Redox Biology, 6,* 617-639.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., y Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*, 777-783.

Nongonierma, A. B., y FitzGerald, R. J. (2015). The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A review. *Journal of Functional Foods, 17*, 640-656.

Nongonierma, A. B., y FitzGerald, R. J. (2016). Strategies for the discovery, identification and validation of milk protein-derived bioactive peptides. *Trends in Food Science & Technology, 50*, 26-43.

Oh, J., Hlatky, L., Jeong, Y. S., y Kim, D. (2016). Therapeutic effectiveness of anticancer phytochemicals on cancer stem cells. *Toxins, 8*, 199.

Ohinata, K., Agui, S., y Yoshikawa, M. (2007). Soymorphins, novel μ opioid peptides derived from soy-β-conglycinin β-subunit, have anxiolytic activities. *Bioscience, Biotechnology & Biochemistry, 71*, 2618-2621.

Ohsawa, K., Satsu, H., Ohki, K., Enjoh, M., Takano, T., y Shimizu, M. (2008). Producibility and digestibility of antihypertensive β-casein tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the gastrointestinal tract: Analyses using an *in vitro* model of mammalian gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 56*, 854-858.

O´Keeffe, M. B., y FitzGerald, R. J. (2014). Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells. *International Dairy Journal, 36*, 128-135.

Pang, G., Xie, J., Chen, Q., y Hu, Z. (2012). How functional foods play critical roles in human health. *Food Science and Human Wellness, 1*, 26-60.

Pantako, O. T., Lemieux, L., y Amiot, J. (2001). The effects of α-lactalbumin and whey protein concentrate on dry matter recovery, TCA soluble protein levels, and peptide distribution in the rat gastrointestinal tract. *Canadian Journal of Physiology and Pharmacology, 79*, 320-328.

Park, J. H., Jeong, H. J., y de Lumen, B. O. (2007). *In vitro* digestibility of the cancer-preventive soy peptides lunasin and BBI. *Journal of Agricultural and Food Chemistry, 55*, 10703-10706.

Payan, D. G., Horvath, K., y Graf, L. (1987). Specific high-affinity binding-sites for a synthetic gliadin heptapeptide on human peripheral-blood lymphocytes. *Life Sciences, 40*, 1229-1236.

Peng, X., Kong, B., Yu, H., y Diao, X. (2014). Protective effect of whey protein hydrolysates against oxidative stress in D-galactose-induced ageing rats. *International Dairy Journal, 34*, 80-85.

Pepe, G., Tenore, G. C., Mastrocinque, R., Stusio, P., y Campiglia, P. (2013). Potential anticarcinogenic peptides from bovine milk. *Journal of Amino Acids, 2013*, 939804.

Perego, S., Cosentino, S., Fiorilli, A., Tettamanti, G., y Ferraretto, A. (2012). Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with voltage-operated L-type calcium channels. *Journal of Nutritional Biochemistry, 23*, 808-816.

Petrilli, P., Picone, D., Caporale, C., Addeo, F., Auricchio, S., y Marino, G. (1984). Does casomorphin have a functional-role? *FEBS Letters, 169*, 53-56.

Pihlanto, A., Rokka, T., y Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Journal of Dairy Science, 81*, 325-331.

Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S., y Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *Journal of Chromatography B, 878*, 295-308.

Picariello, G., Iacomino, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., Di Luccia, A., y Addeo, F. (2013a). Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins. *Food Chemistry, 139*, 203-212.

Picariello, G., Mamone, G., Nitride, C., Addeo, F., y Ferranti, P. (2013b). Protein digestomics: Integrated platforms to study food-protein digestion and derived functional and active peptides. *Trends in Analytical Chemistry, 52*, 120-134.

Picariello, G., Miralles, B., Mamone, G., Sánchez-Rivera, L., Recio, I., Addeo, F., y Ferranti, P. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition & Food Research, 59*, 948-956.

Plaisancié, P., Boutrou, R., Estienne, M., Henry, G., Jardin, J., Paquet, A., y Léonil, J. (2015). β-casein(94-123)-derived peptides differently modulate production of mucin in intestinal goblet cells. *Journal of Dairy Research, 82*, 36-46.

Plaisancié, P., Claustre, J., Estienne, M., Henry, G., Boutrou, R., Paquet, A., y Léonil, J. (2013). A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. *Journal of Nutritional Biochemistry, 24*, 213-221.

Power, O., Jakeman, P., y FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids, 44*, 797-820.

Pratheeshkumar, P., Sreekala, C., Zhang, Z., Budhraja, A., Ding, S., Son, Y. O., Wang, X., Hitron, A., Hyun-Jung, K., Wang, L., Lee, J. C., y Shi, X. (2012). Cancer prevention with promising natural products: Mechanisms of action and molecular targets. *Anticancer Agents in Medicinal Chemistry, 12*, 1159-1184.

Quirós, A., Contreras, M. M., Ramos, M., Amigo, L., y Recio, I. (2009). Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. *Peptides, 30,* 1848-1853.

Quirós, A., Dávalos, A., Lasunción, M. A., Ramos, M., y Recio, I. (2008). Bioavailability of the antihypertensive peptide LHLPLP: Transepithelial flux of HLPLP. *International Dairy Journal, 18*, 279-286.

Rains, J. L., y Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radical Biology & Medicine, 50*, 567-575.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signaling pathways. *Molecular Nutrition & Food Research, 52*, 507-526.

Ray, P. D., Huang, B. W., y Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signaling, 24,* 981-990.

Rayaprolu, S. J., Hettiarachchy, N. S., Chen, P., Kannan, A., y Mauromostakos, A. (2013). Peptides derived from high oleic acid soybean meals inhibit colon, liver and lung cancer cell growth. *Food Research International, 50*, 282-288.

Razzeto, G. S., Lucero López, V. R., Giménez, M. S., y Escudero, N. L. (2015). Soybean flour induces a greater increase of the antioxidant defenses in rats fed with a normocaloric diet compated with a hypercaloric diet. *Journal of the Science of Food and Agriculture, 95*, 607-613.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., y Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer. How are they linked? *Free Radical Biology & Medicine, 49,* 1603-1616.

Rutgeerts, P., Vermeire, S., y Van Assche, G. (2009). Biological therapies for inflammatory bowel diseases. *Gastroenterology, 136*, 1182-1197.

Rutherfurd-Marwick, K. J. (2012). Food proteins as a source of bioactive peptides with diverse functions. *British Journal of Nutrition, 108*, S149-S157.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., y Donkor, O. N. (2015). Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. *Comprehensive Reviews in Food Science and Food Safety, 14*, 123-138.

Sakai, T., Banno, Y., Kato, Y., Nozawa, Y., y Kawaguchi, M. (2005). Pepsin-digested bovine lactoferrin induces apoptotic cell death with JNK/SAPK activation in oral cancer cells. *Journal of Pharmacological Sciences, 98*, 41-48.

Salamat-Miller, N., y Johnston, T. P. (2005). Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium. *International Journal of Pharmaceutics, 294*, 201-216.

Sambruy, Y., Ferruzza, S., Ranaldi, G., y De Angelis, I. (2001). Intestinal cell culture models: Applications in toxicology and pharmacology. *Cell Biology and Toxicology, 17*, 301-317.

Sánchez-Rivera, L., Martínez-Maqueda, D., Cruz-Huerta, E., Miralles, B., y Recio, I. (2014a). Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food Research International, 63*, 170-181.

Sánchez-Rivera, L., Ares, I., Miralles, B., Gómez-Ruiz, J. Á., Recio, I., Martínez-Larrañaga, M. R., Anadón, A., y Martínez, M. A. (2014b). Bioavailability and kinetics of the antihypertensive casein-derived peptide HLPLP in rats. *Journal of Agricultural and Food Chemistry, 62*, 11869-11875.

Sánchez-Rivera, L., Diezhandino, I., Gómez-Ruiz, J. Á., Fresno, J. M., Miralles, B., y Recio, I. (2014c). Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. *Electrophoresis, 35*, 1627-1636.

Sarmadi, B. H., e Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31*, 1949-1956.

Scanff, P., Yvon, M., Thirouin, S., y Pelissffir, J. –P. (1992). Characterization and kinetics of gastric emptying of peptides derived from milk proteins in the preruminant calf. *Journal of Dairy Research, 59*, 437-447.

Schusdziarra, V., Schick, R., Holland, A., de la Fuente, A., Specht, J., Maier, V., Brantl, V., y Pfeiffer, E. (1983a). Effect of opiate-active substances on pancreatic polypeptides levels in dogs. *Peptides, 4*, 205-210.

Schusdziarra, V., Schick, R., de la Fuente, A., Specht, J., Klier, M., Brantl, V., y Pfeiffer, E. (1983b). Effect of β-casomorphins and analogs on insulin release in dogs. *Endocrinology, 112*, 885-889.

Schusdziarra, V., Schick, R., de la Fuente, A., Holland, A., Brantl, V., y Pfeiffer, E. (1983c). Effect of β-casomorphins on somatostatin release in dogs. *Endocrinology, 112*, 1948-1951.

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., y Hernández-Escalante, V. M. (2011). Bioavailability of bioactive peptides. *Food Research International, 27*, 213-226.

Shay, J., Elbaz, H. A., Lee, I., Zielske, S. P., Malek, M. H., y Hüttemann, M. (2015). Molecular mechanisms and therapeutic effects of (−)-epicatechin and other polyphenols in cancer, inflammation, diabetes and neurodegeneration. *Oxidative Medicine and Cellular Longevity, 2015*, 181260.

Shidal, C., Al-Rayyan, N., Yaddanapudi, K., y Davis, K. R. (2016). Lunasin is a novel therapeutic agent for targeting melanoma cancer stem cells. *Oncotarget*, en prensa. Doi 10.18632/oncotarget.11554.

Shimizu, M. (2010). Interaction between food substances and the intestinal epithelium. *Bioscience, Biotechnology & Biochemistry, 74*, 232-241.

Shimizu, M. (2012). Functional food in Japan: Current status and future of gut-modulating food. *Journal of Food and Drug Analysis, 20*, 213-216.

Shimizu, M., y Hachimura, S. (2011). Gut as target for functional food. *Trends in Food Science and Technology, 22*, 646-650.

Singh, R., y Czaja, M. J. (2008). Regulation of hepatocyte apoptosis by oxidative stress. *Journal of Gastroenterology and Hepatology, 23*, 501-502.

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., y Lleonart, M. E. (2013). Oxidative stress and cancer: An overview. *Ageing Research Reviews, 12*, 376-390.

Sprong, R. C., Schonewille, A. J., y van der Meer, R. (2010). Dietary cheese whey protein protects rat against mild dextran sulfate sodium-induced colitis: Role of mucin and microbiota. *Journal of Dairy Science, 93*, 1364-1371.

Staljanssens, D., Van Camp, J., Billiet, A., De Meyer, T., Al Shukor, N., De Vos, W. H., y Smagghe, G. (2012). Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor. *Peptides, 34*, 226-231.

St Clair, W. H., Billings, P. C., Carew, J. A., Keller-McGandy, C., Newberne, P., y Kennedy, A. R. (1990). Suppression of dimethylhydrazine-induced carcinogenesis in mice by dietary addition of the Bowman-Birk protease inhibitor. *Cancer Research, 50*, 580-586.

Svedberg, J., de Haas, J., Leimenstoll, G., Paul, F., y Teschemacher, H. (1985). Demonstration of β-casomorphin immunoreactive materials in *in vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides, 6*, 825-830.

Swaisgood, H. E. (1992). Chemistry of the caseins. In: *Advanced Dairy Chemistry-1: Proteins,* P. F. Fox, Ed. Elsevier Science Publishers, London, UK, pp. 63-110.

Takahashi, M., Moriguchi, S., Yoshikawa, M., y Sasaki, R. (1994). Isolation and characterization of oryzatensin – a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. *Biochemistry and Molecular Biology International, 33*, 1151-1158.

Tani, F., Iio, K., Chiba, H., y Yoshikawa, M. (1990). Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agricultural and Biological Chemistry, 54*, 1803-1810.

Tani, F., Shiota, A., Chiba, H., y Yoshikawa, M. (1994). Serorphin, an opioid peptide from bovine serum albumin. In: *β-Casomorphins and Related Peptides: Recent Developments.* V. Brantl, H. Teschemacher, Eds. VCH, Weinheim, Germany, pp. 49-53.

Taylor, D. A. (2011). *In vitro* opioid receptor assays. *Current protocols in pharmacology, 55*, 1-34.

Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design, 9*, 1331-1344.

Teschemacher, H., Koch, G., y Brantl, V. (1997). Milk protein-derived opioid receptor ligands. *Biopolymers, 43*, 99-117.

Trivedi, M. V., Laurence, J. S., y Siahaan, T. J. (2009). The role of thiols and disulfides on protein stability. *Current Protein and Peptide Science, 10*, 614-625.

Trompette, A., Claustre, J., Caillon, F., Jourdan, G., Chayvialle, J. A., y Plaisancié, P. (2003). Milk bioactive peptides and β-casomorphins induce mucus release in rat jejunum. *Journal of Nutrition, 133*, 3499-3503.

Troost, F. J., Steijns, J., Saris, W. H. M., y Brummer, R. J. M. (2001). Gastric digestion of bovine lactoferrin *in vivo* in adults. *Journal of Nutrition, 131*, 2101-2104.

Umeno, A., Horie, M., Murotomi, K., Nakajima, Y., y Yoshida, Y. (2016). Antioxidative and antidiabetic effect of natural polyphenols and isoflavones. *Molecules, 21*, 708.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., y Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*, 1-40.

van der Pijl, P. C., Kies, A. K., Ten Have, G. A. M., Duchateau, G. S. M. J. E., y Deutz, N. E. P. (2008). Pharmacokinetics of proline-rich tripeptides in the pig. *Peptides, 29*, 2196-2202.

Van der Sluis, M., De Koning, B. A. E., De Bruijn, A. C. J. M., Velcich, A., Meijerink, J. P. P., Van Goudoever, J. B., Büller, H. A., Dekker, J., Van Seuningen, I., Renes, I. B., y Einerhand, A. W. C. (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology*, *131*, 117-129.

Vegarud, G. E., Langsrud, T., y Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *British Journal of Nutrition, 84*, S91-S98.

Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., Kucherlapati, R., Lipkin, M., Yang, K., y Augenlicht, L. (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science*, *295*, 1726-1729.

Vernaza, M. G., Dia, V. P., de Mejia, E. G., y Chang, Y. K. (2012). Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry, 134*, 2217-2225.

Wadley, A. J., Veldhuijzen van Zanten, J. J. C. S., y Aldred, S. (2013). The interactions of oxidative stress and inflammation with vascular dysfunction in ageing: the vascular health triad. *AGE, 35*, 705-718.

Waldhoer, M., Bartlett, S. E., y Whistler, J. L. (2004). Opioid receptors. *The Annual Review of Biochemistry, 73*, 953-990.

Wan, H., Liu, D., Yu, X., Sun, H., y Li, Y. (2015). A Caco-2 cell-based quantitative antioxidant activity assay for antioxidants. *Food Chemistry, 175*, 601-608.

Wang, Z., Li, S., Cao, Y., Tian, X., Zeng, R., Liao, D. –F., y Cao, D. (2016). Oxidative stress and carbonyl lesions in ulcerative colitis and associated colorectal cancer. *Oxidative Medicine and Cellular Longevity, 2016*, 9875298.

Ware, J. H., Wan, X. S., Newberne, P., y Kennedy, A. R. (1999). Bowman-Birk inhibitor concentrate reduces colon inflammation in mice with dextran sulfate sodium-induced ulcerative colitis. *Digestive Diseases & Sciences, 44*, 986-990.

White, C. L., Bray, G. A., y York, D. A. (2000). Intragastric β-casomorphin 1-7 attenuates the suppression of fat intake by enterostatin. *Peptides, 2*, 1377-1381.

Xie, N., Wang, C., Ao, J., y Li, B. (2013). Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its *in vitro* gastrointestinal digest. *Food Research International, 51*, 114-122.

Yang, H. –Y., Tzeng, Y. –H., Chai, C. –Y., Hsieh, A. –T., Chen, J. –R., Chang, L. –S., y Yang, S. –S. (2011). Soy protein retards the progression of non-alcoholic steatohepatitis via improvement of insulin resistance and steatosis. *Nutrition, 27*, 943-948.

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., y Yoshikawa, M. (2001). Rubiscolin, a δ selective opioid peptide derived from plant Rubisco. *FEBS Letters, 509*, 213-217.

Yang, T., Fang, S., Zhang, H. X., Xu, L. X., Zhang, Z. Q., Yuan, K. T., Xue, C. L., Yu, H. L., Zhang, S., Li, Y. F., Shi, H. P., y Zhang, Y. (2013). N-3 PUFAs have antiproliferative and apoptotic effects on human colorectal cancer stem-like cells *in vitro*. *Journal of Nutritional Biochemistry, 24*, 744-753.

Ye, X., y Ng, T. B. (2009). A trypsin-chymotrypsin inhibitor with antiproliferative activity from small glossy black soybeans. *Planta Medica, 75*, 550-556.

Yin, H., Miao, J., y Zhang, Y. (2010). Protective effect of β-casomorphin-7 on type I diabetes rats induced with streptozotocin. *Peptides, 31*, 1725-1729.

Yoshikawa, M., Tani, F., Shiota, H., Suganuma, H., Usui, H., Kurahashi, K., y Chiba, H. (1994). Casoxin D, an opioid antagonist ileum-contracting/vasorelaxing peptide derived from human α_{s1}-casein. In: *β-Casomorphins and Related Peptides: Recent Developments.* V. Brantl, H. Teschemacher, Eds. VCH, Weinheim, Germany, pp. 43-48.

Yoshikawa, M., Tani, F., Yoshimura, T., y Chiba, H. (1986). Opioid peptides from milk proteins. *Agricultural and Biological Chemistry, 50*, 2419-2421.

Yoshikawa, M. (2015). Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides, 72*, 208-225.

Young, D., Ibuki, M., Nakamori, T., Fan, M., y Mine, Y. (2012). Soy-derived di- and tripeptides alleviate colon and ileum inflammation in pigs with dextran sodium sulfate-induced colitis. *Journal of Nutrition, 142*, 363-368.

Yvon, M., y Pelissier, J. P. (1987). Characterization and kinetics of evacuation of peptides resulting from casein hydrolysis in the stomach of the calf. *Journal of Agricultural and Food Chemistry, 35*, 148-156.

Zarkadas, C. G., Gagnon, C., Gleddie, S., Khanizadeh, S., Cober, E. R., y Guillemette, R. J. D. (2007). Assessment of the protein quality of fourteen soybean [*Glycine max* (L.) Merr.] cultivars using amino acid analysis and two-dimensional electrophoresis. *Food Research International, 40*, 129-146.

Zhang, Q. –X., Jin, M. –M., Zhang, L., Yu, H. –X., Sun, Z., y Lu, R. –R. (2015). Hydrophobicity of whey protein hydrolysates enhances the protective effect against oxidative damage on PC-12 cells. *Journal of Dairy Research, 82*, 1-7.

Zhu, H., y Li, R. (2012). Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Experimental Biology and Medicine, 237,* 474-480.

Zoghbi, S., Trompette, A., Claustre, J., El Homsi, M., Garzón, J., Jourdan, G., Scoazec, J. –Y., y Plaisancié, P. (2006). β-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a μ-opioid pathway. *American Journal of Physiology – Gastrointestinal and Liver Physiology, 290*, G1105-G1113.

6. ANNEXES / ANEXOS

Dairy protein hydrolysates: Peptides for health benefits.

INTERNATIONAL DAIRY JOURNAL, 38 (2014), 82-100

Milk proteins, peptides, and oligosaccharides: Effects against the 21st century disorders.

BIOMED RESEARCH INTERNATIONAL, 2015 (2015), 146840

Italian legumes: Effect of sourdough fermentation on lunasin-like polypeptides.

MICROBIAL CELL FACTORIES, 14 (2015), 168

An update on lunasin research, a bioactive seed peptide for health promotion.

RECENT PROGRESS IN MEDICINAL PLANTS, PHYTOTHERAPEUTICS II (2016), J. N. Govil, Ed. Studium Press LLC, New Dehli, India, pp. 331-352

Functionality of soybean compounds in the oxidative stress-related disorders.

GASTROINTESTINAL TISSUE: OXIDATIVE STRESS AND DIETARY ANTIOXIDANTS (in press), J. Gracia-Sancho, M. J. Salvadó, Eds. Elsevier Inc, Amsterdam, The Netherlands

---

International Dairy Journal 38 (2014) 82–100

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Review

Dairy protein hydrolysates: Peptides for health benefits

Blanca Hernández-Ledesma, María José García-Nebot, Samuel Fernández-Tomé, Lourdes Amigo, Isidra Recio*

Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 30 September 2013
Received in revised form
19 November 2013
Accepted 19 November 2013
Available online 3 December 2013

ABSTRACT

During food digestion, proteins are hydrolysed into a large variety of peptides. Some of these peptides are structurally similar to sequences acting in the organism as endogenous signals, or hormones. Therefore, homologous food peptides can interact with the same receptors or enzymes in the organism, and in this form, exert an agonistic or antagonistic activity. The evidence of the potential of these dietary peptides to modulate numerous physiological conditions has been mainly achieved using *in vitro* assays; however, it is accepted that health evidence has to be based on *in vivo* trials (animals or humans) since the activity of these molecules depends on the ability of these peptides to reach the target tissue in an active form, which in turn depends on their structure. This article reviews the literature on the physiological effects of milk-derived bioactive peptides with special emphasis in the evidence achieved in animal and human trials.

Contents

* Corresponding author. Tel.: +34 910017940.
E-mail address: i.recio@csic.es (I. Recio).

0958-6946/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.idairyj.2013.11.004

1. Introduction

It is recognised that diet is one of the environmental factors that influences our health and the development of certain diseases. Among the different food components, protein constitutes a major nutrient with daily ingests between 95 and 120 g of protein, which is hydrolysed into a large variety of peptides during gastrointestinal digestion. Some of these peptides share structural characteristics with endogenous peptides that act in the organism as hormones, neurotransmitters, or regulatory peptides. These exogenous food-derived peptides can interact with the same receptors in the organism and exert an agonistic or antagonistic activity. There are many examples of food-derived peptides structurally similar to endogenous peptides; one of the most representative examples is opioid peptides, which have been demonstrated to behave as opioid receptors ligands (Teschemacher, 2003). This group of food-derived bioactive peptides was the first described in 1979 (Henschen, Lottspeich, Brantl, & Teschemacher, 1979) and since then, and especially during the last two decades, much effort has been dedicated to the identification of peptides with different physiological effects. However, the level of evidence built for these food peptides is diverse. For instance, for antihypertensive peptides, activity has been demonstrated in animal models and clinical trials, but for other peptides, bioactivity has been just proven in cell cultures or *in vitro* assays. In addition, there are many examples of lack of correlation between *in vitro* and *in vivo* results, mainly due to further degradation of peptides during gastrointestinal digestion, or the impossibility to reach the target organ in a sufficient amount to exert the physiological effect.

On the other hand, absorption of food-derived peptides is not a requirement to exert a biological function in the organism. The gut is considered to be the largest endocrine organ in the body with a large range of endogenous peptides secreted and receptors expressed. The gastrointestinal tract is in contact with food digests and therefore, with an important quantity (and variety) of food-derived peptides. For this reason, the effects of peptides on different intestinal functions and health are attracting an increasing interest (Moughan, Fuller, Han, Kies, & Miner-Williams, 2007; Shimizu & Hachimura, 2011). It cannot be disregarded that given the low bioavailability described for some bioactive peptides, certain observed physiological effects might be mediated through interaction with receptors located at the gut.

The aim of this paper is to review the literature on the physiological effects of milk-derived bioactive peptides with special emphasis in the evidence achieved in animal and human trials. A short section deals with the advances on hydrolysis technologies. Finally, a section with future trends, and challenges on dairy peptides research is included.

2. Technology: dairy protein hydrolysates

During the last two decades, there has been a growing interest in the use of dairy hydrolysates containing bioactive peptides as agents for maintaining general health and preventing chronic human diseases. As a result, several technologies, mainly based on the enzymatic hydrolysis, have been developed for the production of these bioactive hydrolysates (Hernández-Ledesma, Recio, Ramos, & Amigo, 2002; Korhonen & Pihlanto, 2006; McDonagh & Fitzgerald, 1998; Phelan, Aherne, Fitzgerald, & O'Brien, 2009). This strategy is the main choice, but some disadvantages in the method have been reported, such as the need for using chemical or thermal processes, to stop the proteolysis reaction that could affect the final attributes of the hydrolysed proteins (Kosseva, Panesar, Kaur, & Kennedy, 2009). Immobilisation of enzyme molecules over different supports could overcome this problem, allowing the separation of the enzyme from the protein mixture reaction and, furthermore making possible its reuse (Madadlou, Sheehan, Emam-Djomeh, & Mousavi, 2011; Marqués et al., 2011; Rocha, Gonçalves, & Texeira, 2011). In the food industry, the use of enzymatic membrane reactors represents an interesting technology that allows protein hydrolysis and the subsequent separation of peptides generated by microfiltration or chromatography techniques, such as size exclusion or ion-exchange chromatography (Welderufael, Gibson, & Jauregui, 2012). Some studies have applied these methodologies for the recovery of caseinophosphopeptides (CPPs), antimicrobial, and angiotensin-converting enzyme (ACE)-inhibitory peptides from bovine casein (CN) hydrolysates (Recio & Visser, 1999a; Wu et al., 2013; Zhao, Xu, Yang, & Katiyo, 2013).

Moreover, alternatively to traditional methods, subcritical water hydrolysis strategies have been proposed for dairy hydrolysates production without the need for acids, bases or enzymes. The study of Espinoza, Morawicki, and Hager (2012) demonstrated successfully subcritical water hydrolysis of a whey protein isolate (WPI) and evaluated how treatment conditions (temperature and reaction time) affected the degree of hydrolysis, and the production, composition and concentration of peptides and free amino acids released. In addition, high hydrostatic pressure causes substantial modifications to milk proteins, and ultimately influences their functional properties. The potential utilities of high pressure treatments in dairy technologies have been reviewed (Chawla, Patil, & Singh, 2011; Da Cruz et al., 2010; López-Fandiño, 2006). As an example, some studies employing high pressure conditions have yielded protein hydrolysates with enhanced antioxidant and anti-inflammatory effects in intestinal epithelial cells exposed to hydrogen peroxide (Piccolomini, Iskandar, Lands, & Kubow, 2012).

3. Health benefits

3.1. Effects on the cardiovascular system

Cardiovascular diseases have become a worldwide health problem that goes beyond socio-economic barriers and equally affects men and women. Diet plays a key role in the development of the most significant risk factors of these diseases, such as hypertension, obesity, diabetes, low-grade systemic inflammation, and atherosclerosis. In recent years, bioactive milk peptides have gained interest because of their notable antihypertensive, antioxidant, anti-inflammatory and hypocholesterolaemic effects. In this section, the most current scientific information from cell culture, animal experiments and clinical studies on the role of milk proteins-derived peptides on cardiovascular diseases is summarised and discussed.

3.1.1. Antihypertensive peptides

Elevated blood pressure is one of the major independent risk factors for cardiovascular disease (Erdmann, Cheung, & Schröder, 2008). ACE (EC 3.4.15.1) is one of the main regulators of blood pressure; thus, inhibition of this enzyme is considered as one of the strategies for the treatment of hypertension. In recent years, antihypertensive effects of some peptides derived from milk proteins have been evaluated by *in vitro* and *in vivo* studies, becoming the best known class of bioactive peptides. One of the greatest challenges in developing milk peptides as antihypertensive food ingredients has been proving their *in vivo* efficacy. It mainly depends on the capacity of peptides, after being orally ingested, to reach the target organs in an intact and active form. In the last years, studies demonstrating the bioavailability of potential antihypertensive peptides have been carried out. Most of these studies aimed to evaluating the resistance of bioactive sequences to gastrointestinal

digestion, and their capacity to be absorbed using cell line models (Contreras, Sancho, Recio, & Mills, 2012; Picariello et al., 2013; Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008).

The dairy peptides have been evaluated using spontaneously hypertensive rats (SHR), and the peptide sequences, doses and maximum decrease of systolic blood pressure (SBP) have been summarised in some reviews (Fitzgerald, Murray, & Walsh, 2004; Hernández-Ledesma, Contreras, & Recio, 2011; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012b).

As shown in Table 1, two strategies have been used to release antihypertensive milk peptides: milk protein hydrolysis and fermentation. Hydrolysates of whole milk protein, caseinates and

Table 1
Antihypertensive activity in spontaneously hypertensive rats of peptide derived milk proteins by enzymatic hydrolysis and fermentation.[a]

Procedure	Enzyme/microorganism	Protein fragment	Peptide sequence	Dose (mg kg^{-1})	Decrease in SBP (mm Hg)	Reference
Hydrolysis	Trypsin	α_{S1}-CN f(23–34)	FFVAPFPGVFGK	100.0	−34.0	Karaki et al. (1990)
		α_{S1}-CN f(194–199)	TTMPLW	100.0	−13.6	Miguel, Manso, López-Fandiño,
		β-CN f(177–183)	AVPYPQR	100.0	−10.0	Alonso, and Salaices (2007)
		CMP f(106–112)	MAIPPKK	10.0	−28.0	
	Pepsin	α_{S1}-CN f(90–94)	RYLGY	5.0	−25.0	Contreras et al. (2009)
		α_{S1}-CN f(143–149)	AYFYPEL	5.0	−20.0	Recio et al. (2006)
		α_{S2}-CN f(89–95)	YQKFPQY	5.0	−15.0	Ruiz-Giménez et al. (2010)
		α_{S2}-CN f(203–208)	PYVRYL	3.0	−23.4	
		Lfcin f(20–25)	RRWQWR	10.0	−16.7	
		Lfcin f(22–23)	WQ	10.0	−11.4	
	Gastric and pancreatic enzymes	α-La f(50–53)	YGLF	0.1	−23.4	Nurminen et al. (2000)
	Pepsin, chymotrypsin and trypsin	κ-CN f(22–24)	IAK	4.0	−20.7	Miguel, Gómez-Ruiz, Recio,
		κ-CN f(61–66)	YAKPVA	6.0	−23.1	and Aleixandre (2010)
		κ-CN f(76–86)	WQVLPNAVPAK	7.0	−18.4	
		κ-CN f(98–105)	HPHPHLSF	10.0	−15.7	
	Proteinase K	β-CN f(59–61)	VYP	8.0	−21.0	Abubakar et al. (1998)
		β-CN f(59–64)	VYPFPG	8.0	−22.0	
		β-CN f(80–90)	TPVVVPPFLQP	8.0	−8.0	
		β-Lg f(78–80)	IPA	8.0	−31.0	
		BSA f(221–222)	FP	8.0	−27.0	
	Proteinase of *Lb. helveticus* CP790	α_{S1}-CN f(104–109)	YKVPQL	2.0	−13.0	Maeno et al. (1996)
		α_{S2}-CN f(189–192)	AMPKPW	2.0	−5.0	
		α_{S2}-CN f(190–197)	MKPWIQPK	2.0	−3.0	
		α_{S2}-CN f(198–202)	TKVIP	2.0	−9.0	
		β-CN f(140–143)	LQSW	2.0	−2.0	
		β-CN f(169–174)	KVLPVP	2.0	−32.2	
		β-CN f(169–175)	KVLPVPQ	2.0	−31.5	
	Thermolysin	β-Lg f(58–61)	LQKW	10.0	−18.1	Hernández-Ledesma et al. (2007)
		β-Lg f(103–105)	LLF	10.0	−29.0	
	AS1.398 neutral protease	κ-CN f(15–18)	DERF	300.0[b]	n.d.[b]	Jiang et al. (2010)
		κ-CN f(25–30)	RYPSYG	–	–	
	Flavourzyme + *S. thermophilus* and *Lb. bulgaricus*	κ-CN f(58–61)	YPYY	3.4	−15.9	Tsai et al. (2008)
	Prozyme 6 and mixture of lactic acid bacteria	α_{S1}-CN f(162–164)	GVW	3.3	−22.0	Chen et al. (2007)
		β-Lg f(17–19)	GTW			
Fermentation	*Lb. helveticus* CPN4	α_{S1}-CN f(146–147)	YP	2.0	−32.1	Maeno et al. (1996)
	Lb. helveticus and *S. cerevisiae*	β-CN f(74–76)	IPP	0.3	−28.3	Nakamura, Yamamoto, Sakai,
		β-CN f(84–86)	VPP	0.6	−32.1	Okubo et al. (1995); Nakamura, Yamamoto, Sakai, and Takano (1995)
	E. faecalis	β-CN f(58–76)	LVYPFPGPIPNSL-	6.0	−14.9	Miguel et al. (2006);
		β-CN f(133–138)	PQNIPP	3.0	−25.3	Quirós et al. (2007)
		β-CN f(133–139)	LHLPLP	10.0	−7.7	
		β-CN f(134–138)	LHLPLPL	7.0	−23.5	
		β-CN f(197–206)	HLPLP	10.0	−16.2	
		β-CN f(201–209)	VLGPVRGPFP VRGPFPIIV	10.0	−16.1	
	Caprine kefir	β-CN f(58–68)	LVYPFTGPIPN	10.0	−28.0	Miguel et al. (2010)
	Manchego cheese	α_{S1}-CN f(102–109)	KKYNVPQL	10.0	−11.5	Miguel et al. (2010); Gómez-Ruiz, Ramos, and Recio (2002)
	Gouda cheese	α_{S1}-CN f(1–9)	RPKHPIKHQ	6.1	−9.3	Saito, Nakamura, Kitazawa,
		β-CN f(60–68)	YPFPGPIPN	7.5	−7.0	Kawai, and Itoh (2000)
	Enzyme-modified cheese	β-CN f(102–104)	MAP	3.0	−17.0	Tonouchi, Suzuki, Uchida,
	Lc. lactis NRRL B-50571	κ-CN f(98–110)	HPHPHLSFMAIPP	35.0	−17.7	and Oda (2008)
	Lc. lactis NRRL B-50572	β-CN f(69–77)	SLPQNIPPL	50.0	−16.7	Rodríguez-Figueroa et al. (2012, 2013)
		κ-CN f(35–40)	YPSYGL	–	–	Rodríguez-Figueroa et al. (2012, 2013)
		β-CN f(194–209)	VLGPVRGPFP			
		κ-CN f(35–40)	YPSYGL			

[a] Adapted from Martínez-Maqueda et al. (2012b); SBP, systolic blood pressure.
[b] Effect observed after administration of casein hydrolysate.

whey proteins using gastric and pancreatic enzymes, alone or in combination, have shown antihypertensive activity in SHR. As an example, the tryptic CN hydrolysate containing the peptide corresponding to α_{S1}-CN f(23–34) has been patented and commercialised as antihypertensive agent under the name of peptide C12®. More recently, in a pepsin casein hydrolysate, two peptides derived from α_{S1}-CN, with sequences RYLGY and AYFYPEL, have demonstrated potent SBP reducing effects in SHR (Contreras, Carrón, Montero, Ramos, & Recio, 2009). The action of pepsin is also required to release the antihypertensive fragments RRWQWR and WQ from peptide lactoferricin (LFcin) (Ruiz-Giménez et al., 2010). Combination of gastric and pancreatic enzymes has been reported to be a successful strategy to produce potent antihypertensive peptides from milk proteins (Gómez-Ruiz, Ramos, & Recio, 2007; Nurminen et al., 2000). Similarly, the use of food-grade enzymes derived from microorganisms have become usual for the release of peptides with demonstrated SBP reducing effects in SHR (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Chen, Tsai, & Pan, 2007; Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007; Jiang, Tian, Brodkorb, & Huo, 2010; Maeno, Yamamoto, & Takano, 1996; Tsai, Chen, Pan, Gong, & Chung, 2008) (Table 1).

Another strategy to produce antihypertensive peptides is the use of the proteolytic system of lactic acid bacteria proteins during the elaboration of fermented milks and cheeses. The most representative example is the production of the β-CN-derived peptides, IPP and VPP, in sour milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Calpis®) with potent decreasing effects on the SBP of SHR (Nakamura et al., 1995; Nakamura, Yamamoto, Sakai, & Takano, 1995). Other peptides derived from β-CN released after milk fermentation with *Enterococcus faecalis*, with sequences LHLPLP and HLPLP, have also shown antihypertensive effect in this rat model (Miguel, Recio, Ramos, Delgado, & Aleixandre, 2006; Quirós et al., 2007). In recent studies, fermented milks with *Lactococcus lactis* NRRLB-50571 and NRRLB-50572 have presented important SBP, diastolic blood pressure (DBP), and heart rate-lowering effects in SHR (Rodríguez-Figueroa, González-Córdova, Torres-Llanez, Garcia, & Vallejo-Cordoba, 2012; Rodríguez-Figueroa, González-Córdova, Astiazaran-Garcia, Hernández-Mendoza, & Vallejo-Cordoba, 2013).

Most of the human intervention studies have focused on the tripeptides IPP and VPP. Two meta-analyses performed with the published data of 17 and 12 clinical trials have been reported by Pripp (2008) and Xu, Qin, Wang, Li, and Chang (2008), respectively. These meta-analyses have reported an average decrease in SBP of 5.1 and 4.8 mm of Hg, respectively. A recent meta-analysis carried out with 28 clinical trials has reported a mean reduction of SBP of 1.66 mm of Hg (Qin et al., 2013). However, other clinical trials have reached controversial results. In fact, Engberink et al. (2008) and Usinger, Jensen, Flambard, Linnneberg, and Ibsen (2010) did not find an effect on human blood pressure in Dutch and Danish subjects when they daily consumed fermented milks containing peptides IPP and VPP. Recently, the meta-analysis of Cicero, Gerocarni, Laghi, and Borghi (2011), which included 18 trials, has reported that IPP and VPP reduce SBP in Asian subjects while this effect was lower in Caucasian individuals. Moreover, Cicero, Aubin, Azais-Braesco, and Borghi (2013) have found that these peptides can significantly decrease SBP in European subjects, as previously shown in Japanese subjects, although the effects were more noteworthy in middle-age adults. These findings suggest that genetics and/or dietary patterns might exert an important influence on the antihypertensive effects of peptides IPP and VPP, with further studies being needed to confirm this hypothesis. Moreover, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) (ESFA, 2009) has concluded that the evidence presented to date on the antihypertensive effects of peptides VPP and IPP is insufficient to establish a cause/effect relationship between the consumption of these peptides and the maintenance of normal blood pressure and, therefore, further studies are required.

Other milk protein-derived hydrolysates and peptides have been tested in clinical trials. The consumption of a whey protein hydrolysate for 6 weeks resulted in a reduction of SBP and DBP of hypertensive subjects, although peptides responsible for the observed effects have not been identified yet (Pins & Keenan, 2006). More recently, it was reported that peptides RYLGY and AYFYPEL could also reduce blood pressure in hypertensive humans (Recio et al., 2011). A yoghurt enriched with these peptides was administrated to 71 hypertensive subjects (divided in placebo and active substances groups), and 50 normotensive volunteers that only received active substance. After 6 weeks of consumption, the hypertensive patients showed a notable reduction in their SBP, while no significant changes in blood pressure were detected in both the placebo and the normotensive groups.

3.1.2. *Anti-inflammatory and antioxidant properties of peptides*

Chronic inflammation is involved in many age-related diseases, such as atherosclerosis, vascular diseases, arthritis, cancer, diabetes, osteoporosis, dementia, obesity, and metabolic syndrome (Yu & Chung, 2006). Different cytokines play a pivotal role as mediators in the production of biomarkers implied in the progression of inflammation and the endothelial dysfunction. The down-regulation of these cytokines by food components, including peptides, may retard or alleviate inflammation, thus exerting beneficial effects against cardiovascular diseases (Tompa et al., 2010). To date, only the commercial peptide NOP-47, derived from whey, has demonstrated in a clinical study to enhance the vascular function by modulating glucose levels and biomarkers of inflammation and oxidative stress in healthy individuals (Ballard et al., 2009).

In vitro, a preliminary study using lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages suggests the potential role of a yak CN hydrolysate with alcalase to prevent inflammation-related disorders because of its ability to reduce the secretion of pro-inflammatory cytokines, and the production of nitric oxide (NO). Also, this hydrolysate has demonstrated to exert radical scavenging activity that might contribute to its beneficial properties (Mao, Cheng, Wang, & Wu, 2011).

Obesity is accompanied by low-grade chronic inflammation in different tissues such as adipose tissue, liver, pancreas, muscle and brain (Troncon Rosa, Zulet, Marchini, & Martínez, 2012). The inflammatory response associated to obesity provokes the activation of cytokines and transcriptional factors, and the penetration of macrophages in the adipose tissue, resulting in an unresolved response associated with insulin resistance, endothelial dysfunction and subsequent cardiovascular disorders (Chung et al., 2009). Dietary interventions targeted at reducing adipose tissue inflammation can be a valuable alternative for improving the general metabolic profile (Siriwardhana et al., 2013). However, Pal and Ellis (2010, 2011) did not observe significant reductions in inflammatory biomarkers of overweight and obese subjects after neither acute nor chronic supplementation with sodium caseinate or WPI compared with the controls. These results are in agreement with those reported by Lee, Skurk, Hennig, and Hauner (2007) that did not show any significant difference after administration of skimmed milk to mild hypertensive subjects. These authors suggested that potential bioactive peptides could be hydrolysed by intestinal or plasma peptidases, losing their anti-inflammatory activity. The lack of concluding findings makes difficult guaranteeing the anti-inflammatory activity of peptides derived from CN or whey proteins, and more studies are needed to confirm it.

Oxidative stress is another responsible factor for the initiation or evolution of cardiovascular diseases. Natural antioxidants provide additional benefits to the endogenous defence strategies in the battle against oxidative stress (Erdmann et al., 2008). Food-derived peptides have shown to be potent antioxidants without important side effects (Sarmadi & Ismail, 2010), with milk proteins being one of the most frequently studied sources. To date, many antioxidant peptides derived from both CN and whey proteins have been characterised by *in vitro* chemical assays (Pihlanto, 2006; Power, Jakeman, & Fitzgerald, 2013). These assays are restricted because of their limited similarity to physiological conditions, and further studies are needed to confirm the demonstrated effects. In the last few years, cell cultures have being used to assess the potential health effects of antioxidant milk-derived hydrolysates and/or peptides. These models are useful because they allow evaluation of additional factors such as peptide bioavailability and metabolism besides biological activity. However, to our knowledge, no cell culture experiments demonstrating the antioxidant activity on the cardiovascular system have been conducted.

The results of animal experiments and human trials suggest that fermented milk products may exert antioxidant effects associated to cardiovascular benefits. Zommara, Tougo, Sakanao, and Imaizumi (1998) found that fermented milk produces an anti-peroxidative action on rats fed a vitamin-E deficient diet. Moreover, the consumption of fermented goat milk by healthy individuals improved the total plasma antioxidant activity, lowered the levels of oxidised low density lipoprotein, isoprostanes and the glutathione redox ratio, and prolonged the resistance of the lipoprotein fraction to oxidation, resulting in the improvement of the anti-atherogenicity (Kullisaar et al., 2003). Although the compounds responsible for these effects have not been identified yet, peptides released during milk fermentation might have a key function. Consequently, further studies elucidating the role of antioxidant peptides in the protective activity of fermented milks on the cardiovascular system are needed.

3.1.3. Hypocholesterolaemic peptides

Diet plays a key role in plasma lipids profile, and consequently it is used as a strategy to prevent or decrease the incidence of cardiovascular diseases (Erdmann et al., 2008). It was observed that whey protein displayed a hypocholesterolaemic effect in rats after the ingestion of cholesterol free or cholesterol-enriched diets compared to CN or soybean proteins enriched diets (Nagaoka, Kanamaru, & Kuzuya, 1991; Nagaoka, Kanamaru, Kuzuya, Kojima, and Kuwata, 1992). This effect was confirmed when a tryptic hydrolysate of β-lactoglobulin (β-Lg) was administered to rats fed a diet rich in cholesterol. A notable reduction in the cholesterol level as well as an increase of the high density lipoproteins–cholesterol content, and of the excretion of fecal steroid was observed (Nagaoka et al., 2001).

A peptide called lactostatin, IIAEK, corresponding to fragment f(71–75) of β-Lg, obtained from its tryptic hydrolysate, showed hypocholesterolaemic activity in rats, being this effect higher than that showed by β-sitosterol (Nagaoka et al., 2001). Another β-Lg derived peptide, β-lactotensin (HIRL), obtained by hydrolysis with chymotrypsin, decreased total cholesterol and low density lipoproteins plus very low density lipoproteins–cholesterol content in mice fed a cholesterol-enriched diet (Yamauchi, Ohinata, & Yoshikawa, 2003). The exact mechanisms involved in the hypocholesterolaemic activity of these peptides have not been elucidated yet, but the preliminary results suggested that the amino acid composition may play a key influence (Erdmann et al., 2008). However, more studies are clearly needed to confirm the results, especially in humans determining the exact mechanism of this action.

3.2. Effects on intestinal health

The intestine is an organ responsible for nutrient absorption, barrier functions, signal recognition, and the production of endogenous active molecules. Many physiological actions are known to be regulated by hormones or cytokines, but substances contained in the diet are also thought to play a role as modulators of intestinal functions. Peptides derived from milk proteins are a group of substances with potential effects at gastrointestinal level through different mechanisms of action. They have been shown to exert protective actions on intestinal mucus, modulatory effects of mineral absorption, and anti-inflammatory, antidiabetic, and satiating activities.

3.2.1. Protective effect on the gut mucosa

The protective properties of the gastrointestinal mucus gel are attributed to its principal component, mucins, high molecular weight glycoproteins secreted by goblet cells. Disruption of the structure of this mucus has important physiological implications (Corfield et al., 2000; Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008), and strong evidence was reported when spontaneous colitis and colon cancer were developed in mice genetically deficient in the mucin gene *Muc2* (Van der Sluis et al., 2006; Velcich et al., 2002).

Although intestinal epithelial cells functions are usually controlled by endogenous agents, it is worth mentioning that these cells come into continuous contact with high concentrations of food components and substances along the gut luminal surface; hence, they might be also regulated by external factors, such as nutrients (Gibson & Muir, 2005; Moughan et al., 2007). Interestingly, some dietary compounds have proven capacity to positively influence the protective properties of the mucus gel layer (Montagne, Piel, & Lalles, 2004). Dairy proteins, hydrolysates, and peptides have been demonstrated to modify the dynamics of mucus mainly via influencing the mucin secretion and expression, and the number of goblet cells. The published studies showing evidence of these bioactivities in *ex-vivo*, *in vitro* and/or *in vivo* assays are summarised in Table 2.

In the first studies carried out using an *ex-vivo* preparation of rat jejunum, CN and α-lactalbumin (α-La) hydrolysates evoked an increase in the mucin secretion, while native CN did not produce any effect (Claustre et al., 2002; Trompette et al., 2003). The peptide β-casomorphin 7, derived from β-CN, showed a strong rise in the mucin glycoprotein secretion. Since the observed effects were abolished by pre-treatment with opioid antagonist naloxone, it has been suggested that the activity might be mediated by interaction with opioid receptors. Additionally, Zoghbi et al. (2006) demonstrated that β-casomorphin 7 significantly enhanced the mucin secretion and stimulated the expression of mucin *Muc2* and *Muc3* genes in rat intestinal cells DHE, and *MUC5AC* gene in human intestinal cells HT29-MTX. Recently, a whey protein hydrolysate rich in β-Lg-derived peptides was found to induce mucin secretion and *MUC5AC* gene expression in HT29-MTX cells (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013b). Among the peptides contained in this hydrolysate, sequence β-lactorphin was identified as the major responsible for the observed effects. Cells exposed to β-lactorphin increased their mucin synthesis, whereas no significant differences were observed in the *MUC5AC* expression analysis (Martínez-Maqueda et al., 2013b). The stimulatory effect of a CN hydrolysate has been also assessed in HT29-MTX cells by Martínez-Maqueda, Miralles, Cruz-Huerta, and Recio (2013a). The hydrolysate and two α_{S1}-CN-peptides contained in it, promoted the mucin output via inducing mucin-like glycoprotein secretion and *MUC5AC* over-expression. Similarly, Plaisancié et al. (2013) have evaluated the impact of a total peptide pool from commercial yoghurt in

Table 2
Dairy proteins, hydrolysates and peptides carrying biological activities related to gastrointestinal mucosal protection.

Ex-vivo/in vitro/in vivo assays	Protein fragment/ hydrolysate[a]	Peptide sequence	Dose[b]	Mechanisms of action/effects	% of controls[c]	Reference
Preparation of isolated vasculary perfused rat jejunum	CN hydrolysate	–	0.5[d]	Induction of jejunal mucin secretion	417	Claustre et al. (2002)
	α-La hydrolysate	–	5.0[d]	Induction of jejunal mucin secretion	335	Claustre et al. (2002)
	β-CN f(60–63)	YPFP	0.001	Induction of jejunal mucin secretion	N.R.	Trompette et al. (2003)
	β-CN f(60–63)	YPFP-NH_2	0.001	Induction of jejunal mucin secretion	445	Trompette et al. (2003)
	β-CN f(60–66)	YPFPGPI	0.120	Induction of jejunal mucin secretion	563	Claustre et al. (2002)
	β-CN f(60–66)	YPFPGPI	0.120	Induction of jejunal mucin secretion	555	Trompette et al. (2003)
Rat intestinal mucin-producing cells (DHE)	β-CN f(60–66)	YPFPGPI	0.100	Induction of mucinlike glycoprotein secretion Mucin genes *Muc2* and *Muc3* overexpression	227 225/208	Zoghbi et al. (2006)
Human intestinal mucin-producing cells (HT29-MTX)	CN hydrolysate	–	0.1[d]	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	210 180	Martínez-Maqueda et al. (2013a)
	WPC hydrolysate	–	0.1[d] 1.0[d]	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	152 153	Martínez-Maqueda et al. (2013b)
	α_{S1}-CN f(143–149)	AYFYPEL	0.050 0.100	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	162 174	Martínez-Maqueda et al. (2013a)
	α_{S1}-CN f(144–149)	YFYPEL	0.050 0.500	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	166 179	Martínez-Maqueda et al. (2013a)
	β-CN f(51–55)	YPFVE	0.100	Induction of mucinlike glycoprotein secretion	234	Martínez-Maqueda et al. (2012a)
	β-CN f(60–66)	YPFPGPI	0.100	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	163 176	Zoghbi et al. (2006)
	β-CN f(94–123)	GVSKVKEAM APKHKEMPF PKYPVEPFTESQ	0.10[e]	Induction of mucinlike glycoprotein secretion Mucin genes *MUC2* and *MUC4* overexpression	N.R. N.R.	Plaisancié et al. (2013)
	α-La f(50–53)	YGLF-NH_2	0.10 0.50	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	201 160	Martínez-Maqueda et al. (2013a)
	β-Lg f(102–105)	YLLF	0.50	Induction of mucinlike glycoprotein secretion	174	Martínez-Maqueda et al. (2013b)
	β-Lg f(102–105)	YLLF-NH_2	0.10 0.50	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	453 222	Martínez-Maqueda et al., 2012a Martínez-Maqueda et al., 2013b
Rat gastric epitelial cells (RGM1)	α-La protein	–	3.0[f]	Stimulate mucin synthesis, mucin secretion and prostaglandin E_2 synthesis	123/119/140	Ushida, Shimokawa, Toida, Matsui, and Takase (2007)
Supplementation in the diet of rats	CN hydrolysate	–	114[g]	Average daily gain, average daily food intake, gain:feed ratio and ileal endogenous nitrogen flow enhanced Upregulation of mucin genes *Muc3* in the small intestine and *Muc4* in the colon	410/149/270 277/325	Han et al. (2008)
	Cheese whey protein	–	160[g]	Protection against DSS-induced colitis: decrease in mucosal and luminal markers of colitis, and faecal mucin excretion and faecal microbiota enhanced	N.R.	Sprong et al. (2010)
Intragastric gavage in rats	WPI	–	0.75[g]	Protection against ethanol-induced ulcerative lesions: reduction of ulcerative lesion index and plasma gastrin, and stimulation of gastric mucus production	−74/−38 168	Castro, Carvalho, Tinti, Possenti, and Sgarbieri (2010)
	α-La protein	–	0.30[g]	Increase the thickness of the mucus gel layer in corpus and antrum gastric mucosa	163/164	Ushida et al. (2007)
	α-La hydrolysate	–	0.20[g]	Protection against indomethacin-induced ulcerative lesions: induction of gastric mucus production and prostaglandin E2 biosynthesis	134/536	Mezzaroba et al. (2006)
	β-CN f(94–123)	GVSKVKEAMA PKHKEMPFPK YPVEPFTESQ	0.01[e] 0.10[e] 0.100	Upregulation of duodenal and ileal *Muc4* mRNA expression Increase in the duodenal goblet cells recount Upregulation of jejunal *Muc2* mRNA expression Increase in the jejunal and ileal goblet cells recounts Upregulation of jejunal *Muc4* mRNA expression	156/157 133 152 143/134 161	Plaisancié et al. (2013)

[a] WPC, whey protein concentrate.
[b] Dose given in mM except where otherwise indicated.
[c] Maximum effect within the different treatment conditions assessed; N.R., non-reported.
[d] Dose is weight/volume.
[e] Dose is μM.
[f] Dose is mg mL^{-1}.
[g] Dose is g kg^{-1}.

HT29-MTX cells, observing an increase of the secreted mucin, and the mRNA levels of *MUC2* and *MUC4* genes. The β-CN fragment f(94–123) was suggested to be responsible for the reported bioactivities after evaluating its effects in intestinal cells and in rats. Oral administration of this peptide to rats yielded an increase in the goblet cells counts, modified *Muc2* and *Muc4* mRNA expression, and ultimately improved gut mucosa protection by enhancing crypts containing Paneth cells and increasing the number of rat defensin 5 and lysozyme (LZ) mRNA transcripts in the rat small intestine (Plaisancié et al., 2013).

Other *in vivo* studies have also pointed to the regulation of the protective mucus layer by dairy proteins and their degradation products. A diet containing a CN hydrolysate as the exclusive source of nitrogen was given to rats in which enhancements in the ileal endogenous nitrogen flow and in the expression of *Muc3* in the small intestine, and Muc4 in the colon were observed (Han, Deglaire, Sengupta, & Moughan, 2008). In the study of Sprong, Schonewille, and van der Meer (2010), the protective effect of cheese whey protein was examined in the dextran sulphate sodium (DSS)-induced model of rat colitis. After seven days of DSS consumption, rats fed the cheese whey protein diet showed lower levels for both mucosal and luminal markers of colitis. Although faecal mucin excretion was higher, expression of *Muc2* in the colon was not statistically different from controls. Incorporation of cheese whey protein to the diet also resulted in an increase of the counts of protective microbiota species. Interestingly, one group of rats fed a diet consisting in CN with threonine/cysteine supplementation was included in the study, and the observed protection against DSS-induced colitis was similar to the rats fed the cheese whey protein diet (Sprong et al., 2010). It was therefore suggested that reported beneficial effects of cheese whey protein might be due to its high threonine and cysteine content. In this context, availability of specific amino acids like threonine, cysteine, proline and serine has been shown to be important to increase the number of *Muc2*-containing goblet cells, up-regulate the mucin synthesis, and restore the bowel microbiota, thus helping colonic defences and mucosal healing in DSS-treated rats (Faure et al., 2006).

3.2.2. Modulatory peptides of mineral absorption

Mineral deficiencies are the most important nutritional problems worldwide, with the iron deficiency being the most common. This disorder happens when the mineral ingestion from diet does not meet a daily need of minerals. In this context, the mineral fortification is one of the best and most common strategies to prevent this deficiency (Zimmermann & Hurrell, 2007). It has been proposed that the phosphorylated regions released from CN during digestion, CPPs, could increase mineral solubility at intestinal pH, modulating its bioavailability (Meisel & Fitzgerald, 2003). Several authors have demonstrated the release of CPPs in rats and minipigs after the ingestion of CN (Kasai, Iwasaki, Tanaka, & Kiriyama, 1995; Meisel & Frister, 1988, 1989; Naito & Suzuki, 1972, 1974; Sato, Noguchi, & Naito, 1986; Sato, Shindo, Gunshin, Noguchi, & Naito, 1991). In humans, the liberation of CPPs has been reported after consumption of milk, yogurt, and CN (Boutrou et al., 2013; Chabance et al., 1998; Meisel et al., 2003). Also, CPPs have been demonstrated to resist further degradation by digestive enzymes and enteric bacteria in both rats and humans (Brommage, Juillerat, & Jost, 1991; Hirayama, Toyota, Hidaka, & Naito, 1992; Kasai et al., 1995; Meisel et al., 2003). Regarding the *in vivo* studies with CPP preparations, several authors have reported an increase of the soluble calcium concentration in the intestine of rats due to CPPs released from CN contained in the diet, which should improve the absorption of this mineral (Kitts, Yuan, Nagasawa, & Moriyama, 1992; Lee, Noguchi, & Naito, 1979, 1980, 1983; Sato, Noguchi, & Naito, 1983; Sato et al., 1986). However, the results of studies on the influence of CPPs upon calcium absorption in humans and animal models are controversial.

The addition of a pool of CPPs to aqueous solutions and infant foods containing phytate increased calcium absorption in rat pups after gastric intubation, being able to overcome the negative effect of phytate on its absorption (Hansen, Sandström, & Lönnerdal, 1996). In agreement with this result, in rats fed an isolated soy protein-based diet supplemented with CPPs and calcium, an increase of calcium absorption was reported, although the effect was not dose dependent (Saito, Lee, & Kimura, 1998). In contrast, diet supplementation with CPPs and calcium did not improve calcium absorption in growing pigs fed a soy-based diet (Pointillart & Guéguen, 1989), in rats fed a whey protein-based diet (Brommage et al., 1991; Kopra, Scholz-Ahrens, & Barth, 1992; Scholz-Ahrens, Kopra, & Barth, 1990), or in rats fed an experimental diet containing mainly egg albumin, corn starch, and glucose (Bennett et al., 2000; Tsuchita, Goto, Yonehara, & Kuwata, 1995). In ovariectomised rats, used as a postmenopausal bone loss model, calcium absorption was enhanced in the first three days when CPPs derived from β-CN and calcium (at higher doses) were supplemented (Tsuchita, Sekiguchi, Kuwata, Igarashi, & Ezawa, 1993). No effects of CPPs upon calcium absorption was observed in rats after administration by gastric intubation of non-fortified milk while an increase was observed when administered milk was fortified with calcium (Mora-Gutierrez, Farrell, Attaie, McWhinney, & Wang, 2007; Tsuchita, Suzuki, & Kuwata, 2001). Using a ligated duodenal loop technique, CPPs increased calcium absorption in rachitic and normal chicks (Mykkänen & Wasserman, 1980), and rats (Kitts et al., 1992; Sato et al., 1986). These results are in disagreement with the observations of Yuan and Kitts (1991) in the same model, who did not observe any difference on calcium absorption after addition of CPPs to CN or soybean enriched diets. The controversial results obtained in the above mentioned studies could be caused by several factors influencing the effect of CPPs, such as the animal model, physiological status of the individuals, type of diet and its calcium or vitamin D content, source and composition of CPPs, and the length of the assay, among others.

In humans, an increase of calcium absorption has been shown due to the addition of CPPs to rice-based cereal, although the effect was not observed after addition to whole-grain cereal or to three different meals with different calcium and phytate contents (Hansen, Sandström, Jensen, & Sorensen, 1997a,b). It has been not observed influence of CPPs upon calcium bioavailability after their ingestion with milk (López-Huertas et al., 2006; Narva, Kärkkäinen, Poussa, Lamberg-Allardt, & Korpela, 2003) or with a drink of calcium lactate (Teucher et al., 2006). In contrast, Heany, Saito, and Orimo (1994) showed a positive role of CPPs in postmenopausal women with low calcium absorption capacity, despite the fact that this effect was not observed in women with normal absorption capacity of this mineral.

CPPs bound to calcium salts have been also found to be useful to remineralise the teeth of mammals, displaying anticariogenic effects when added to dentifrices, oral care products, chewing gum or other confectionery foods (Luo & Wong, 2004). In human trials, synthetic nanocomplexes of CPPs and calcium salts incorporated in mouth rinses and sugar-free chewing gums have been proven to be potential anticariogenic agents (Reynolds, Cai, Shen, & Walker, 2003).

Concerning the iron bioavailability, a positive influence of CPPs on this parameter has been reported, reflected in an increase in the haemoglobin and haematocrit levels, as well as in the iron liver storage when iron deficient rats were supplemented with iron and hydrolysed β-CN or β-CN fragment f(1–25)4P (Aït-Oukhatar et al., 1997, 1999). Likewise, the blood iron levels were increased in rats after ingestion of iron bound to hydrolysed CN (Chaud et al., 2002).

These results were confirmed using a perfused rat intestinal model loop system, in which the iron bound to β-CN f(1–25)4P increased the absorption of this mineral in iron deficient and non-iron deficient rats (Aït-Oukhatar et al., 2002; Ani-Kibangou et al., 2005; Bouhallab et al., 2002; Pérès et al., 1997; Pérès, Bouhallab, Bureau, Neuville et al., 1999; Pérès, Bouhallab, Bureau, Maubois et al., 1999). In addition, diverse fractions of CPPs exerted different effects on iron absorption, being this effect higher when the iron was bound to β-CN fractions than to α_S-CN fractions (Bouhallab et al., 2002; Kibangou et al., 2005), suggesting the influence of the structural characteristics and conformations of CPPs on their capacity to improve the iron bioavailability.

In humans, the effect of CPPs on iron bioavailability is controversial. Whereas Hurrell, Lynch, Trinidad, Dassenko, and Cook (1989) reported an improvement of iron absorption after the intake of hydrolysed CN, Aït-Oukhatar et al. (2002) did not observe any effect after the ingestion of iron bound to β-CN f(1–25). *In vivo* studies with other minerals, such as zinc, have also found variable results. Hansen et al. (1996) reported an increased zinc absorption in rat pups after the addition of CPPs to aqueous solutions and infant foods containing phytate, avoiding the negative effect of this compound on zinc bioavailability. However, no effect was reported from soybean protein based diet containing CPPs in rats (Matsui, Okumura, & Yano, 2002). As observed for calcium, zinc absorption was reported to increase when CPPs were added to rice-based cereal. However, no effects were observed when CPPs were added to whole-grain cereal or three different meals with different calcium and phytate contents (Hansen et al., 1997a,b). These results indicate that the effects of CPPs can be influenced by factors, such as the food matrix, i.e., liquid or solid diet, as well as the phytate content (Miquel & Farré, 2007).

3.2.3. *Anti-inflammatory peptides at gastrointestinal level*

Inflammatory bowel disease is a chronic and degenerative condition comprising a group of disorders, being the ulcerative colitis and Crohn's disease the most relevant both in incidence and prevalence. Although the aetiology of this disease remains unknown, it has been demonstrated that genetic, immunological, environmental, and lifestyle factors are implied (Bernstein et al., 2009). Currently, inflammatory bowel disease is mainly treated with drugs that suppress the immune system. However, the multiple adverse effects shown by these drugs have made that their use is limited and selective to a small number of patients. To avoid these limitations, new and natural treatments without side effects are being searched and developed (Sánchez de Medina et al., 2010). The most extensively studied peptide for its activity against intestinal inflammation is the bovine caseinomacropeptide (CMP). In recent years, different animal models of intestinal inflammation have been used to provide evidence on the anti-inflammatory effect of CMP at gastrointestinal level. The activity of this peptide in mice with colitis and ileitis induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS), a model that shares important similarities with human Crohn's disease has been established (Daddaoua et al., 2005; Requena et al., 2008). Recently, the effects have been assessed in the DSS-induced model of rat colitis (López Posadas et al., 2010). These studies also evaluated the mechanism of action of CMP indicating that it mainly acts on the inflammatory/immune response by the activation of macrophages, favouring the differentiation of regulatory T cells and limiting the activation of T helper 1 cells (Sánchez de Medina et al., 2010).

Preliminary studies have shown the anti-inflammatory effect of β-CN hydrolysates by *Lactobacillus delbrueckii* ssp. *lactis* CRL581 in a murine TNBS-induced colitis model (Espeche Turbay, de Moreno de LeBlanc, Perdigón, Savoy de Giori, & Hebert, 2012). Thus, these results suggest that other milk peptides in addition to CMP might act as protective agents on intestinal inflammation, and further studies focused on the identification of potentially responsible sequences, and the evaluation of their mechanism of action should be designed.

3.2.4. *Peptides against type 2 diabetes*

Ingestion of a meal provokes the secretion, at gastrointestinal level, of incretins hormones, such as glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP). These hormones are involved in the modulation of gut motility, secretion of gastric and pancreatic enzymes, nutrient absorption, and stimulation of the insulin secretion from the pancreas that allows the disposal of the absorbed glucose (Drucker & Nauck, 2006). Type 2 diabetes, representing 90–95% of the diagnosed cases of diabetes, is characterised by multiple pathophysiologic defects including progressive dysfunction of pancreatic cells, insulin resistance, and increased production of hepatic glucose (Bharatam, Patel, Adane, Mittal, & Sundriyal, 2007). It has been demonstrated that continuous intravenous administration of GLP-1 normalises blood glucose levels in diabetic patients (Nauck et al., 1993). However, the rapid degradation of this hormone by the enzyme dipeptidyl-peptidase IV (DPP-IV) makes this strategy for the type 2 diabetes treatment unfeasible. Currently, incretin-based therapies include a combination of GLP-1 analogues resistant to DPP-IV action, and orally bioavailable DPP-IV inhibitors (Fadini & Avogaro, 2011).

Diet supplementation with whey protein has been demonstrated, by preclinical and clinical studies, to potentiate insulin release and ameliorate postprandial glucose control in both healthy and type 2 diabetic subjects (Sousa et al., 2012). Initially, it was suggested that these effects were due to its rapid digestion and the consequent fast increase of the levels of different amino acids in plasma (Luhovyy, Akhavan, & Anderson, 2007). However, the intake of free amino acid mixtures did not produce the same effects (Nilsson, Holst, & Bjorck, 2007). Recently, it has been hypothesised that peptides released from whey proteins during their transit through gastrointestinal tract might be responsible for the post-meal glycaemic response produced after whey intake (Akhavan, Luhovyy, Brown, Cho, & Anderson, 2010). Even the acute ingestion of a whey protein hydrolysate by rats resulted in a higher transient leucine response with a sequential increase in insulin (Toedebusch et al., 2012). A recent study has demonstrated the dose-dependent insulinotropic effect of whey protein hydrolysates in a cell-based co-culture approach using pancreatic BRIN-BD11 beta cells and Caco-2 cells monolayers (Gaudel et al., 2013). These authors confirmed these effects in obese (ob/ob) mice, observing, after the oral administration of those hydrolysates, an improvement of blood glucose clearance, reduction of hyperinsulinemia, and restoration of the pancreatic islet capacity to secrete insulin in response to glucose.

In addition, a significant reduction of DPP-IV activity was observed in the proximal small bowel of rodents fed whey proteins (Gunnarsson et al., 2006), suggesting that peptides released from these proteins might exert DPP-IV inhibitory effects at intestinal level. Recent studies have demonstrated the *in vitro* DPP-IV inhibitory activity of hydrolysates derived from whey proteins, and peptides contained in these hydrolysates, and potentially responsible for the observed effects have been identified (Nongonierma & Fitzgerald, 2013). Among these peptides, sequences IPA (β-lactosin) and IPAVF, derived from β-Lg are amongst the most effective DPP-IV inhibitory peptides described to date (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Tulipano, Sibilia, Caroli, & Cocchi, 2011). A β-Lg-derived peptide, with the sequence VAGTWY, presented hypoglycemic effects in the oral glucose tolerance test in mice (Uchida, Ohshiba, & Mogami, 2011). This *in vivo* effect has been also demonstrated for the peptide LPQNIPPL,

a CN-derived fragment described as DPP-IV inhibitor (Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). In fact, a recent in silico study has shown that both CN and whey proteins serve as precursors of DPP-IV inhibitory peptides because of the high number of fragments contained within them that match DPP-IV inhibitory sequences described in the literature (Lacroix & Li-Chan, 2012).

3.2.5. Effect on satiety

It is recognised that protein is the most satiating macronutrient of food (Fromentin et al., 2012). Although it is not clear if the source of protein can influence this satiating effect, the consumption of dairy foods and calcium has been recommended in several diets aiming the regulation of the body weight. Several clinical studies have proposed a certain satiating effect for dairy products. For instance, in a 6-month trial with an energy-restricted diet, the group receiving milk supplementation showed a decreased desire to eat and hunger versus the placebo group, although no differences in the level of ghrelin or leptin were found (Gilbert et al., 2011). In a recent study with 49 overweight and obese adults, the high-dairy group had similar weight loss than the low-dairy group, but high-dairy group showed slightly higher peptide YY concentrations in plasma and enhanced sensation of satisfaction (Jones et al., 2013).

The mechanisms of action of different protein types on satiety are still under investigation, but it has been proposed that it could be related to a slowdown of the gastric emptying, an increase in brain amino acids, or the presence of specific peptides or amino acids. In this respect, it has been highlighted that in animal or human trials, diet has to be well equilibrated in its essential amino acid content in order to prevent depression in food intake due to conditioned food aversion (Fromentin et al., 2012). In particular, it has been proposed that whey proteins contribute to short term appetite suppression, with this effect being higher than that obtained with CN, soy protein and egg albumin (Anderson, Tecimer, Shah, & Zafar, 2004). This effect can be attributed to the high content in branched-chain amino acids (mainly, L-leucine), the presence of certain peptides, such as CMP, or it can be mediated by the release of satiety hormones (cholecystokinin, GLP-1, GIP, peptide YY or ghrelin). A review that summarises the evidence built in animal and human trials on the effect of whey protein consumption on satiety and intake regulatory mechanisms has been previously published (Luhovy et al., 2007). Later reports have compared the effect of whey protein and a whey protein hydrolysate, when consumed before a meal, on glucose and insulin levels and pre- and post-meal satiety. Interestingly, it was found that the whey protein, but not the whey protein hydrolysate, reduced post-meal blood glucose and insulin concentrations in a dose-dependent manner, and diminished food intake (Akhavan et al., 2010). Therefore, it seems relevant the form in which the protein is ingested, or in other words, the peptides that reach the gut during gastrointestinal digestion of whey proteins. Peptide composition and characterisation of hydrolysates is definitive in this kind of studies to extract cause-effect conclusions. Other studies have confirmed that whey proteins enhance satiety in humans in a short time period of time compared with carbohydrates but the effect could not be attributed to CMP since same effect was achieved with a WPI without CMP (Lam, Moughan, Awati, & Morton, 2009). Similarly, in a double-blind acute study with 20 overweight/obese males, no differences were found on plasma cholecystokinin levels or subjective satiety after ingestion of CMP with different glycosylation levels (Keogh et al., 2010). However, a positive effect was shown in a study with individuals with phenylketonuria where the consumption of CMP at breakfast was compared with an amino acid-based formula. Lower postprandial ghrelin levels after breakfast containing CMP associated with a greater feeling of fullness were found (MacLeod, Clayton, van Calcar, & Ney, 2010). Consequently, more work is needed to confirm the effect or certain whey components on satiety, and to elucidate the mechanism of action implicated in the activity of the whey protein fraction. Finally, it has been pointed out that the difference in satiety by various proteins can be also influenced by the physical properties of food in the gut, and factors, such as, viscosity, or clotting of proteins in the gut play an important role. For instance, in a comparative study with whey protein drinks and alginate-based drinks with different viscosities and protein contents, it was found that subjects reported reduced hunger with protein drinks with higher viscosity independently of the protein content, suggesting that viscosity can exert a higher effect than protein concentration (Solah et al., 2010).

Concerning the CN fraction of milk, it is known that peripheral opioid and cholecystokinin receptors are activated by ingestion of CN, and blocking receptors with antagonists reduces their effect on food intake (Froetschel, Azain, Edwards, Barb, & Amos, 2001; Pupovac & Anderson, 2002). Therefore, the presence of peptides able to interact with these receptors will determine the activity of CN hydrolysates. Because the composition of the CN hydrolysates varies with the hydrolysis process, peptidomic characterisation of the products is essential.

In a comparative study of CN and whey on gastrointestinal hormone secretion and appetite, it was found a greater subjective satiety after the whey load. Whey also produced a higher increase in postprandial plasma amino acid concentration, and higher levels of cholecystokinin, GLP-1 and GIP than CN. These results emphasise the importance of the type of protein, and confirm that post-absorptive increases in plasma amino acids and gastrointestinal hormones are involved in the appetite response (Hall, Millward, Long, & Morgan, 2003).

3.3. Effects on body defences

Apart from their nutritional value, milk proteins are able to exert physiological activities aiming to protect the newborn mammal. Furthermore, throughout the course of digestion of milk, bioactive peptides are released from consumed proteins. These peptides may possess not only immunomodulatory, but also antimicrobial and antiproliferative properties that help improving the defence system of the neonate.

3.3.1. Antimicrobial peptides

The antimicrobial activities of milk protein-derived peptides are very diverse, ranging from those with a prebiotic effect, peptides with the ability to prevent the attachment or invasion of pathogen microorganisms, to peptides killing or inhibiting the growth of microorganisms. This section will refer to this latter group of peptides with special emphasis on those peptides with antibacterial activity. The antiviral activity of dairy proteins, modifications thereof, and derived peptides has been reviewed by different authors (Berlutti et al., 2011; Pan et al., 2006).

Milk-derived antimicrobial peptides are characterised by a highly positively charged sequence and certain amphiphilic character. It has been proposed that the net positive charge will aid binding to negatively charged bacterial membranes and the amphiphilic character will help membrane disruption. Some peptides, like LFcin and lactoferrampin, contain also a hydrophobic region with a tryptophan residue, which is involved in membrane insertion (Schiffer, Chang, & Stevens, 1992). However, recent reports have also described the activity of anionic peptides-enriched extracts from Cheddar cheese against *Listeria monocytogenes* (Demers-Mathieu et al., 2013a). The origin and activity of some of

the most relevant antibacterial peptides derived from milk proteins are summarised in Table 3.

The antimicrobial peptides that have received most attention in the last two decades are derived from lactoferrin (LF). Hydrolysis of bovine LF with pepsin generates several protein fragments with antibacterial activity. Among them, fragment f(17–41/42), known as LFcin (Bellamy et al., 1992), is one of the most potent with a broad spectrum against Gram-positive and Gram-negative bacteria; but also other active fragments from the N-lobe of the protein, i.e., f(267–285), f(267–288), and f(277–288), are released by the action of this enzyme (Recio & Visser, 1999b). A peptide from this region of the protein, f(268–288), called lactoferrampin, was later chemically synthesised, and it has demonstrated activity against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Van der Kraan et al., 2004). Recently, it has been shown that chimerisation of LFcin and lactoferrampin increases activity against various microorganisms, such as *Candida albicans*, and *Burkholderia pseudomallei* (Bolscher, Nazmi, van Marle, van 't Hof, & Veerman, 2012; Puknun et al., 2013).

Digestion of the major bovine whey proteins, β-Lg and α-La, with trypsin and chymotrypsin, respectively, releases peptides with moderate activity against Gram-positive bacteria (Pellegrini, Dettling, Thomas, & Hunziker, 2001; Pellegrini, Thomas, Bramaz, Hunziker, & Von Fellenberg, 1999). Recently, Demers-Mathieu et al. (2013b) obtained an antibacterial fraction rich in anionic peptides of about 8 amino acids long by nanofiltration of a tryptic hydrolysate of a WPI. The same research group has recently demonstrated the antibacterial activity of a peptic hydrolysate of a

Table 3
Fragments, origin, antibacterial, and other biological activities of the most relevant antibacterial peptides.[a]

Fragment[b]	Isolation	Antibacterial activity	Other activities[c]	References
α_{S1}-CN f(1–23)	Bovine CN digested with chymosin	Gram-positive bacteria, fungi and yeast	Immunomodulatory	Lahov and Regelson (1996)
α_{S1}-CN f(99–109)	Bovine sodium caseinate digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	McCann et al. (2006)
α_{S1}-CN f(21–29) α_{S1}-CN f(30–38)	Bovine sodium caseinate fermented with *Lactobacillus acidophilus* DPC6026	Several Gram-positive and Gram-negative bacteria	N.R.	Hayes, Ross, Fitzgerald, Hill, and Stanton (2006)
α_{S2}-CN f(164–169) α_{S2}-CN f(183–207)	Bovine α_{S2}-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	Growth promoter	Recio and Visser (1999b) Smith, Wilkinson, and Liu (1997)
$^{O}\alpha_{S2}$-CN f(165–170) $^{O}\alpha_{S2}$-CN f(165–181) $^{O}\alpha_{S2}$-CN f(184–208) $^{O}\alpha_{S2}$-CN f(203–208)	Ovine α_{S2}-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	Antihypertensive Antioxidant	López-Expósito, Gómez-Ruiz, Amigo, and Recio (2006), Recio et al. (2006)
^{H}κ-CN f(43–97)	Human milk digested with pepsin	Several Gram-positive and Gram-negative bacteria, yeasts	N.R.	Liepke, Zucht, Frossmann, and Stöndker (2001)
κ-CN f(106–169)	Bovine CN digested with chymosin	*S. mutans* *P. gingivalis* *E. coli*	Bifidogenic Immunomodulatory	Malkoski et al. (2001) Prouxl, Gauthier, and Roy (1992) Brody (2000)
κ-CN f(18–24) κ-CN f(30–32) κ-CN f(139–146)	Bovine κ-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	López-Expósito, Minervini, Amigo, and Recio (2006)
^{H}β-CN f(184–210)	Human β-CN digested with a proteinase of *Lactobacillus helveticus* PR4	Several Gram-positive and Gram-negative bacteria	N.R.	Minervini et al. (2003)
LF f(17-41/42)	Bovine LF digested with pepsin or chymosin	Several Gram-positive and Gram-negative bacteria, viruses, fungi, parasites	Antitumoral Antiinflamatory	Bellamy et al. (1992) Hoek, Milne, Grieve, Dionoysius, and Smith (1997) ligo et al. (1999) Levay and Viljoen (1995)
^{H}LF f(1–47)	Human LF digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	Bellamy et al. (1992)
LF f(1–48) LF f(1–47) LF f(277–288) LF f(267–285) LF f(267–288)	Bovine LF digested with pepsin	*Micrococcus flavus*	N.R.	Recio and Visser (1999b)
^{C}LF f(14–42)	Caprine LF digested with pepsin	*Micrococcus flavus* *Escherichia coli*	N.R.	Recio and Visser (2000)
α-La f(1–5) α-La f(17–31)S-S(109–114) α-La f(61–68)S-S(75–80)	Bovine α-La digested with chymotrypsin	Several Gram-positive bacteria	N.R.	Pellegrini et al. (1999)
β-Lg f(15–20) β-Lg f(25–40) β-Lg f(78–83) β-Lg f(92–100)	Bovine β-Lg digested with trypsin	Several Gram-positive bacteria	N.R.	Pellegrini et al. (2001)
LZ f(98–112)	Hen egg white LZ digested with clostripain	Several Gram-positive and Gram-negative bacteria	N.R.	Pellegrini et al. (1997) Ibrahim, Thomas, and Pellegrini (2001)
LZ f(98–108) LZ f(15–21)	Hen egg white LZ digested with pepsin and trypsin	Gram-positive and Gram-negative bacteria	N.R.	Mine, Ma, and Lauriau (2004)
LZ (46–61)	Hen egg white LZ digested with papain and trypsin	Gram-positive and Gram-negative bacteria	N.R.	Memarpoor-Yazdi, Assodeh, and Chamani (2012)

[a] Fragments are bovine unless otherwise indicated; peptides obtained by chemical synthesis are not included. Adapted from López-Expósito and Recio (2008).
[b] Upper case superscripts define the origin of the precursor protein if not bovine: ^{O}, ovine; ^{H}, human; ^{C}, caprine origin.
[c] N.R., Non-reported.

WPI, and identified various active sequences derived from β-Lg and α-La (Theolier, Hammami, Labelle, Fliss, & Jean, 2013).

Although CN has been traditionally considered important from the nutritional point of view, it is also source of antibacterial peptides. Most relevant peptides from the CN fraction correspond to the N-terminal region of α_{S1}-CN, several fragments from the C-terminal domain of α_{S2}-CN and some peptides from κ-CN (Table 3). All of them are active against Gram-positive and -negative bacteria, and most of them have a net positive charge. More details about the formation and the *in vitro* activity of these sequences have been reviewed by López-Expósito & Recio (2008).

Because evidence of the activity of these antibacterial fragments should be based on *in vivo* studies, several animal assays and few clinical trials with some of these sequences or hydrolysates have been conducted. Most of the *in vivo* studies have used the entire protein LF, but the observed activity can be attributed to the protein or some of the generated peptides during digestion, such as LFcin-containing fragments (Kuwata, Yip, Tomita, & Hutchens, 1998; Kuwata, Yip, Yamauchi et al., 1998). *In vivo* studies with bovine LF orally administered to mice have demonstrated protective effects against infections or septic shock, although it is not clear whether these effects can be attributed to the antibacterial activity of the protein or to immunity modulation (reviewed by Legrand, 2012; Van Hooijdonk, Kussendrager, & Steijns, 2000). It has been demonstrated that orally administered LF or its hydrolysate can play a role on gastrointestinal health and intestinal flora of newborns (animals or humans). For instance, a recent clinical trial with 472 infants in neonatal care showed that LF reduced fungal late-onset sepsis, especially in low weight infants (Manzoni et al., 2012). Several clinical studies on the role of LF in children and neonates have been recently reviewed (Embleton, Berrington, McGuire, Stewart, & Cummings, 2013; Ochoa, Pezo, Cruz, Chea-Woo, & Cleary, 2012). Other studies with LF or hydrolysates thereof have shown their *in vivo* protective effect in urinary tract infection (Håversen et al., 2000), and hepatic colonisation of *L. monocytogenes* in mice (Lee et al., 2005).

The *in vivo* antibacterial activity of a α_{S1}-CN derived antibacterial fragment, isracidin, against *L. monocytogenes*, *Streptococcus pyogenes*, and *Staphylococcus aureus*, has been demonstrated in mice. Protection of other mammals (rabbits, guinea pigs and sheeps) against this latter microorganism has been also confirmed. Later on, a tryptic CN hydrolysate that exerts antimicrobial activity through stimulation of the bacterial autolytic system was assayed in newborn calves affected with colibacillosis. The hydrolysate acted as antimicrobial but also as immunostimulant and growth promoter (Biziulevičius, Žukaité, Normantienė, Biziulevičienė, & Arestov, 2003).

3.3.2. Immunomodulatory peptides

Milk contains several components, essential during neonatal development, that exhibit an important protective role against infection by modulating the immune system of the newborn. Among these components, it is found that CN, its fractions, whey proteins, LF, and their different hydrolysates show modulatory effects on the immune system in both *in vitro* and *in vivo* studies (Gauthier, Pouliot, & Saint-Sauveur, 2006; Gill, Doull, Rutherfurd, & Cross, 2000). However, most of these studies have been conducted by *in vitro* assays, focussing on the specific immune system, which requires an antigen-specific response (Cross & Gill, 2000).

It has been reported that whey protein and its peptide fractions, obtained from hydrolysis with trypsin and chymotrypsin, modulated the immune system when they were administered to mice (Saint-Sauveur, Gauthier, Boutin, Montoni, & Fliss, 2009). These authors showed an increase in serum immunoglobulin (Ig) A levels in non-infected and *E. coli* O157:H7 infected mice. In relation to whey proteins, it has been shown that α-La enhanced humoural immune response in mice, with this effect being higher than that shown for CN, soy or wheat diet (Bounous & Kongshavn, 1985; Bounous, Létourneau & Kongshavn, 1983; Bounous, Shenouda, Kongshavn, & Osmond, 1985). These results were confirmed when mice were fed α-La hydrolysate (Bounous, 1981; Bounous & Kongshavn, 1982).

LF is considered one of the main host defences against infections, displaying different biological activities including immunomodulation (González-Chávez, Erévalo-Gallegos, & Rascón-Cruz, 2009; Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002; Wakabayashi, Yamauchi, & Takase, 2006). Thus, the ingestion of LF accelerated the improvement of immune system in immunocompromised mice (Artym, Zimecki, Paprocka, & Kruzel, 2003; Takahura et al., 2004). In this sense, it was observed a higher IgA level in the bile and intestine of mice orally immunised with cholera toxin and fed a pepsin LF hydrolysate (Miyauchi, Kaino, Shinoda, Fukuwatari, & Hayasawa, 1997), indicating that this hydrolysate improved mucosal immunity. It has to be pointed out that IgA production is the main humoural immune response given by gut-associated lymphoid tissue, which prevents the entry of potentially harmful antigens that could enter through the oral route. Wang et al. (2000) showed that the administration of LF and its pepsin hydrolysate increased the mucosal immune response in mice, and this increase was higher in tumour bearing mice, suggesting an anti-metastasis and anti-carcinogenesis activity mediated by interleukine (IL)-18 production. In piglets, the ingestion of a LFcin-lactoferrampin mixture increased serum IgA, IgG and IgM levels, thus improving the immune function and gut health (Tang et al., 2009).

Several studies have shown that peptides released from milk fermented with *L. helveticus* R389 are able to improve the mucosal immune system in non-infected (LeBlanc, Matar, Valdéz, LeBlanc, & Perdigón, 2002; Vinderola, Matar, Palacios, & Perdigón, 2007; Vinderola, Matar, & Perdigón, 2007) or *E. coli* O157:H7 infected mice (LeBlanc, Fliss, & Matar, 2004), as well as to decrease the development of induced fibrosarcoma in mice (LeBlanc et al., 2002). On the other hand, a tryptic CN hydrolysate has also shown an immunostimulatory activity in newborn calf partly due to the capacity of this hydrolysate to stimulate the autolytic microbial system (Biziulevičius et al., 2003). Likewise, an immunomodulatory activity of CPPs was shown in mice fed ovalbumin-based diet supplemented with these peptides that was mediated by an increase of IgA levels in serum, intestine, and faeces towards β-Lg injected peritoneally or bacterial LPS of *Salmonella typhimurium* orally administered (Otani, Kihara, & Park, 2000; Otani, Nakano, & Kawahara, 2003). These authors attributed these effects to an increase in IL-5 and IL-6 levels (Otani et al., 2003). A previous study reported increased IgA level in human faeces after the ingestion of cake supplemented with CPPs (Kitamura & Otani, 2002), with this effect being higher in people that ingested dairy products with low frequency.

Despite all these studies where an immunomodulant activity of different hydrolysates obtained from milk proteins was observed, the peptides responsible for this activity remain unidentified. Thus, further studies intended to identify these sequences, elucidate their mechanism of action, and discover new bioactive sequences are needed.

3.3.3. Antiproliferative peptides

Currently, cancer is the second leading cause of mortality worldwide. It is well known that 90–95% of cancer cases are attributed to environment and lifestyle factors, including tobacco and alcohol consumption, diet, sun exposure, pollutants, infections, stress, obesity, and physical inactivity (Anand et al., 2008). Among these factors, dietary patterns and food components are closely

associated with several types of cancer, being responsible for the 35% of cancer deaths (Manson, 2003). However, in vitro and in vivo studies have revealed that a large number of food compounds could lower cancer risk and even sensitise tumour cells to anti-cancer therapies (De Kok, van Breda, & Manson, 2008).

In the last few years, food protein-derived peptides have become a group of nutraceuticals with demonstrated activity preventing the different stages of cancer, including initiation, promotion, and progression (De Mejia & Dia, 2010). Among the milk derived peptides, LFcin B is the most extensively studied. As shown in Table 4, LFcin has demonstrated, by cell culture experiments, a potent activity against different types of cancer cell lines, including breast, colon, fibrosarcoma, leukaemia, oral, and ovarian cancer cells, without harming normal lymphocytes, fibroblasts, endothelial, or epithelial cells (Furlong, Mader, & Hoskin, 2010). The selectivity of action of this peptide seems to be due to its strongly cationic character that allows it to interact with negatively charged structures on cancer cells, resulting in the destabilisation of cancer cell membranes (Hoskin & Ramamoorthy, 2008). However, the net neutral charge of healthy non-transformed cells keeps them spared. In addition to this mechanism of action related to its cationic nature, LFcin has been shown *in vitro* to act inducing apoptosis, arresting cell cycle, modulating gene expression, and preventing the angiogenesis (De Mejia & Dia, 2010).

In vivo evidence has revealed that subcutaneous administration of LFcin B resulted in a significant inhibition of tumour growth and liver and lung metastases of lymphoma and melanoma cells in spontaneous and experimental metastasis mice models (Yoo, Watanabe, Koike et al., 1997; Yoo, Watanabe, Watanabe et al., 1997). Moreover, these authors demonstrated the capacity of this peptide to reduce the number of tumour-induced blood vessels inhibiting tumour angiogenesis. Subcutaneous treatment of Meth A fibrosarcoma mice xenografts with LFcin B significantly inhibited tumour growth (Eliassen et al., 2002). Same effect was observed after treating established neuroblastoma xenografts with repeated injections of this peptide (Eliassen et al., 2006).

The ability of CPPs to bind calcium has been demonstrated to be responsible for the protective effect of these peptides from toxicity caused by calcium overload in differentiated intestinal cells, preventing their apoptosis (Perego et al., 2013). In addition, CPPs act against intestinal tumour HT-29 and AZ-97 cells inhibiting their proliferation and inducing programmed cell death through activation of voltage-activated calcium channels, which mediate the calcium flood according to the depolarisation state of the cell (Perego, Cosentino, Fiorilli, Tettamanti, & Ferraretto, 2012).

Opioid CN-derived peptides, β-casomorphin 7 and β-casomorphin 5, have also shown antiproliferative and cell cycle arresting activity on breast cancer cells (Hatzglou, Bakogeorgou, Hatzoglou, Martin, & Castanas, 1996; Maneckjee, Biswas, & Vonderhaar, 1990). It has been suggested that these effects are mediated by interaction with opioid receptors. Similarly, the interaction with specific opioid and somatostatin receptors present in the intestinal

Table 4
Effects of peptide lactoferricin against cancer demonstrated by cell culture experiments and animals models.

Type of cancer	Cell line/animal model	Effects/mechanisms of action	Reference
Breast cancer	MCF-7, T-47D, MDA-MB-435 cells	Cytotoxic activity/induction of apoptosis	Mader, Salsman, Conrad, and Hoskin (2005)
Colon carcinoma	C26 cells Caco2 cells Ultraviolet-irradiated Caco2 cells Colo-35 and HT-29 cells	Cytotoxic activity Reduction of cell proliferation/cell cycle arrest at S phase by down regulation of cyclin E1 Reduction of DNA damage Cytotoxic activity/induction of apoptosis	Eliassen et al. (2002) Freiburghaus, Janicke, Lindmark-Månsson, Oredsson, and Paulsson (2009) Freiburghaus, Lindmark-Månsson, Oredsson, and Paulsson (2012) Mader et al. (2005)
Fibrosarcoma	Meth A cells	Cytotoxic activity/tumour cell membrane disruption	Eliassen et al. (2002)
Leukaemia	THP-1 human monocytic leukaemic cells Jurkat T leukaemia cells	Induction of apoptosis mediated by production of intracellular reactive oxygen species (ROS) and activation of Ca^{2+}/Mg^{2+}-dependent endonucleases Induction of apoptosis mediated by production of ROS, activation of caspases-3 and -9, and sequential permeabilisation of the cell membrane Reduction of DNA methyltransferases expression	Yoo, Watanabe, Koike et al. (1997) Mader et al. (2005, 2007) Zhang and Liu (2010)
Lymphoma	Subcutaneous inoculation of A20 cell lymphomas in syngeneic mice Spontaneous metastasis models (L5178Y-ML25) in syngeneic mice Raji and Ramos Burkitt's B-lymphoma cells Deficient mice bearing B-lymphoma xenografts	Tumour necrosis and regression of the tumors/induction of long-term specific cellular immunity against the A20 lymphoma Inhibition of tumour metastasis Induction of apoptosis/DNA fragmentation, chromatin condensation, and nuclear disintegration Extension of survival of mice	Berge et al. (2010) Yoo, Watanabe, Watanabe et al. (1997) Furlong et al. (2010)
Melanoma	B16F10 cells Spontaneous metastasis models (B16-BL6 cells) in syngeneic mice	Cytotoxic activity Inhibition of tumour metastasis	Eliassen et al. (2002) Yoo, Watanabe, Watanabe et al. (1997)
Neuroblastoma	Human MYCN-amplified and non-MYCN amplified neuroblastoma cell lines Neuroblastoma xenografts in nude rats	Cytotoxic activity through destabilisation of the cytoplasmic membrane, and activation of caspases-6, -7 and -9 Reduction of the growth of neuroblastoma xenografts	Eliassen et al. (2006)
Oral cancer	Oral squamous cell carcinoma cell line SAS	Induction of apoptosis by cleavage of caspase-3 and poly-ADP ribose polymerase. Phosphorylation of extracellular signal-regulated kinase, and c-Jun N-terminal kinase/stress activated protein kinase	Sakai, Banno, Kato, Nozawa, and Kawaguchi (2005)
Ovarian cancer	Skov3 and Caov3	Cytotoxic activity/induction of apoptosis	Mader et al. (2005)

tract of mammals might be responsible for the antiproliferative effects on colon cancer cells demonstrated for these β-casomorphins and other milk-derived opioid peptides (Pepe, Tenore, Mastrocinque, Stusio, & Campiglia, 2013).

3.4. Effects on the nervous system

3.4.1. Relaxing peptides

The popular sedative and calming properties of milk can be attributed to various compounds including proteins and peptides released during digestion. In fact, α-casozepine (YLGYLEQLLR), a peptide derived from tryptic hydrolysis of α_{S1}-CN, with benzodiazepine-like activity has shown anticonvulsant and anxiolytic activities in rats (Miclo et al., 2001). However, the mechanism of action of the tryptic hydrolysate differs from that of diazepam. While diazepam induced a disinhibition state in rats, the tryptic hydrolysate did not display such a side effect (Violle et al., 2006) despite its affinity for gamma-aminobutyric acid (GABA) type A receptors. Specific linking of bovine α_{S1}-CN tryptic hydrolysate-derived peptides on GABA receptor is involved in anxiolysis, but not on that implied in memory-impairing effects (Messaoudi, Lalonde, Schroeder, & Desor, 2009). A fragment, corresponding to sequence α_{S1}-CN f(91–97) could be the responsible for the *in vivo* activity of α-casozepine (Cakir-Kiefer et al., 2011). Oral intake of an encapsulated α_{S1}-CN tryptic hydrolysate, containing this peptide, before a stressing situation, decreases the blood pressure increase induced by the stress. Moreover, the plasma cortisol concentration decreased in the treated subjects compared with those who have taken a placebo (Messaoudi, Lefranc-Millot, Desor, Demagny, & Bourdon, 2005). In another study, the oral intake of the encapsulated hydrolysate by female volunteers significantly reduced their digestive, cardiovascular, intellectual, emotional and social stress-related symptoms (Kim et al, 2007).

Moreover, it has been reported an anti-stress effect of LF in the neonatal maternal separation model (Takeuchi et al., 2003), and in an elevated plus-maze test combined with an electric foot-shock in adult rats (Kamemori, Takeuchi, Hayashida, & Harada, 2004).

3.4.2. Antinociceptive peptides

Pain receptors or nociceptors are nerve endings found in the tissues of various organs and systems, characterised by distinguishing securely and efficiently a harmless event from another. It has been reported that milk proteins such as LF reduces nociceptive activity mediated by μ-opioid receptor in several models of pain in rats (Hayashida, Takeuchi, Shimizu, Ando, & Harada, 2003a). Moreover, these authors have demonstrated that the analgesia induced by spinal administration of morphine was greatly potentiated by coadministration of LF (Hayashida, Takeuchi, Shimizu, Ando, & Harada, 2003b), and that LF may block the development of tolerance to morphine in mice, possibly via the selective activation of NO synthase (Tsuchiya et al., 2006). Raju, Kumar, Arutselvan, Thejomoorthy, and Puvanakrishan (2005) have found that PEEP1261, a tetrapeptide corresponding to f(39–42) of human LF possesses antinoceptive activity with optimal effect at 40 mg kg^{-1} body weight in both tail-flick model and acetic acid induced writhing in rats. Furthermore, it was observed that this peptide exhibited also antipyretic activity.

4. Future prospects

Beyond their well-known nutritional value, milk proteins may exhibit a plethora of biological activities that influence the growth, development and function of specific organs, metabolic responses to absorbed nutrients, and defence systems, among others. Many of these activities could be exerted by peptides released from parent protein during gastrointestinal digestion or food processing. The research area of bioactive peptides is only at its beginning and more sequences along with additional physiological effects will be discovered in the future. Most of the properties of milk bioactive peptides have been demonstrated by *in vitro* assays and/or animal model systems. However, data obtained from these studies are insufficient to demonstrate efficacy of those peptides in humans. Evaluation of the peptide doses and treatment durations, and elucidation of the molecular mechanisms of actions are still required for many bioactive peptides. So far, evidence for such effect exists only for a few proteins and peptides, and once the beneficial effect has been demonstrated in acute human intervention studies the results cannot be always translated to longitudinal effects. To generate evidence of these beneficial effects, further long-term human trials using sufficient number of subjects, controlled doses and formulations are needed. It has been proposed that discrepancies found in some clinical trials could be due to human diversity and phenotypic differences between individuals. In this context, future clinical trials of food bioactives could benefit from data on genotype, metabolomic profiles and proteomics or transcriptomics data of the volunteers. In these studies, there is also a need to identify and validate biomarkers that conclusively be related to a certain health benefit. It is expected that new biomarkers will be developed using novel approaches including functional genomics, food metabolomics, microbiomics and epigenetics, and by exploring markers in human studies based on foods and diets, and not just their individual ingredients. Bioavailability of food peptides is also crucial in these studies, since it is dependent of numerous factors such as the food matrix, food composition but also it may vary between individuals. Finally, it has to be taken into account that the pharmacological approach is not always applicable to food bioactives where the physiological effects are smaller. Scientific progress in the field must be targeted at a better understanding of how these food-derived peptides interact with the human body and can prevent the initiation, development or progression of risk factors for diet-related chronic diseases.

Acknowledgements

This work has received financial support from projects AGL2011-24643, Consolider Ingenio 2010 FUN-C-Food CSD2007-00063, PIE 201270E076, FEDER-INNTERCONECTA-GALICIA (ENVELEFUN), and FP7-SME-2012-315349 (FOFIND). The authors are participant in the FA1005COST Action INFOGEST on food digestion. B. H. -L. and S. F. -T. acknowledge CSIC and the Ministry of Economy and Competitiveness for their Ramón y Cajal contract and FPI fellowship, respectively. The authors thank B. Miralles for text revision.

References

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *Journal of Dairy Science, 81*, 3131–3138.

Aït-Oukhatar, N., Bouhallab, S., Arhan, P., Maubois, J. L., Drosdowsky, M., & Bouglé, D. (1999). Iron tissue storage and hemoglobin levels of deficient rats repleted with iron bound to the caseinophophopeptides 1-25 of β-casein. *Journal of Agricultural and Food Chemistry, 47*, 2786–2790.

Aït-Oukhatar, N., Bouhallab, S., Bureau, F., Arhan, P., Maubois, J. L., Drosdowsky, M. A., et al. (1997). Bioavailability of caseinophosphopeptide bound iron in the young rat. *Nutritional Biochemistry, 8*, 190–194.

Aït-Oukhatar, N., Pèrés, J. M., Bouhallab, S., Neuville, D., Bureau, F., Bouvard, G., et al. (2002). Bioavailability of caseinophopshopeptide-bound iron. *Journal of Laboratory and Clinical Medicine, 140*, 290–294.

Akhavan, T., Luhovyy, B. L., Brown, P. H., Cho, C. E., & Anderson, G. H. (2010). Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. *American Journal of Clinical Nutrition, 91*, 966–975.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., et al. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research, 25*, 2097–2116.

Anderson, G. H., Tecimer, S. N., Shah, D., & Zafar, T. A. (2004). Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. *Journal of Nutrition, 134*, 3011–3015.

Ani-Kibangou, B., Bouhallab, S., Mollé, D., Henry, G., Bureau, F., Neuville, D., et al. (2005). Improved absorption of caseinophosphopeptide-bound iron: role of alkaline phosphatase. *Journal of Nutritional Biochemistry, 16*, 398–401.

Artym, J., Zimecki, M., Paprocka, M., & Kruzel, M. L. (2003). Orally administered lactoferrin restores humoral immune response in immunocompromised mice. *Immunology Letters, 89*, 9–15.

Ballard, K. D., Bruno, R. S., Seip, R. L., Quann, E. E., Volk, B. M., Freidenreich, D. J., et al. (2009). Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: a randomized, placebo controlled trial. *Nutrition Journal, 8*. Article No 34.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochimica et Biophysica Acta, 1121*, 130–136.

Bennett, T., Desmond, A., Harrington, M., McDonagh, D., FitzGerald, R., Flynn, A., et al. (2000). The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *British Journal of Nutrition, 83*, 673–680.

Berge, B., Eliassen, L. T., Camilio, K. A., Bartnes, K., Sveinbjørnsson, B., & Rekdal, Ø. (2010). Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide. *Cancer Immunology and Immunotherapy, 59*, 1285–1294.

Berlutti, F., Pantanella, F., Natalizi, T., Frioni, A., Paesano, R., Polimeni, A., et al. (2011). Antiviral properties of lactoferrin. A natural immunity molecule. *Molecules, 16*, 6992–7018.

Bernstein, C. N., Fried, M., Krabshuis, J. H., Cohen, H., Eliakim, R., Fedail, S., et al. (2009). World gastroenterology organisation practice guidelines for the diagnosis and management of IBD in 2010. *Inflammatory Bowel Disease, 16*, 112–124.

Bharatam, P. V., Patel, D. S., Adane, L., Mittal, A., & Sundriyal, S. (2007). Modeling and informatics in designing anti-diabetic agents. *Current Pharmaceutical Design, 13*, 3518–3530.

Biziulevičius, G. A., Žukaitė, V., Normantienč, T., Biziulevičienè, G., & Arestov, I. G. (2003). Non-specific immunity-enhancing effects of tryptic casein hydrolysate versus Fermosorb for treatment/prophylaxis of newborn calf colibacillosis. *FEMS Immunology and Medical Microbiology, 39*, 155–161.

Bolscher, J., Nazmi, K., van Marle, J., van 't Hof, W., & Veerman, E. (2012). Chimerization of lactoferricin and lactoferrampin peptides strongly potentiates the killing activity against *Candida albicans*. *Biochemistry and Cell Biology, 90*, 378–388.

Bouhallab, S., Cinga, V., Aït-Oukhatar, N., Bureau, F., Neuville, D., Arhan, P., et al. (2002). Influence of various phosphopeptides of caseins on iron absorption. *Journal of Agricultural and Food Chemistry, 50*, 7127–7130.

Bounous, G. (1981). Influence of dietary lactalbumin hydrolysate on the immune system of mice and resistance to Salmonellosis. *Journal of Infectious Diseases, 144*, 281.

Bounous, G., & Kongshavn, P. A. L. (1982). Influence of dietary protein on the immune system of mice. *Journal of Nutrition, 112*, 1747–1755.

Bounous, G., & Kongshavn, P. A. L. (1985). Differential effect of dietary protein type on B-cell and T-cell immune response in mice. *Journal of Nutrition, 115*, 1403–1408.

Bounous, G., Létourneau, L., & Kongshavn, P. L. A. (1983). Influence of dietary protein type on the immune system of mice. *Journal of Nutrition, 113*, 1415–1421.

Bounous, G., Shenouda, N., Kongshavn, P. A. L., & Osmond, D. (1985). Mechanism of altered B-cell response induced by changes in dietary protein type in mice. *Journal of Nutrition, 115*, 1409–1417.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., et al. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy human. *American Journal of Clinical Nutrition, 97*, 1314–1323.

Brody, E. P. (2000). Biological activities of bovine glycomacropeptide. *British Journal of Nutrition, 84*, S39–S46.

Brommage, R., Juillerat, M. A., & Jost, R. (1991). Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Lait, 71*, 173–180.

Cakir-Kiefer, C., Le Roux, Y., Balandras, F., Trabalon, M., Dary, A., Laurent, F. M., et al. (2011). In vitro digestibility of α-casozepine, a benzodiazepine-like peptide from bovine casein, and biological activity of its main proteolytic fragment. *Journal of Agricultural and Food Chemistry, 59*, 4464–4472.

Castro, G. A., Carvalho, J. E., Tinti, S. V., Possenti, A., & Sgarbieri, V. C. (2010). Anti-ulcerogenic effect of a whey protein isolate and collagen hydrolysates against ethanol ulcerative lesions on oral administration to rats. *Journal of Medicinal Food, 13*, 83–90.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., et al. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie, 80*, 155–156.

Chaud, M. V., Izumi, C., Nahaal, Z., Shuhama, T., Pires Bianchi, M. L., & De Freitas, O. (2002). Iron derivates from casein hydrolysates as a potential source in the treatment of iron deficiency. *Journal of Agricultural and Food Chemistry, 50*, 871–877.

Chawla, R., Patil, G. R., & Singh, A. K. (2011). High hydrostatic pressure technology in dairy processing: a review. *Journal of Food Science and Technology, 48*, 260–268.

Chen, G. W., Tsai, J. S., & Pan, B. S. (2007). Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. *International Dairy Journal, 17*, 641–647.

Chung, H. Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A. Y., et al. (2009). Molecular inflammation: underpinnings of aging and age-related diseases. *Ageing Research Reviews, 8*, 18–30.

Cicero, A. F. G., Aubin, F., Azais-Braesco, V., & Borghi, C. (2013). Do the lactotripeptides isoleucine-proline-proline and valine-proline-proline reduce systolic blood pressure in European subjects? A meta-analysis of randomized controlled trials? *American Journal of Hypertension, 25*, 425–436.

Cicero, A. F. G., Gerocarni, B., Laghi, L., & Borghi, C. (2011). Blood pressure lowering effect of lactotripeptides assumed as functional foods: a meta-analysis of current available clinical trials. *Journal of Human Hypertension, 25*, 425–436.

Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., et al. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology – Gastrointestinal and Liver Physiology, 283*, G521–G528.

Contreras, M. M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal, 19*, 566–573.

Contreras, M. M., Sancho, M. A., Recio, I., & Mills, C. (2012). Absorption of casein antihypertensive peptides through an *in vitro* model of intestinal epithelium. *Food Digestion, 3*, 16–24.

Corfield, A. P., Myerscough, N., Longman, R., Sylvester, P., Arul, S., & Pignatelli, M. (2000). Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut, 47*, 589–594.

Cross, M. L., & Gill, H. S. (2000). Immunomodulatory properties of milk. *British Journal of Nutrition, 84*, S81–S89.

Da Cruz, A. G., de Assis Fonseca Faria, J., Isay Saad, S. M., Andre Bolini, H. M., Sant'Ana, A. S., et al. (2010). High pressure processing and pulsed electric fields: potential use in probiotic dairy foods processing. *Trends in Food Science and Technology, 21*, 483–493.

Daddaoua, A., Puerta, V., Zarzuelo, A., Suárez, M. D., Sánchez de Medina, F., & Martínez-Augustin, O. (2005). Bovine glycomacropeptide is anti-inflammatory in rats with hapten induced colitis. *Journal of Nutrition, 135*, 1164–1170.

De Kok, T. M., van Breda, S. G., & Manson, M. M. (2008). Mechanisms of combined action of different chemopreventive dietary compounds. *European Journal Nutrition, 47*, 51–59.

De Mejia, E. G., & Dia, V. P. (2010). The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Review, 29*, 511–528.

Demers-Mathieu, V., Gauthier, S. F., Britten, M., Fliss, I., Robitaille, G., & Jean, J. (2013a). Inhibition of *Listeria monocytogenes* growth in Cheddar cheese by an anionic peptides-enriched extract from whey proteins. *International Dairy Journal, 32*, 6–12.

Demers-Mathieu, V., Gauthier, S. F., Britten, M., Fliss, I., Roobitaille, G., & Jean, J. (2013b). Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration. *International Dairy Journal, 28*, 94–101.

Drucker, D. J., & Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet, 368*, 1696–1705.

EFSA. (2009). EFSA Panel on Dietetic Products Nutrition and Allergies (NDA). Scientific opinion on the substantion of health claims related to isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) and maintenance of normal blood pressure (ID 615, 661, 1831, 1832, 2891), and maintenance of the elastic properties of the arteries (ID 1832,) pursuant to Article 13(1) of regulation (EC) No 1924/2006 on request from the European Commission. *EFSA Journal, 7*, 1259–1277.

Eliassen, L. T., Berge, G., Leknessund, A., Wilkman, M., Lindin, I., Løkke, C., et al. (2006). The antimicrobial peptide, Lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth *in vivo*. *International Journal of Cancer, 119*, 493–500.

Eliassen, L. T., Berge, G., Sveinbjørnsson, B., Svendsen, J. S., Vorland, L. H., & Rekdal, Ø. (2002). Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Research, 22*, 2703–2710.

Embleton, N. D., Berrington, J. E., McGuire, W., Stewart, C. J., & Cummings, S. P. (2013). Lactoferrin: antimicrobial activity and therapeutic potential. *Seminars in Fetal and Neonatal Medicine, 18*, 143–149.

Engberink, M. F., Schouten, E. G., Kok, F. J., Van Mierlo, L. A. J., Brouwer, I. A., & Geleijnse, J. M. (2008). Lactotripeptides show no effect on human blood pressure: results from a double-blind randomized controlled trial. *Hypertension, 51*, 399–405.

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*, 643–654.

Espeche Turbay, M. B., de Moreno de LeBlanc, A., Perdigón, G., Savoy de Giori, G., & Hebert, E. M. (2012). β-casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. *Journal of Dairy Science, 95*, 1108–1118.

Espinoza, A. D., Morawicki, R. O., & Hager, T. (2012). Hydrolysis of whey protein isolate using subcritical water. *Journal of Food Science, 71*, C20–C26.

Fadini, G. P., & Avogaro, A. (2011). Cardiovascular effects of DPP-4 inhibition: beyond GLP-1. *Vascular Pharmacology, 55*, 10–16.

Faure, M., Mettraux, C., Moennoz, D., Godin, J. P., Vuichoud, J., Rochat, F., et al. (2006). Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *Journal of Nutrition, 136*, 1558–1564.

Fitzgerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *Journal of Nutrition, 134*, S980–S988.

Freiburghaus, C., Janicke, B., Lindmark-Månsson, H., Oredsson, S. M., & Paulsson, M. A. (2009). Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. *Journal of Dairy Science, 92*, 2477–2484.

Freiburghaus, C., Lindmark-Månsson, H., Oredsson, S. M., & Paulsson, M. A. (2012). Reduction of ultraviolet light-induced DNA damage in human colon cancer cells treated with a lactoferrin-derived peptide. *Journal of Dairy Science, 95*, 5552–5560.

Froetschel, M. A., Azain, M. J., Edwards, G. L., Barb, C. R., & Amos, H. E. (2001). Opioid and cholecystokinin antagonists alleviate gastric inhibition of food intake by premeal loads of casein in meal-fed rats. *Journal of Nutrition, 131*, 3270–3276.

Fromentin, G., Darcel, N., Chaumontet, C., Marsset-Baglieri, A., Nadkarni, N., & Tomé, D. (2012). Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutrition Research Reviews, 25*, 29–39.

Furlong, S. J., Mader, J. S., & Hoskin, D. W. (2010). Bovine lactoferricin induces caspase-independent apoptosis in human B-lymphoma cells and extends the survival of immune-deficient mice bearing B-lymphoma xenografts. *Experimental and Molecular Pathology, 88*, 371–375.

Gaudel, C., Nongonierma, A. B., Maher, S., Flynn, S., Krause, M., Murray, B., et al. (2013). A whey protein hydrolysate promotes insulinotropic activity in a clonal pancreatic beta-cell line and enhances glycemic function in ob/ob mice. *Journal of Nutrition, 143*, 1109–1114.

Gauthier, S. F., Pouliot, Y., & Saint-Sauveur, D. (2006). Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *International Dairy Journal, 16*, 1315–1323.

Gibson, P. R., & Muir, J. G. (2005). Reinforcing the mucus: a new therapeutic approach for ulcerative colitis? *Gut, 54*, 900–903.

Gilbert, J. A., Joanisse, D. R., Chaput, J. P., Miegueu, P., Cianflone, K., Almeras, N., et al. (2011). Milk supplementation facilitates appetite control in obese women during weight loss: a randomised, single-blind, placebo-controlled trial. *British Journal of Nutrition, 105*, 133–143.

Gill, H. S., Doull, F., Rutherfurd, K. J., & Cross, M. L. (2000). Immunoregulatory peptides in bovine milk. *British Journal of Nutrition, 84*, S111–S117.

Gómez-Ruiz, J. A., Ramos, M., & Recio, I. (2002). Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. *International Dairy Journal, 12*, 697–706.

Gómez-Ruiz, J. A., Ramos, M., & Recio, I. (2007). Identification of novel angiotensin converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques. *Electrophoresis, 28*, 4202–4211.

González-Chávez, S. A., Erévalo-Gallegos, S., & Rascón-Cruz, Q. (2009). Lactoferrin: structure, function and applications. *International Journal of Antimicrobial Agents, 33*, 301e1–301e8.

Gunnarsson, P. T., Winzell, M. S., Deacon, C. F., Larsen, M. O., Jelic, K., Carr, R. D., et al. (2006). Glucose-induced incretin hormone release and inactivation are differently modulated by oral fat and protein in mice. *Endocrinology, 147*, 3173–3180.

Hall, W. L., Millward, D. J., Long, S. J., & Morgan, L. M. (2003). Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *British Journal of Nutrition, 89*, 239–248.

Han, K. S., Deglaire, A., Sengupta, R., & Moughan, P. (2008). Hydrolyzed casein influences mucin gene expression in the rat. *Journal of Agricultural and Food Chemistry, 56*, 5572–5576.

Hansen, M., Sandström, B., & Lönnerdal, B. (1996). The effect of casein phosphopeptides on zinc and calcium absorption from high phytate infant diets assessed in rat pups and Caco-2 cells. *Pediatric Research, 40*, 547–552.

Hansen, M., Sandström, B., Jensen, M., & Sorensen, S. S. (1997a). Casein phosphopeptides improve zinc and calcium absorption from rice-based but not from whole-grain infant cereal. *Journal of Pediatric Gastroenterology and Nutrition, 24*, 56–62.

Hansen, M., Sandström, B., Jensen, M., & Sorensen, S. S. (1997b). Effect of casein phosphopeptides on zinc and calcium absorption from bread meals. *Journal of Trace Elements in Medicine and Biology, 11*, 143–149.

Hatzoglou, A., Bakogeorgou, E., Hatzoglou, C., Martin, P. M., & Castanas, E. (1996). Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line. *European Journal of Pharmacology, 310*, 217–223.

Håversen, L. A., Engberg, I., Baltzer, L., Dolphin, G., Hanson, L.Å., & Mattsby-Baltzer, I. (2000). Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary infection in mice. *Infection and Immunity, 68*, 5816–5823.

Hayashida, K., Takeuchi, T., Shimizu, H., Ando, K., & Harada, E. (2003a). Novel function of bovine milk derived lactoferrin on antinociception mediated by μ-opioid receptor in the rat spinal cord. *Brain Research, 965*, 239–245.

Hayashida, K., Takeuchi, T., Shimizu, H., Ando, K., & Harada, E. (2003b). Lactoferrin enhances opioid-mediated analgesia via nitric oxide in the rat spinal cord. *American Journal of Physiology, 285*, R306–R312.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Applied and Environmental Microbiology, 72*, 2260–2264.

Heany, R. P., Saito, Y., & Orimo, H. (1994). Effect of caseinophosphopeptide on absorbability of co-ingested calcium in normal postmenopausal women. *Journal of Bone and Mineral Metabolism, 12*, 77–81.

Henschen, A., Lottspeich, F., Brantl, V., & Teschemacher, H. (1979). Beta-casomorphins – exorphins derived from casein peptone. *Hoppe-Seylers Zeitschrift fur Physiologische Chemie, 360*, 1157–1158.

Hernández-Ledesma, B., Contreras, M. M., & Recio, I. (2011). Antihypertensive peptides: production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*, 23–35.

Hernández-Ledesma, B., Miguel, M., Amigo, L., Aleixandre, M. A., & Recio, I. (2007). Effect of simulated gastrointestinal digestion of the antihypertensive properties of β-lactoglobulin-derived peptides. *Journal of Dairy Research, 74*, 336–339.

Hernández-Ledesma, B., Recio, I., Ramos, M., & Amigo, L. (2002). Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. *International Dairy Journal, 12*, 805–812.

Hirayama, M., Toyota, K., Hidaka, H., & Naito, H. (1992). Phosphopeptides in rat intestinal digests after ingesting casein phosphopeptides. *Bioscience, Biotechnology and Biochemistry, 56*, 1128–1129.

Hoek, K., Milne, J. M., Grieve, P. A., Dionoysius, D. A., & Smith, R. (1997). Antibacterial activity of bovine lactoferrin-derived peptides. *Antimicrobial Agents and Chemotherapy, 41*, 54–59.

Hoskin, D. W., & Ramamoorthy, A. (2008). Studies on anticancer activities of antimicrobial peptides. *Biochimica et Biophysica Acta, 1778*, 357–385.

Hurrell, R. F., Lynch, S. R., Trinidad, T. P., Dassenko, S. A., & Cook, J. D. (1989). Iron absorption in humans as influenced by bovine milk proteins. *American Journal of Clinical Nutrition, 49*, 546–552.

Ibrahim, H. R., Thomas, U., & Pellegrini, A. (2001). A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *Journal of Biological Chemistry, 276*, 43767–43774.

Iigo, M., Kuhara, T., Ushida, Y., Sekine, K., Moore, M. A., & Tsuda, H. (1999). Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. *Clinical and Experimental Metastasis, 17*, 35–40.

Jiang, Z., Tian, B., Brodkorb, A., & Huo, G. (2010). Production, analysis and *in vivo* evaluation of novel angiotensin-I-converting enzyme inhibitory peptides from bovine casein. *Food Chemistry, 123*, 779–786.

Jones, K. W., Eller, L. K., Parnell, J. A., Doyle-Baker, P. K., Edwards, A. L., & Reimer, R. A. (2013). Effect of a dairy- and calcium-rich diet on weight loss and appetite during energy restriction in overweight and obese adults: a randomized trial. *European Journal of Clinical Nutrition, 67*, 371–376.

Kamemori, N., Takeuchi, T., Hayashida, K., & Harada, E. (2004). Suppressive effects of milk-derived lactoferrin on the psychological stress in adult rats. *Brain Research, 1029*, 34–40.

Karaki, H., Doi, K., Sugano, S., Uchiwa, H., Sugai, R., Murakami, U., et al. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comparative Biochemistry and Physiology. Part C. Pharmacology, Toxicology, and Endocrinology, 96*, 367–371.

Kasai, T., Iwasaki, R., Tanaka, M., & Kiriyama, S. (1995). Caseinphosphopeptides (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. *Bioscience, Biotechnology and Biochemistry, 59*, 26–30.

Keogh, J. B., Woonton, B. W., Taylor, C. M., Janakievski, F., Desilva, K., & Clifton, P. M. (2010). Effect of glycomacropeptide fractions on cholecystokinin and food intake. *British Journal of Nutrition, 104*, 286–290.

Kibangou, I. B., Bouhallab, S., Henry, G., Bureau, F., Allouche, S., Blais, A., et al. (2005). Milk proteins and iron absorption: contrasting effects of different caseinophosphopeptides. *Pediatric Research, 58*, 731–734.

Kim, J. H., Desor, D., Kim, Y. T., Yoon, W. J., Kim, K. S., Jun, J. S., et al. (2007). Efficacy of α_{S1}-casein hydrolysate on stress-related symptoms in women. *European Journal Clinical Nutrition, 61*, 536–541.

Kitamura, H., & Otani, H. (2002). Fecal IgA levels in healthy persons who ingested cakes with or without bovine casein phosphopeptides. *Milchwissenschaft, 57*, 11–12.

Kitts, D. D., Yuan, Y. V., Nagasawa, T., & Moriyama, Y. (1992). Effect of casein, casein phosphopeptides and calcium intake on ileal ^{45}Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. *British Journal of Nutrition, 68*, 765–781.

Kopra, N., Scholz-Ahrens, K., & Barth, C. A. (1992). Effect of casein phosphopeptides on utilization of calcium in vitamin D-replete and vitamin D-deficient rats. *Milchwissenschaft, 47*, 488–493.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*, 945–960.

Kosseva, M. R., Panesar, P. S., Kaur, G., & Kennedy, J. F. (2009). Use of immobilised biocatalysts in the processing of cheese whey. *International Journal of Biological Macromolecules, 45*, 437–447.

Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T., & Zilmer, M. (2003). Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects. *British Journal of Nutrition, 90*, 449–456.

Kuwata, H., Yip, T. T., Tomita, M., & Hutchens, T. W. (1998). Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochimica et Biophysica Acta, 1429*, 129–141.

Kuwata, H., Yip, T. T., Yamauchi, K., Teraguchi, S., Hayasawa, H., Tomita, M., et al. (1998). The survival of ingested lactoferrin in the gastrointestinal tract of adult mice. *Biochemistry Journal, 334*, 321–323.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. *Journal of Functional Foods, 4*, 403–422.

Lahov, E., & Regelson, W. (1996). Antibacterial and inmunostimulating casein-derived substances from milk: casecidin, isracidin peptides. *Federal Chemistry Toxicology, 34*, 131–145.

Lam, S. M. S. C. C., Moughan, P. J., Awati, A., & Morton, H. R. (2009). The influence of whey protein and glycomacropeptide on satiety in adult humans. *Physiology and Behavior, 96*, 162–168.

LeBlanc, J. G., Matar, C., Valdéz, J. C., LeBlanc, J., & Perdigón, G. (2002). Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. *Journal of Dairy Science, 85*, 2733–2742.

LeBlanc, J., Fliss, I., & Matar, C. (2004). Induction of a humoral immune response following an *Escherichia coli* O157:H7 infection with an immunomodulatory peptidic fraction derived from *Lactobaciluus helveticus*-fermented milk. *Clinical and Diagnostic Laboratory Immunology, 11*, 1171–1181.

Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, B. H., et al. (2005). Potencial antimicrobial effects of human lactoferrin against oral infection with *Listeria monocytogenes* in mice. *Journal of Medical Microbiology, 54*, 1049–1054.

Lee, Y. M., Skurk, T., Hennig, M., & Hauner, H. (2007). Effect of a milk drink supplemented with whey peptides on blood pressure in patients with mild hypertension. *European Journal of Nutrition, 46*, 21–27.

Lee, Y. S., Noguchi, T., & Naito, H. (1979). An enhanced intestinal absorption of calcium in the rat directly attributed to dietary casein. *Agricultural and Biological Chemistry, 43*, 2009–2011.

Lee, Y. S., Noguchi, T., & Naito, H. (1980). Phosphopeptides and soluble calcium in the intestine of rats given a casein diet. *British Journal of Nutrition, 43*, 457–467.

Lee, Y. S., Noguchi, T., & Naito, H. (1983). Intestinal absorption of calcium in rats given rats containing casein or amino acid mixture: the role of casein phosphopeptides. *British Journal of Nutrition, 49*, 67–76.

Legrand, D. (2012). Lactoferrin, a key molecule in immune and inflammatory processes. *Biochemistry and Cell Biology, 90*, 252–268.

Levay, P. F., & Viljoen, M. (1995). Lactoferrin, a general review. *Haematologica, 80*, 252–267.

Liepke, C., Zucht, H. D., Forssmann, W. G., & Ständker, L. (2001). Purification of novel peptide antibiotics from human milk. *Journal of Chromatography B, 752*, 369–377.

Linden, S. K., Sutton, P., Karlsson, N. G., Korolik, V., & McGuckin, M. A. (2008). Mucins in the mucosal barrier to infection. *Mucosal Immunology, 1*, 183–197.

López-Expósito, I., & Recio, I. (2008). Protective effect of milk peptides: antibacterial and antitumor properties. *Advances in Experimental Medicine and Biology, 606*, 271–293.

López-Expósito, I., Gómez-Ruiz, J. A., Amigo, L., & Recio, I. (2006). Identification of antibacterial peptides from ovine α_{S2}-casein. *International Dairy Journal, 16*, 1072–1080.

López-Expósito, I., Minervini, F., Amigo, L., & Recio, I. (2006). Identification of antibacterial peptides from bovine κ-casein. *Journal of Food Protection, 69*, 2992–2997.

López-Fandiño, R. (2006). High pressure-induced changes in milk proteins and possible applications in dairy technology. *International Dairy Journal, 16*, 1119–1131.

López-Huertas, E., Teucher, B., Boza, J. J., Martínez-Férez, A., Majsak-Newman, G., Baró, L., et al. (2006). Absorption of calcium from milks enriched with fructo-oligosacharides, caseinophosphopeptides, tricalcium phosphate, and milk solids. *American Journal of Clinical Nutrition, 83*, 310–316.

López-Posadas, R., Requena, P., González, R., Suárez, M. D., Zarzuelo, A., Sánchez de Medina, F., et al. (2010). Bovine glycomacropeptide has intestinal antiinflammatory effects in rats with dextran sulfate-induced colitis. *Journal of Nutrition, 140*, 2014–2019.

Luhovyy, B. L., Akhavan, T., & Anderson, G. H. (2007). Whey proteins in the regulation of food intake and satiety. *Journal of the American College of Nutrition, 26*, S704–S712.

Luo, S. J., & Wong, L. L. (2004). *Oral care confections and method of using*. US Patent, 6733818.

MacLeod, E. L., Clayton, M. K., van Calcar, S. C., & Ney, D. M. (2010). Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria. *Molecular Genetics and Metabolism, 100*, 303–308.

Madadlou, A., Sheehan, D., Emam-Djomeh, Z., & Mousavi, M. E. (2011). Ultrasound-assisted generation of ACE-inhibitory peptides from casein hydrolyzed with nanoencapsulated protease. *Journal of the Science of Food and Agriculture, 91*, 2112–2116.

Mader, J. S., Richardson, A., Salsman, J., Top, D., de Antueno, R., Duncan, R., et al. (2007). Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria. *Experimental Cell Research, 313*, 2634–2650.

Mader, J. S., Salsman, J., Conrad, D. M., & Hoskin, D. W. (2005). Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. *Molecular Cancer Therapeutics, 4*, 612–624.

Maeno, M., Yamamoto, N., & Takano, T. (1996). Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *Journal of Dairy Science, 79*, 1316–1321.

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., et al. (2001). Kappacin, a novel antimicrobial peptide from bovine milk. *Antimicrobial Agents and Chemotherapy, 45*, 2309–2315.

Maneckjee, R., Biswas, R., & Vonderhaar, B. K. (1990). Binding of opioides to human MCF-7 breast-cancer cells and their effects on growth. *Cancer Research, 50*, 2234–2238.

Manson, M. (2003). Cancer prevention – the potential for diet to modulate molecular signaling. *Trends in Molecular Medicine, 9*, 11–18.

Manzoni, P., Stolfi, I., Messner, H., Cattani, S., Laforgia, N., Romeo, M. G., et al. (2012). Bovine lactoferrin prevents invasive fungal infections in very low birth weight infants: a randomized controlled trial. *Pediatrics, 129*, 116–123.

Mao, X.-Y., Cheng, X., Wang, X., & Wu, S.-J. (2011). Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chemistry, 126*, 484–490.

Marqués, D., Pessela, B. C., Betancor, L., Monti, R., Carrascosa, A. V., Rocha-Martín, J., et al. (2011). Protein hydrolysis by immobilized and stabilized trypsin. *Biotechnology Progress, 27*, 677–683.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., & Recio, I. (2013a). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *International Dairy Journal, 32*, 13–19.

Martínez-Maqueda, D., Miralles, B., de Pascual-Teresa, S., Reverón, I., Muñoz, R., & Recio, I. (2012a). Food-derived peptides stimulate mucin secretion and gene expression in intestinal cells. *Journal of Agricultural and Food Chemistry, 60*, 8600–8605.

Martínez-Maqueda, D., Miralles, B., Ramos, M., & Recio, I. (2013b). Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells. *Food Research International, 54*, 1287–1291.

Martínez-Maqueda, D., Miralles, B., Recio, I., & Hernández-Ledesma, B. (2012b). Antihypertencive peptides from food proteins: a review. *Food and Function, 3*, 350–361.

Matsui, T., Okumura, H., & Yano, H. (2002). Absorption of zinc from dietary casein phosphopeptide complex with zinc in rats given a soybean protein-based diet. *Journal of Nutritional Science and Vitaminology, 48*, 247–250.

McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H., et al. (2006). Isolation and characterisation of a novel antibacterial peptide from bovine α_{s1}-casein. *International Dairy Journal, 16*, 316–323.

McDonagh, D., & Fitzgerald, R. J. (1998). Production of caseinophosphopeptides (CPPs) from sodium caseinate using a range of commercial protease preparations. *International Dairy Journal, 8*, 39–45.

Meisel, H., & Fitzgerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design, 9*, 1289–1295.

Meisel, H., & Frister, H. (1988). Chemical characterization of a casein-ophosphopeptide isolated from in vivo digests of a casein diet. *Biological Chemistry Hoppe-Seyler, 369*, 1275–1279.

Meisel, H., & Frister, H. (1989). Chemical characterization of bioactive peptides from *in vivo* digests of casein. *Journal of Dairy Research, 56*, 343–349.

Meisel, H., Bernard, H., Fairweather-Tait, S., Fitzgerald, R. J., Hartmann, R., Lane, C. N., et al. (2003). Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjets. *British Journal of Nutrition, 89*, 351–358.

Memarpoor-Yazdi, M., Asoodeh, A., & Chamani, J. (2012). A novel antioxidant and antimicrobial peptide from egg white lysozyme hydrolysates. *Journal of Functional Foods, 4*, 278–286.

Messaoudi, M., Lalonde, R., Schroeder, H., & Desor, D. (2009). Anxiolytic-like effects and safety profile of a tryptic hydrolysate from bovine α_{s1}-casein in rats. *Fundamental and Clinical Pharmacology, 23*, 323–330.

Messaoudi, M., Lefranc-Millot, C., Desor, D., Demagny, B., & Bourdon, L. (2005). Effects of a tryptic hydrolysate from bovine milk α_{S1}-casein on hemodynamic responses in healthy human volunteers facing successive mental and physical situations. *European Journal of Nutrition, 44*, 128–132.

Mezzaroba, L. F. H., Carvalho, J. E., Ponezi, A. N., Antônio, M. A., Monteiro, K. M., Possenti, A., et al. (2006). Antiulcerative properties of bovine α-lactalbumin. *International Dairy Journal, 16*, 1005–1012.

Miclo, L., Perrin, E., Driou, A., Papadopoulos, V., Boujrad, N., Vanderesse, R., et al. (2001). Characterization of α-casozepine, a tryptic peptide from bovine α_{S1}-casein with benzo diazepine-like activity. *FASEB Journal, 15*, 1780–1782.

Miguel, M., Gómez-Ruiz, J. A., Recio, I., & Aleixandre, A. (2010). Changes in arterial blood pressure after single oral administration of milk-casein derived peptides in spontaneously hypertensive rats. *Molecular Nutrition and Food Research, 54*, 1422–1427.

Miguel, M., Manso, M. A., López-Fandiño, R., Alonso, M. J., & Salaices, M. (2007). Vascular effects and antihypertensive properties of kappa-casein macropeptide. *International Dairy Journal, 17*, 1473–1477.

Miguel, M., Recio, I., Ramos, M., Delgado, M. A., & Aleixandre, A. (2006). Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats. *Journal of Dairy Science, 89*, 3352–3359.

Mine, Y., Ma, F. P., & Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *Journal of Agricultural and Food Chemistry, 52*, 1088–1094.

Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., & Gobetti, M. (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. *Applied and Environmental Microbiology, 69*, 5297–5305.

Miquel, E., & Farré, R. (2007). Effects and future trends of casein phosphopeptides on zinc bioavailability. *Trends in Food Science and Technology, 18*, 139–143.

Miyauchi, H., Kaino, A., Shinoda, I., Fukuwatari, Y., & Hayasawa, H. (1997). Immunomodulatory effect of bovine lactoferrin pepsin hydrolysate on murine splenocytes and Peyer's patch cells. *Journal of Dairy Science, 80*, 2330–2339.

Montagne, L., Piel, C., & Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: nutrition and health implications. *Nutrition Reviews, 62*, 105–114.
Mora-Gutierrez, A., Farrell, H. M., Attaie, R., McWhinney, V. J., & Wang, C. (2007). Influence of bovine and caprine casein phosphopeptides differing in α_{s1}-casein content in determining the absorption of calcium from bovine and caprine calcium-fortified milks in rats. *Journal of Dairy Research, 75*, 356–366.
Moughan, P. J., Fuller, M. F., Han, K. S., Kies, A. K., & Miner-Williams, W. (2007). Food-derived bioactive peptides influence gut function. *International Journal of Sport Nutrition and Exercise Metabolism, 17*, S5–S22.
Mykkänen, H. M., & Wasserman, R. H. (1980). Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *Journal of Nutrition, 110*, 2141–2148.
Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., et al. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochemical and Biophysical Research Communications, 281*, 11–17.
Nagaoka, S., Kanamaru, Y., & Kuzuya, Y. (1991). Effects of whey protein and casein on the plasma and liver lipids in rats. *Agricultural and Biological Chemistry, 55*, 813–818.
Nagaoka, S., Kanamaru, Y., Kuzuya, Y., Kojima, T., & Kuwata, T. (1992). Comparative studies on the serum cholesterol lowering action of the whey protein and soybean protein in rats. *Bioscience, Biotechnology and Biochemistry, 56*, 1484–1485.
Naito, H., & Suzuki, H. (1972). In vivo formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. *Agricultural and Biological Chemistry, 36*, 409–415.
Naito, H., & Suzuki, H. (1974). Further evidence for the formation in vivo of phosphopeptide in the intestinal lumen from dietary β-casein. *Agricultural and Biological Chemistry, 38*, 1543–1545.
Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of antiotensin-I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*, 777–783.
Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors of angiotensing I-converting enzyme. *Journal of Dairy Science, 78*, 1253–1257.
Narva, M., Kärkkäinen, M., Poussa, T., Lamberg-Allardt, C., & Korpela, R. (2003). Caseinphosphopeptides in milk and fermented milk do not affect calcium metabolism acutely in postmenopausal women. *Journal of the American College Nutrition, 22*, 88–93.
Nauck, M. A., Kleine, N., Ørskov, C., Holst, J. J., Willms, B., & Creutzfeldt, C. (1993). Normalization of fasting hyperglycemia by exogenous-1 (7–36 amide) in type 2 GLP diabetic patients. *Diabetologia, 36*, 741–744.
Nilsson, M., Holst, J. J., & Bjorck, I. M. (2007). Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose equivalent drinks. *American Journal of Clinical Nutrition, 85*, 996–1004.
Nongonierma, A. B., & Fitzgerald, R. J. (2013). Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides, 39*, 157–163.
Nurminen, M. L., Sipola, M., Kaarto, H., Pihlanto-Leppälä, A., Piilola, K., Korpela, R., et al. (2000). Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. *Life Science, 66*, 1535–1543.
Ochoa, T. J., Pezo, A., Cruz, K., Chea-Woo, E., & Cleary, T. G. (2012). Clinical studies of lactoferrin in children. *Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire, 90*, 457–467.
Otani, H., Kihara, Y., & Park, M. (2000). The immunoenhancing property of a dietary casein phosphopeptide preparation inn mice. *Food and Agricultural Immunology, 12*, 165–173.
Otani, H., Nakano, K., & Kawahara, T. (2003). Stimulatory effect of a dietary casein phosphopeptide preparation on the mucosal IgA response of mice to orally ingested lipopolysaccharide from *Salmonella typhimurium*. *Bioscience, Biotechnology and Biochemistry, 67*, 729–735.
Pal, S., & Ellis, V. (2010). The chronic effects of whey proteins on blood pressure, vascular function, and inflammatory markers in overweight individuals. *Obesity (Silver Spring), 18*, 1354–1359.
Pal, S., & Ellis, V. (2011). Acute effects of whey protein isolate on blood pressure, vascular function and inflammatory markers in overweight postmenopausal women. *British Journal of Nutrition, 105*, 1512–1519.
Pan, Y., Lee, A., Wan, J., Coventry, M. J., Michalski, W. P., Shiell, B., et al. (2006). Antiviral properties of milk proteins and peptides. *International Dairy Journal, 16*, 1252–1261.
Pellegrini, A., Dettling, C., Thomas, U., & Hunziker, P. (2001). Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochimica et Biophysica Acta, 1526*, 131–140.
Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., & Von Fellenberg, R. (1999). Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochimica et Biophysica Acta, 1426*, 439–448.
Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Humziker, P., & von Fellenberg, R. (1997). Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *Journal of Applied Microbiology, 82*, 372–378.
Pepe, G., Tenore, G. C., Mastrocinque, R., Stusio, P., & Campiglia, P. (2013). Potential anticarcinogenic peptides from bovine milk. *Journal of Amino Acids, 2013*, 939804.
Perego, S., Cosentino, S., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2012). Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with voltage-operated L-type calcium channels. *Journal of Nutritional Biochemistry, 23*, 808–816.
Perego, S., Zabeo, A., Marasco, E., Giussani, P., Fiorilli, A., Tettamanti, G., et al. (2013). Casein phosphopeptides modulate calcium uptake and apoptosis in Caco2 cells through their interaction with the TRPV6 calcium channel. *Journal of Functional Foods, 5*, 847–857.
Pérès, J. M., Bouhallab, S., Bureau, F., Maubois, J. L., Arhan, P., & Bouglé, D. (1997). Absorption digestive du fer lié au caséinophosphopeptide 1-25 de la β-caséine. *Lait, 77*, 433–440.
Pérès, J. M., Bouhallab, S., Bureau, F., Maubois, J. L., Arhan, P., & Bouglé, D. (1999). Reduction of iron/zinc interactions using metal bound to the casein-ophospeptide 1-25 of β-casein. *Nutrition Research, 19*, 1655–1663.
Pérès, J. M., Bouhallab, S., Bureau, F., Neuville, D., Maubois, J. L., Devroede, G., et al. (1999). Mechanisms of absorption of caseinophosphopeptide bound iron. *Journal of Nutritional Biochemistry, 10*, 215–222.
Phelan, M., Aherne, A., Fitzgerald, R. J., & O'Brien, N. M. (2009). Casein-derived bioactive peptides: biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal, 19*, 643–654.
Picariello, G., Iacomino, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., et al. (2013). Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food Chemistry, 139*, 203–212.
Piccolomini, A. F., Iskandar, M. M., Lands, L. C., & Kubow, S. (2012). High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food and Nutrition Research, 56*. Article No 17549.
Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*, 1306–1314.
Pins, J. J., & Keenan, M. D. (2006). Effects of whey peptides on cardiovascular disease risk factors. *Journal of Clinical Hypertension, 8*, 775–782.
Plaisancié, P., Claustre, J., Estienne, M., Henry, G., Boutrou, R., Paquet, A., et al. (2013). A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of globet cells and Paneth cells along the small intestine. *Journal of Nutritional Biochemistry, 24*, 213–221.
Pointillart, A., & Guéguen, L. (1989). Absence d'effect de l'incorporation d'un phosphopeptide du lait sur l'utlisation du calcium et du phosphore chez le jeune porc. *Reproduction, Nutrition, Development, 29*, 477–486.
Power, O., Jakeman, P., & Fitzgerald, R. J. (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids, 44*, 797–820.
Pripp, A. H. (2008). Effect of peptides derived from food proteins on blood pressure: a meta-analysis of randomized controlled trials. *Food and Nutrition Research, 52*, 1–9.
Prouxl, M., Gauthier, S. F., & Roy, D. (1992). Effect of casein hydrolysates on the growth of Bifidobacteria. *Lait, 72*, 393–404.
Puknun, A., Bolscher, J. G. M., Nazmi, K., Veerman, E. C. I., Tungpradabkul, S., Wongratanacheewin, S., et al. (2013). A heterodimer comprised of two bovine lactoferrin antimicrobial peptides exhibits powerful bactericidal activity against *Burkholderia pseudomallei*. *World Journal of Microbiology and Biotechnology, 29*, 1217–1224.
Pupovac, J., & Anderson, G. H. (2002). Dietary peptides induce satiety via cholecystokinin-A and peripheral opioid receptors in rats. *Journal of Nutrition, 132*, 2775–2780.
Qin, L.-Q., Xu, J.-Y., Dong, J.-Y., Zhao, Y., van Bladeren, P., & Zhang, W. (2013). Lactotripeptides intake and blood pressure management: a meta-analysis of randomized controlled clinical trials. *Nutrition, Metabolism & Cardiovascular Diseases, 23*, 395–402.
Quiros, A., Davalos, A., Lasuncion, M. A., Ramos, M., & Recio, I. (2008). Bioavailability of the antihypertensive peptide LHLPLP: transepithelial flux of HLPLP. *International Dairy Journal, 18*, 279–286.
Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., et al. (2007). Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*. *International Dairy Journal, 17*, 33–41.
Raju, K. V. S. N., Kumar, D. A., Arutselvan, N., Thejomoorthy, P., & Puvanakrishan, R. (2005). Antinociceptive and antipyretie effects of derivatized tetrapeptide from lactoferrinin rats. *Peptides, 26*, 615–619.
Recio, I., & Visser, S. (1999a). Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin. In situ enzymatic hydrolysis on an ion-exchange membrane. *Journal of Chromatography A, 831*, 191–201.
Recio, I., & Visser, S. (1999b). Identification of two distinct antibacterial domains within the sequence of bovine α_{S2}-casein. *Biochimica et Biophysica Acta, 1428*, 314–326.
Recio, I., & Visser, S. (2000). Antibacterial bing characteristics of bovine, ovine, caprine lactoferrins: a comparative study. *International Dairy Journal, 10*, 597–605.
Recio, I., Contreras, M. M., Gomez-Sala, B., Vázquez, C., Fernández-Escribano, H., & del Campo, R. (2011). Effect of a casein hydrolysate containing novel peptides in hypertensive subjects. *Annals of Nutrition and Metabolism, 58*, 16–17.
Recio, I., Quirós, A., Hernández-Ledesma, B., Gómez-Ruiz, J. A., Miguel, M., Amigo, L., et al. (2006). *Bioactive peptides in enzymatic hydrolysates milk caseins and method of obtaining same*. International WO Patent, 131586.
Requena, P., Daddaoua, A., Martínez-Plata, E., González, M., Zarzuelo, A., Suarez, M. D., et al. (2008). Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17. *British Journal of Pharmacology, 154*, 825–832.

Reynolds, E. C., Cai, F., Shen, P., & Walker, G. D. (2003). Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. *Journal of Dental Research, 82*, 206–211.
Rocha, C., Gonçalves, M. P., & Teixeira, J. A. (2011). Immobilization of trypsin on spent grains for whey protein hydrolysis. *Process Biochemistry, 46*, 505–511.
Rodríguez-Figueroa, J. C., González-Córdova, A. F., Astiazaran-García, H., & Vallejo-Cordoba, B. (2013). Hypotensive and heart rate-lowering effects in rats receiving milk fermented by specific *Lactococcus lactis* strains. *British Journal of Nutrition, 109*, 827–833.
Rodríguez-Figueroa, J. C., González-Córdova, A. F., Torres-Llanez, M. J., García, H. S., & Vallejo-Cordoba, B. (2012). Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild *Lactococcus lactis* strains. *Journal of Dairy Science, 95*, 5536–5543.
Ruiz-Giménez, P., Ibáñez, A., Salom, J. B., Marcos, J. F., López-Díez, J. J., Vallés, S., et al. (2010). Antihypertensive properties of lactoferricin B-derived peptides. *Journal of Agricultural and Food Chemistry, 58*, 6721–6727.
Saint-Sauveur, D., Gauthier, S. F., Boutin, Y., Montoni, A., & Fliss, I. (2009). Effect of feeding whey peptide fractions on the immune response in the healthy and *Escherichia coli* infected mice. *International Dairy Journal, 19*, 537–544.
Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *Journal of Dairy Science, 83*, 1434–1440.
Saito, Y., Lee, Y. S., & Kimura, S. (1998). Minimum effective dose of casein phosphopeptides (CPP) for enhancement of calcium absorption in growing rats. *International Journal for Vitamin and Nutrition Research, 68*, 335–340.
Sakai, T., Banno, Y., Kato, Y., Nozawa, Y., & Kawaguchi, M. (2005). Pepsin-digested bovine lactoferrin induces apoptotic cell death with JNK/SAPK activation in oral cancer cells. *Journal of Pharmacological Science, 98*, 41–48.
Sánchez de Medina, F., Daddaoua, A., Requena, P., Capitán-Cañadas, F., Zarzuelo, A., Suárez, M. D., et al. (2010). New insights into the immunological effects of food bioactive peptides in animal models of intestinal inflammation. *Proceedings of the Nutrition Society, 69*, 454–462.
Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides, 31*, 1949–1956.
Sato, R., Noguchi, T., & Naito, H. (1983). The necessity for the phosphate protion of casein molecules to enhance Ca absorption from the small intestine. *Agricultural and Biological Chemistry, 47*, 2415–2417.
Sato, R., Noguchi, T., & Naito, H. (1986). Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *Journal of Nutritional Science and Vitaminology, 32*, 67–76.
Sato, R., Shindo, M., Gunshin, H., Noguchi, T., & Naito, H. (1991). Characterization of phosphopeptide derived from bovine β-casein – an inhibitor to intra-intestinal precipitation of calcium-phosphate. *Biochimica et Biophysica Acta, 1077*, 413–415.
Schiffer, M., Chang, C. H., & Stevens, F. J. (1992). The functions of tryptophan residues in membrane proteins. *Protein Engineering, 5*, 213–214.
Scholz-Ahrens, K. E., Kopra, N., & Barth, C. A. (1990). Effect of casein phosphopeptides on utilization of calcium in minipigs and vitamin-D-deficient rats. *Zeitschrift für Ernährunswissenschaft, 29*, 295–298.
Shimizu, M., & Hachimura, S. (2011). Gut as target for functional food. *Trends in Food Science and Technology, 22*, 646–650.
Silveira, S. T., Martínez-Maqueda, D., Recio, I., & Hernández-Ledesma, B. (2013). Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. *Food Chemistry, 141*, 1072–1077.
Siriwardhana, N., Kalupahanab, N. S., Cekanovac, M., LeMieuxa, M., Greerd, B., & Moustaid-Moussa, N. (2013). Modulation of adipose tissue inflammation by bioactive food compounds. *Journal of Nutritional Biochemistry, 24*, 613–623.
Smith, J. A., Wilkinson, M. C., & Liu, Q. M. (1997). *Casein fragments having growth promoting activity*. International patent WO 1997016460 A1.
Solah, V. A., Kerr, D. A., Adikara, C. D., Meng, X. Q., Binns, C. W., Zhu, K., et al. (2010). Differences in satiety effects of alginate- and whey protein-based foods. *Appetite, 54*, 485–491.
Sousa, G. T. D., Lira, F. S., Rosa, J. C., de Oliveira, E. P., Oyama, L. M., et al. (2012). Dietary whey protein lessens several risk factors for metabolic diseases: a review. *Lipids in Health and Disease, 11*, 67–75.
Sprong, R. C., Schonewille, A. J., & van der Meer, R. (2010). Dietary cheese whey protein protects rats against mild dextran sulfate sodium-induced colitis: role of mucin and microbiota. *Journal of Dairy Science, 93*, 1364–1371.
Takahura, N., Wakabayashi, H., Ishibashi, H., Yamauchi, K., Teraguchi, S., Tamura, Y., et al. (2004). Effect of orally administered bovine lactoferrin on the immune response in the oral candidiasis murine model. *Journal of Medical Microbiology, 54*, 495–500.
Takeuchi, T., Hayashida, K., Inagaki, H., Kuwahara, M., Tsubone, H., & Harada, E. (2003). Opioid mediated suppressive effect of milk-derived lactoferrin on distress induced by maternal separation in the rat pups. *Brain Research, 979*, 216–224.
Tang, Z., Yin, Y., Zhang, Y., Huang, R., Sun, Z., Li, T., et al. (2009). Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin-lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. *British Journal of Nutrition, 101*, 998–1005.
Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design, 9*, 1331–1344.
Teucher, B., Majsak-Newman, G., Dainty, J. R., McDonagh, D., Fitzgerald, R. J., & Fairweather-Tait, S. J. (2006). Calcium absorption is not increased by casein-ophosphopeptides. *American Journal of Clinical Nutrition, 84*, 162–166.
Theolier, J., Hammami, R., Labelle, P., Fliss, I., & Jean, J. (2013). Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. *Journal of Functional Foods, 5*, 706–714.
Toedebusch, R. G., Childs, T. E., Hamilton, S. R., Crowley, J. R., Booth, F. W., & Roberts, M. D. (2012). Postprandial leucine and insulin responses and toxicological effects of a novel whey protein hydrolysate-based supplement in rats. *Journal of the International Society of Sports Nutrition, 9*, 24–33.
Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S., & Hayasawa, H. (2002). Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology, 80*, 109–112.
Tompa, G., Laine, A., Pihlanto, A., Korhonen, H., Rogelj, I., & Marnilab, P. (2010). Chemiluminescence of non-differentiated THP-1 promonocytes: developing an assay for screening anti-inflammatory milk proteins and peptides. *The Journal of Biological and Chemical Luminiscence, 26*, 251–258.
Tonouchi, H., Suzuki, M., Uchida, M., & Oda, M. (2008). Antihypertensive effect of an angiotensin converting enzyme inhibitory peptide from enzyme modified cheese. *Journal of Dairy Research, 75*, 284–290.
Trompette, A., Claustre, J., Caillon, F., Jourdan, G., Chayvialle, J. A., & Plaisancié, P. (2003). Milk bioactive peptides and β-casomorphins induce mucus release in rat jejunum. *Journal of Nutrition, 133*, 3499–3503.
Troncon Rosa, F., Zulet, M. A., Marchini, J. S., & Martínez, J. A. (2012). Bioactive compounds with effects on inflammation markers in humans. *International Journal of Food Sciences and Nutrition, 63*, 749–765.
Tsai, J. S., Chen, T. J., Pan, B. S., Gong, S. D., & Chung, M. Y. (2008). Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk. *Food Chemistry, 106*, 552–558.
Tsuchita, H., Goto, T., Yonehara, Y., & Kuwata, T. (1995). Calcium and phosphorus availability from casein phosphopeptides in male growing rats. *Nutrition Research, 15*, 1657–1667.
Tsuchita, H., Sekiguchi, I., Kuwata, T., Igarashi, C., & Ezawa, I. (1993). The effect of casein phosphopeptides on calcium utilization in young ovariectomized rats. *Zeitschrift für Ernährunswissenschaft, 32*, 121–130.
Tsuchita, H., Suzuki, T., & Kuwata, T. (2001). The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *British Journal of Nutrition, 85*, 5–10.
Tsuchiya, T., Takeuchi, T., Hayashida, K., Shimizu, H., Ando, K., & Harada, E. (2006). Milk-derived lactoferrin may block tolerance to morphine analgesia. *Brain Research, 1068*, 102–108.
Tulipano, G., Sibilia, V., Caroli, A. M., & Cocchi, D. (2011). Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides, 32*, 835–838.
Uchida, M., Ohshiba, Y., & Mogami, O. (2011). Novel dipeptidyl peptidase-4-inhibiting peptide derived from b-lactoglobulin. *Journal of Pharmacological Sciences, 117*, 63–66.
Uenishi, H., Kabuki, T., Seto, Y., Serizawa, A., & Nakajima, H. (2012). Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-IV)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats. *International Dairy Journal, 22*, 24–30.
Ushida, Y., Shimokawa, Y., Toida, T., Matsui, H., & Takase, M. (2007). Bovine α-lactalbumin stimulates mucus metabolism in gastric mucosa. *Journal of Dairy Science, 90*, 541–546.
Usinger, L., Jensen, L. T., Flambard, B., Linnneberg, A., & Ibsen, H. (2010). The anti-hypertensive effect of fermented milk in individuals with prehypertension or borderline hypertension. *Journal of Human Hypertension, 24*, 678–683.
Van der Kraan, M. I. A., Groenink, J., Nazmi, K., Veerman, E. C. I., Bolscher, J. G. M., & Nieuw Amerongen, A. V. (2004). Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. *Peptides, 25*, 177–183.
Van der Sluis, M., De Koning, B. A., De Bruijn, A. C., Velcich, A., Meijerink, J. P., Van Goudoever, J. B., et al. (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology, 131*, 117–129.
Van Hooijdonk, A. C. M., Kussendrager, K. D., & Steijns, J. M. (2000). In vivo antimicrobial and antiviral activity of components in bovine milk and colostrums involved in non-specific defence. *British Journal of Nutrition, 84*, 127–134.
Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., et al. (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science, 295*, 1726–1729.
Vinderola, G., Matar, C., Palacios, J., & Perdigón, G. (2007). Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *Lactobacillus helveticus* R389. *International Journal of Food Microbiology, 115*, 180–186.
Vinderola, G., Matar, C., & Perdigón, G. (2007). Milk fermentation products of *L. helveticus* R389 active calineurin as a signal to promote gut mucosal immunity. *BMC Immunology, 8*, 19–28.
Violle, N., Messaoudi, M., Lefranc-Millot, C., Desor, D., Nejdi, A., Demagny, B., et al. (2006). Ethological comparison of the effects of a bovine α_{S1}-casein tryptic hydrolysate and diazepam on the behaviour of rats in two models of anxiety. *Pharmacology, Biochemistry and Behaviour, 84*, 517–523.
Wakabayashi, H., Yamauchi, K., & Takase, M. (2006). Lactoferrin research, technology and applications. *International Dairy Journal, 16*, 1241–1251.
Wang, W.-P., Iigo, M., Sato, J., Sekine, K., Adachi, I., et al. (2000). Activation of intestinal mucosal immunity in tumor-bearing mice by lactoferrin. *Japanese Journal of Cancer Research, 91*, 1022–1027.

Welderufael, F. T., Gibson, T., & Jauregi, P. (2012). Production of angiotensin-I-converting enzyme inhibitory peptides from β-lactoglobulin and casein-derived peptides: an integrative approach. *Biotechnology Progress, 28*, 746–755.

Wu, S., Qi, W., Li, T., Lu, D., Su, R., & He, Z. (2013). Simultaneous production of multi-functional peptides by pancreatic hydrolysis of bovine casein in an enzymatic membrane reactor via combinational chromatography. *Food Chemistry, 141*, 2944–2951.

Xu, J. Y., Qin, L. Q., Wang, P. Y., Li, W., & Chang, C. (2008). Effect of milk tripeptides on blood pressure: a meta-analysis of randomized controlled trials. *Nutrition, 24*, 933–940.

Yamauchi, R., Ohinata, K., & Yoshikawa, M. (2003). β-Lactotensin and neurotensin rapidly reduce serum cholesterol via NT_2 receptor. *Peptides, 24*, 1955–1961.

Yoo, Y. C., Watanabe, R., Koike, Y., Mitobe, M., Shimazaki, K.-I., et al. (1997). Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species. *Biochemical and Biophysical Research Communications, 237*, 624–628.

Yoo, Y. C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., & Azuma, I. (1997). Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Japanese Journal of Cancer Research, 88*, 184–190.

Yu, B. P., & Chung, H. Y. (2006). The inflammatory process in aging. *Reviews in Clinical Gerontology, 16*, 179–187.

Yuan, Y. V., & Kitts, D. D. (1991). Confirmation of calcium absorption and femoral utilization in spontaneously hypertensive rats fed casein phosphopeptide supplemented diets. *Nutrition Research, 11*, 1257–1272.

Zhang, T.-N., & Liu, N. (2010). Effect of bovine lactoferricin on DNA methyltransferase 1 levels in Jurkat T-leukemia cells. *Journal of Dairy Science, 93*, 3925–3930.

Zhao, W., Xu, G., Yang, R., & Katiyo, W. (2013). Preparation of casein phosphopeptides using a novel continuous process of combining an enzymatic membrane reactor with anion-exchange chromatography. *Journal of Food Engineering, 117*, 105–112.

Zimmermann, M. B., & Hurrell, R. F. (2007). Nutritional iron deficiency. *Lancet, 370*, 511–520.

Zoghbi, S., Trompette, A., Claustre, J., El Homsi, M., Garzón, J., Jourdan, G., et al. (2006). β-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a μ-opioid pathway. *American Journal of Physiology – Gastrointestinal and Liver Physiology, 290*, G1105–G1113.

Zommara, M., Tougo, H., Sakanao, M., & Imaizumi, K. (1998). Prevention of peroxidative stress in rats fed on a low vitamin E containing diet by supplementing with a fermented bovine milk whey preparation: effect of lactic acid and β-lactoglobulin on the antiperoxidative action. *Bioscience, Biotechnology and Biochemistry, 62*, 710–717.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 146840, 16 pages
http://dx.doi.org/10.1155/2015/146840

Review Article

Milk Proteins, Peptides, and Oligosaccharides: Effects against the 21st Century Disorders

Chia-Chien Hsieh,[1] Blanca Hernández-Ledesma,[2] Samuel Fernández-Tomé,[2] Valerie Weinborn,[3,4] Daniela Barile,[3,4] and Juliana María Leite Nobrega de Moura Bell[3,4]

[1]*Department of Human Development and Family Studies (Nutritional Science & Education), National Taiwan Normal University, No.162, Section 1, Heping East Road, Taipei 106, Taiwan*
[2]*Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain*
[3]*Food Science Department, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA*
[4]*Foods for Health Institute, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA*

Correspondence should be addressed to Blanca Hernández-Ledesma; b.hernandez@csic.es

Received 21 October 2014; Accepted 21 December 2014

Academic Editor: Jinsong Ren

Milk is the most complete food for mammals, as it supplies all the energy and nutrients needed for the proper growth and development of the neonate. Milk is a source of many bioactive components, which not only help meeting the nutritional requirements of the consumers, but also play a relevant role in preventing various disorders. Milk-derived proteins and peptides have the potential to act as coadjuvants in conventional therapies, addressing cardiovascular diseases, metabolic disorders, intestinal health, and chemopreventive properties. In addition to being a source of proteins and peptides, milk contains complex oligosaccharides that possess important functions related to the newborn's development and health. Some of the health benefits attributed to milk oligosaccharides include prebiotic probifidogenic effects, antiadherence of pathogenic bacteria, and immunomodulation. This review focuses on recent findings demonstrating the biological activities of milk peptides, proteins, and oligosaccharides towards the prevention of diseases of the 21st century. Processing challenges hindering large-scale production and commercialization of those bioactive compounds have been also addressed.

1. Introduction: Role of Milk in Human Health

Milk, as the first food for mammals, supplies all the energy and nutrients needed for the proper growth and development of the neonate. For all mammalians, the consumption of milk ends at the weaning period with the exception of humans that continue consuming milk throughout their life. Milk and derived dairy products are considered an important constituent of a balanced diet. Moreover, it is a source of many bioactive components, such as high-quality proteins, lipids, carbohydrates, lactose, vitamins, minerals, enzymes, hormones, immunoglobulins, and growth factors, among others. These components not only help meeting human nutritional requirements, but also play a relevant role in preventing various disorders such as hypertension and cardiovascular diseases [1], obesity [2], osteoporosis [3], dental caries [4], poor gastrointestinal health [5], colorectal cancer [6], ageing [7], and others [8].

Milk proteins supply nitrogen and amino acids to young mammals and possess multiple physiological properties in the intact form. Moreover, studies carried out in the past decades have demonstrated the role of these proteins as a source of biologically active peptides. Bioactive peptides are inactive within the sequence of the parent protein but, once released by *in vitro* processing conditions or by *in vivo* gastrointestinal digestion, are capable of acting as regulatory compounds exerting a positive impact on body functions and ultimately promoting health benefits to the consumer [9].

Human milk is undoubtedly the most complete source of nourishment for the newborn. Breastfed infants have been

Table 1: Commercial milk products containing peptides with proven antihypertensive activity.

Commercial name	Obtention process	Protein source	Active sequence(s)	Publication number [reference]
Peptide C12	Hydrolysis with trypsin	α_{s1}-Casein	FFVAPFPEVFGK	JP62270533 [31]
Biozate	Hydrolysis with trypsin	Whey proteins	Whey peptides	US6998259 [157]
Lowpept	Hydrolysis with pepsin	α_{s1}-Casein	RYLGY, AYFYPEL	WO2005012355 [158]
Calpis	Fermentation	β-Casein	VPP, IPP	US5449661A [37, 38]
Evolus	Fermentation	β-Casein	VPP, IPP	US6972282 [159]

shown to be less susceptible to diseases (i.e., diarrhea and respiratory diseases) than those that were not breastfed. This protective effect, which was previously attributed to human milk antibodies, is today strongly correlated with the presence of complex oligosaccharides (OS), the third most abundant component of human milk [10]. Human milk is composed of OS in concentrations varying according to different stages of lactation: 20–23 g/L in colostrum and 12–14 g/L in mature milk [11], being even more abundant than proteins (12 g/L) [12]. Human milk oligosaccharides (HMO) are complex sugars having 3 to 20 monosaccharide units [13] that are not digestible by human enzymes [14]. These compounds have important functions related to the newborn's development and health at local and systemic levels, including prebiotic probifidogenic effects and antiadherence of pathogenic bacteria [15], brain development [16], and immunomodulatory properties [17], among others.

In the last fifty years, chronic disorders have become the leading cause of morbidity and mortality in industrialized countries, with increasing incidence also observed in developing countries. Chronic disorders include cardiovascular and neurological diseases, stroke, cancers, immune disorder and chronic respiratory disease, obesity, diabetes, and metabolic syndrome [18]. In Europe, 87% of all deaths occur due to chronic diseases and the number of people affected is expected to rise considerably over the next few decades. The majority of chronic diseases are caused by risk factors which are mostly preventable. Diet and lifestyle are two environmental factors that strongly affect these diseases; thus modifications of these habits are becoming a new strategy for disease prevention/treatment.

The aim of this paper is to review the recent literature on the physiological effects of proteins, peptides, and oligosaccharides with special emphasis on animal and human trials. Other aspects such as the limited availability of *in vivo* studies demonstrating the biological activities of OS from bovine and caprine milk and the current challenges associated with the recovery and commercial production of these compounds have also been addressed.

2. Impact of Milk Proteins and Peptides on the 21st Century Diseases

2.1. Milk-Derived Peptides against Cardiovascular Diseases. Cardiovascular diseases (CVD) have become the leading cause of morbidity and mortality worldwide, representing an important medical and public health issue [19]. Although earlier studies associated the consumption of whole milk with higher incidence of CVD, it has been demonstrated that milk contains a plethora of bioactive substances which may contribute to the prevention of most of the risk factors of CVD [20]. Recently, bioactive milk peptides have gained interest because of their notable antihypertensive, antioxidant, anti-inflammatory, and hypocholesterolaemic effects. In this section, the most current scientific information regarding *in vitro* and *in vivo* studies on the role of milk proteins-derived peptides on CVD is summarized and discussed.

2.1.1. Milk Peptides with Antihypertensive Activity. Epidemiological studies suggest that the dietary intake of milk and dairy foods is related to decreased risk of hypertension [21]. In addition to their high mineral content (e.g., calcium, potassium, and magnesium) that can lower blood pressure [22], other milk components, such as proteins and their hydrolyzed products, have been also linked to the antihypertensive effect of milk and dairy products. Angiotensin-converting enzyme (ACE) is a multifunctional enzyme that acts as one of the main regulators of blood pressure. Thus, ACE inhibition is currently considered as one of the best strategies for hypertension treatment. Most biologically active peptides generated from milk proteins have demonstrated ACE inhibitory activity. In the last two decades, antihypertensive effects of some of these peptides have been evaluated in spontaneously hypertensive rats (SHR) and hypertensive humans, and the peptide sequences, doses, and maximum decreases of systolic blood pressure (SBP) have been summarized in several reviews [23–25]. The hydrolyzate obtained by the action of pepsin on casein, containing the α_{s1}-casein-derived peptides RYLGY and AYFYPEL, has been patented and commercialized under the name of Lowpept by its antihypertensive properties demonstrated in both SHR [26] and hypertensive humans [27] (Table 1). Pepsin has been also used to hydrolyze whey protein lactoferrin, with the release of peptides containing ACE activity and ACE-dependent vasoconstriction inhibitory properties [28]. Antihypertensive effects in SHR after short-term and long-term treatments have been also observed for those peptides [29, 30]. Trypsin is another gastrointestinal enzyme used to release the antihypertensive peptide α_{s1}-casein peptide f(23–34) from casein during the manufacture of the commercial

ingredient peptide C12 [31, 32] (Table 1). In addition to the use of gastric and pancreatic enzymes, alone or in combination, to produce antihypertensive peptides, the use of food-grade enzymes derived from microorganisms has become common for the release of peptides with demonstrated SBP lowering effects in SHR [33–36].

Milk fermentation is another strategy to produce antihypertensive peptides by the proteolytic action of lactic acid bacteria on milk proteins. The most representative peptides are those derived from β-casein and identified in sour milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Calpis, Table 1). These tripeptides, with sequences VPP and IPP, have demonstrated an ability to exert potent decreasing effects on the SBP of SHR [37, 38]. A number of clinical trials have been conducted to confirm their antihypertensive properties in humans although controversial results have been found. Three meta-analyses performed with the published data of 17 [39], 12 [40], and 28 [41] clinical trials have reported an average decrease in SBP of 5.1, 4.8 mm, and 1.7 mm of Hg, respectively. However, no effects were found in Dutch and Danish subjects consuming fermented milk containing peptides VPP and IPP [42, 43]. A recent meta-analysis including 18 trials has reported higher antihypertensive effects for these two tripeptides in Asian than in Caucasian people [44]. Those findings suggest that genetics and/or dietary patterns might exert an important influence on the antihypertensive effects of peptides IPP and VPP. Similarly, the age has been described as another major influencing factor [45]. With the evidence presented to date, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) [46] concluded that there are no sufficient data to establish a cause/effect relationship between the consumption of peptides VPP and IPP and the control of hypertension, and further studies are thus required. Other peptides derived from β-casein during milk fermentation with *Enterococcus faecalis*, in which sequences are LHLPLP and HLPLP, have also shown antihypertensive effects in SHR [47]. In recent studies, fermented milk with *Lactococcus lactis* NRRLB-50571 and NRRLB-50572 has presented important SBP, diastolic blood pressure (DBP), and heart rate-lowering effects in SHR [48, 49] although the peptides responsible for the activity have not been identified.

Accumulating evidence built in animal and clinical studies is currently available on the antihypertensive activity of milk-derived peptides. However, much work is still needed. Identification of the active form reaching the target organs and elucidation of its bioavailability after oral ingestion and its complete mechanism of action are two of the main aspects required to be deeply investigated in the future to support health claims.

2.1.2. Antioxidant and Anti-Inflammatory Milk-Derived Peptides. Oxidative stress is one of the main responsible factors for the initiation or evolution of CVD. The search of natural antioxidants providing additional benefits to the endogenous antioxidant defense system is gaining interest [50]. Among food-derived peptides with antioxidant properties without harm side effects, those derived from milk proteins are most frequently studied. The majority of the studies carried out to characterize antioxidant peptides derived from casein and whey proteins have only used *in vitro* chemical assays [51, 52]. However, their limited similarity to physiological conditions makes the *in vitro* assays very restrictive, and reported effects need to be confirmed by animal models and/or human trials. Nevertheless, to date, just few *in vivo* trials have been carried out to demonstrate the antioxidant effects of milk-derived peptides related to benefits on cardiovascular health. Zommara et al. [53] reported the antiperoxidative action of fermented milk on rats fed a vitamin-E deficient diet. The consumption of fermented milk by healthy subjects has been also demonstrated to lower the levels of oxidized low-density lipoprotein, isoprostanes, and the glutathione redox ratio. Improvements of total plasma antioxidant activity and of the resistance of the lipoprotein fraction to oxidation have resulted in enhanced antiatherogenicity [54]. The compounds responsible for the observed effects have not been identified yet, although milk peptides liberated during fermentation process might have a crucial role. Thus, further studies focused on evaluating the potential of milk-derived peptides as antioxidant at cardiovascular level should be of great relevance.

Chronic inflammation is another responsible factor for the development of CVD. The downregulation of cytokines involved in the inflammation-associated endothelial dysfunction by food components, including peptides, may delay or alleviate inflammation, thus exerting favorable effects against CVD [55]. A recent study using lipopolysaccharide-(LPS-) stimulated mouse macrophages has reported the ability of a yak casein hydrolyzate to reduce the secretion of proinflammatory cytokines and the production of nitric oxide and to scavenge free radicals, suggesting a potential role as preventive agent against inflammation related disorders [56]. To date, only one human trial has been conducted to demonstrate the anti-inflammatory properties of milk peptides. This study reported an improvement in the vascular function through modulation of the glucose levels and inflammation and oxidative stress biomarkers after the consumption of the commercial whey derived peptide NOP-47 by healthy individuals [57]. This finding opens a new door towards searching of new milk-derived peptides with antioxidant and anti-inflammatory activity.

2.1.3. Hypocholesterolaemic Milk Peptides. Blood lipids are represented in various forms including total cholesterol, triglycerides, lipoproteins (high-density lipoproteins or HDL, low-density lipoproteins or LDL, and very-low-density lipoproteins or VLDL), and free fatty acids. An inappropriate ratio of these lipids is one of the most important risk factors for developing CVD. Therefore, CVD therapy/prevention strategies focus on reaching an optimal lipid balance in order to achieve a positive cardiovascular health. Those therapies aim at increasing the physiological levels of desirable lipids (e.g., HDL cholesterol) while reducing the others associated with atherogenic functions (e.g., LDL cholesterol, triglycerides). Milk proteins, mainly whey proteins

and derived hydrolyzates or peptides, have been reported to exert hypocholesterolaemic effects in different animal models. The ingestion of whey protein was correlated with a significant reduction of total cholesterol levels in rats fed with cholesterol-free and cholesterol-enriched diets [58, 59]. Nagaoka et al. [60] have reported similar effects for a β-lactoglobulin tryptic hydrolyzate administered to rats fed with a diet rich in cholesterol. The hydrolyzate reduced total cholesterol and increased HDL cholesterol and fecal steroid excretion. The fragment f(71–75) of this whey protein, known as lactostatin, with sequence IIAEK, has been reported as the main factor responsible for the observed effects [60]. β-Lactotensin, another β-lactoglobulin peptide, released by chymotrypsin hydrolysis, decreased total cholesterol, LDL, and VLDL cholesterol content in mice fed with a cholesterol-enriched diet [61]. Although the mechanism of action of those peptides has not been completely elucidated, preliminary results suggest a key role played by the amino acid composition [50]. Further studies are clearly needed to corroborate those results. The exact mode of this hypocholesterolaemic action needs to be determined in clinical trials.

2.2. Milk-Derived Hydrolyzates and Peptides on Intestinal Health. The gastrointestinal tract (GIT) serves as a specialized interface between the body and the external environment. The GIT is strategically covered by a monolayer of specially designed epithelial cells continually exposed to a high concentration of food components and substances along the gut luminal surface. Hence, the modulator effect of the diet on GIT functions has been accepted as essential for maintaining and improving the general health of the host [62]. Interestingly, more than 70% of the current "food for specified health uses products" (FOSHU) are related to GIT functions [63].

Dairy proteins, hydrolyzates, and peptides have been demonstrated to transform the dynamics of mucus mainly via influencing the mucin secretion and expression and the number of goblet cells. In *ex vivo* preparations of rat jejunum, casein hydrolyzates increased mucin secretion [64, 65]. The β-casein derived peptide β-casomorphin 7 produced the same effects which have been suggested to be mediated by interaction with opioid receptors. Also, this peptide has been reported to stimulate the expression of mucin *Muc2* and *Muc3* genes in rat intestinal DHE cells and *MUC5AC* gene in human intestinal HT29-MTX cells [66]. Another β-casein fragment, f(94–123), identified in commercial yoghurt, also had the ability to increase the mucin output and the mRNA levels of *MUC2* and *MUC4* genes in HT29-MTX cells [67]. Casein and whey proteins hydrolyzates have been reported to be a source of peptides with capacity to induce mucin secretion and *MUC5AC* gene expression in HT29-MTX cells [68]. Among these peptides, the α_{s1}-casein fragments f(143–149) and f(144–149) and the β-lactoglobulin fragment f(102–105) known as β-lactorphin were suggested as the major peptides responsible for the observed effects.

A few *in vivo* studies have also pointed out the regulation of the protective mucus layer by dairy proteins and products thereof. Rats fed with a diet based on casein hydrolyzates, as the exclusive source of nitrogen, were found to enhance their endogenous nitrogen flow and expression of mucin genes *Muc3* and *Muc4* in the small intestine and colon, respectively [69]. Plaisancié et al. [67] reported the capacity of the β-casein fragment f(94–123), once orally ingested by rats, to upregulate the *Muc2, Muc4, rat defensing 5* and *lysozyme* mRNA transcripts expression, the goblet cells recounts, and the number of crypts containing Paneth cells in the rat small intestine. In the dextran sulphate sodium- (DSS-) induced model of rat colitis, the studies of Sprong et al. [70] and Faure et al. [71] demonstrated the gut-protective effects exerted by a cheese whey protein diet and a diet supplemented with Thr, Ser, Cys, and Pro residues, respectively. Moreover, this protection has been reported for a whey protein isolate and α-lactalbumin hydrolyzate against chemical-induced ulcerative gastric lesions [72, 73].

Enhancement of the mucosal immune response is also a dietary modulating strategy of the defense systems protecting the GIT. Animal models have proved the improvement of the mucosal immunity by promotion of gut-related immunoglobulin (Ig) levels after ingestion of lactoferrin or its derived peptides, lactoferricin and lactoferrampin [74, 75]. Likewise, immunomodulatory effects have been reported for a trypsin casein hydrolyzate in newborn calves [76] and casein phosphopeptides (CPPs) and peptides released from *Lactobacillus helveticus* R389-fermented milk in mice [77, 78]. Furthermore, Kitamura and Otani [79] demonstrated that ingestion by healthy humans of CPPs-enriched cakes induced an increase in the faecal IgA content, suggesting a positive effect on mucosal immunity.

Oxidative and inflammatory imbalances are both involved in the etiology of several human chronic gut-related disorders such as ulcerative colitis and Crohn's disease. The search of natural preventive treatments against these imbalances is being prompted [80, 81]. Whey protein has been suggested to exert beneficial effects through enhancement of antioxidant enzymes and downregulation of both oxidative markers and proinflammatory cytokines [82]. These protective findings were found in animal [83, 84] and humans trials [85, 86]. The whey-derived peptide caseinomacropeptide has been proven to have protective properties in the 2,4,6-trinitrobenzene sulphonic acid (TNBS) and DSS-induced model of rat ileitis and colitis, through immunomodulation of the regulatory T helper cells activation and interleukin secretions [87, 88]. Turbay et al. [89] demonstrated, in the TNBS-induced murine colitis model, the anti-inflammatory effects exerted by β-casein hydrolyzates generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581. However, peptides released and responsible for the observed bioactivity have not been identified yet.

2.3. Milk Proteins and Peptides against Metabolic Disorders. Diabetes mellitus is considered one of the most common metabolic disorders and one of the major health problems worldwide. It affects almost 6% of the world's population, with type 2 diabetes representing approximately 90–95% of the diagnosed cases [90]. Diet and lifestyle interventions

are the preferred treatment strategies for this metabolic disorder, with pharmacotherapy being prescribed only if supervised lifestyle intervention fails [91]. Epidemiological evidence supports that consumption of milk and dairy foods is associated with a lower incidence of type 2 diabetes. These beneficial effects on metabolic and inflammation factors linked to diabetes and insulin resistance have been also demonstrated by cell and animal models, being multiple milk components, such as calcium, medium-chain fatty acids, linoleic conjugated acid, lactose, citrate, proteins, and peptides characterized as the main responsible factors for the observed effects acting through different mechanisms of action [92].

During the ingestion of a meal, the presence of nutrients at gastrointestinal level stimulates the secretion of two incretins hormones, the glucagon-like peptide-1 (GLP-1) and the glucose-dependent insulinotropic polypeptide (GIP). Both hormones are implicated in the stimulation of the insulin secretion from the pancreatic β-cells, secretion of gastric and pancreatic enzymes, and modulation of gut motility and nutrient absorption, allowing the clearance of the absorbed glucose [93]. Type 2 diabetes is characterized by different disorders including progressive dysfunction of pancreatic cells, insulin resistance, and augmented production of hepatic glucose [94]. Continuous intravenous administration of GLP-1 has been demonstrated to normalize blood glucose levels in diabetic subjects [95]. However, the rapid degradation of this hormone by the enzyme dipeptidyl peptidase-IV (DPP-IV) and its consequent inactivation makes this type 2 diabetes treatment strategy impracticable. Currently, specific DPP-IV inhibitors are thus incorporated to GLP-1 analogues in new oral therapies against this metabolic disease [96].

Diet supplementation with whey protein is currently under preclinical and clinical trials as a promising alternative in the prevention and/or treatment of type 2 diabetes and related diseases [97, 98]. Several mechanisms of action have been suggested for whey protein, including the stimulation of insulin release, improvement of glucose tolerance in diabetic patients, reduction of body weight, and modulation of gut hormones such as cholecystokinin, leptin, and GLP-1 [99]. In the last years, the role of peptides released during the transit of whey proteins through the GIT on the observed effects has been hypothesized [100]. Cell culture and animal models have been used to confirm this hypothesis. A dose-dependent insulinotropic activity of whey protein hydrolyzates has been observed in a cell-based coculture using pancreatic BRIN-BD11 cells and Caco-2 cells monolayers [101]. These authors also observed that the oral administration of the hydrolyzates to obese mice evoked an improvement of blood glucose clearance, reduction of hyperinsulinemia, and restoration of the pancreatic capacity to secrete insulin in response to glucose. The main mechanism of action suggested for these hydrolyzates is the DPP-IV inhibitory activity exerted by the peptides contained in them [102]. Among the bioactive peptides described to date, sequences derived from β-lactoglobulin IPA and IPAVF are the most potent as DPP-IV inhibitors [103, 104]. Another β-lactoglobulin fragment with sequence VAGTWY has been also demonstrated to exert hypoglycemic effects in the oral glucose tolerance test in mice [105]. Likewise, both *in vitro* DPP-IV inhibitory and *in vivo* hypoglycemic effects have been reported for peptides released from caseins [106]. Recent *in silico* studies have shown that both caseins and whey proteins might serve as precursors of DPP-IV inhibitory peptides because of the high number of fragments contained within them that match DPP-IV inhibitory sequences [107, 108]. Thus, this research area holds a great potential, and currently a number of investigations are focused on the identification of new milk proteins-derived peptide with capacity to prevent diabetes and associated metabolic syndromes.

2.4. Chemopreventive Role of Milk Proteins and Peptides. Cancer is the second leading cause of mortality worldwide, and its incidence will continue rising in the next few years in spite of the important advances achieved in the development of cancer therapies. It has been estimated that, by 2020, approximately 15 million new cancer cases will be diagnosed, and 12 million cancer patients will die [109]. It is well known that 35% of cancer deaths are attributed to diet and its food components [110]. However, cell culture and animal and human trials results have shown that an important number of food constituents can lower cancer risk and even sensitize tumor cells against anticancer therapies [111]. In the last few years, food proteins and derived peptides have become one of the food components with the most promising preventive properties against cancer initiation, promotion, and progression stages [112].

Among the milk proteins, lactoferrin and its derived peptide lactoferricin are the most studied. For both compounds, their antioxidant, immunomodulatory, and anti-inflammatory activities are closely linked to their protective effects against cancer (Table 2). Lactoferrin acts by inducing apoptosis, inhibiting angiogenesis, and modulating carcinogen metabolizing enzymes, in addition to its antioxidant and immunomodulatory properties [113]. Moreover, lactoferricin has shown potent anticancer properties in different cell lines, including breast, colon, fibrosarcoma, leukemia, and oral and ovarian cancer cells, without harming normal lymphocytes, fibroblasts, or endothelial or epithelial cells [114]. Also, animal models have confirmed the beneficial properties of this milk-derived peptide. The possible mechanism of bovine lactoferricin in anticarcinogenesis has been shown to be related to its ability to induce apoptosis. It is its strongly cationic nature that allows this peptide to target negatively charged cancer cells with the outer membrane [115]. The suppressed ability in angiogenesis of bovine lactoferricin was *in vitro* and *in vivo* demonstrated to contribute to its chemopreventive properties [116]. A significant inhibition of tumor growth and of liver and lung metastasis was reported after subcutaneous administration of bovine lactoferricin in both spontaneous and experimental metastasis mice models [117]. Similar results were observed after subcutaneous treatment and repeated injections of this peptide on Meth A fibrosarcoma xenografts mice and established neuroblastoma xenografts, respectively [118, 119].

α-Lactalbumin is a whey protein with anticancer properties which has been reported when it forms a complex with

Table 2: Chemopreventive properties of lactoferrin and its derived peptide lactoferricin against cancer demonstrated by cell culture experiments and animals models.

Type of cancer	Animal species/protein-peptide	Cell line/animal model	Effects/mechanisms of action	Reference
Breast cancer	Human lactoferrin	MDA-MB-231 cells	Inhibition of cell growth Cell cycle arrest	[160]
	Bovine lactoferrin	4T1 xenograft Balb/c mice	Improvement of tamoxifen chemopreventive effects Downregulation of proinflammatory cytokines	[161]
	Bovine lactoferrin-oleic acid complex	MCF-7 cells	Inhibition of proliferation Induction of apoptosis	[162]
	Bovine lactoferricin	MCF-7, T-47D, and MDA-MB-435 cells	Cytotoxic activity Induction of apoptosis	[163]
Colon cancer	Camel lactoferrin	HCT-116 cells	Inhibition of cell proliferation Antioxidant activity Inhibition of DNA damage	[164]
	Bovine lactoferrin	Caco-2 xenograft mouse model	Inhibition of tumor growth	[165]
	Bovine lactoferrin-oleic acid complex	HT-29 cells	Inhibition of proliferation Induction of apoptosis	[166]
		C26 cells	Cytotoxic activity	[118]
		Caco-2 cells	Inhibition of cell proliferation Cell cycle arrest by downregulation of cyclin E1	[166]
	Bovine lactoferricin	Ultraviolet-irradiated Caco-2 cells	Reduction of DNA damage Cell cycle arrest by downregulation of cyclin E1	[167]
		Colo-35 and HT-29 cells	Cytotoxic activity/induction of apoptosis	[163]
Cervical cancer	Bovine lactoferrin	HeLa cells	Inhibition of cell growth Induction of nuclear accumulation of Smad-2	[168]
Fibrosarcoma	Bovine lactoferricin	Meth A cells	Cytotoxic activity Tumor cell membrane disruption	[118]
Head and neck cancer	Human lactoferrin	Squamous carcinoma O12 tumor bearing mice	Reduction of tumor Immunomodulatory effects	[169]
Hepatocarcinoma	Bovine lactoferrin-oleic acid complex	HepG2 cells	Inhibition of proliferation Induction of apoptosis	[162]

Table 2: Continued.

Type of cancer	Animal species/protein-peptide	Cell line/animal model	Effects/mechanisms of action	Reference
Leukemia	Bovine lactoferricin	THP-1 human monocytic leukemic cells	Induction of apoptosis Activation of ROS generation and Ca^{2+}/Mg^{2+}-dependent endonucleases	[170]
		Jurkat T leukemia cells	Induction of apoptosis by triggering mitochondrial swelling and release of cytochrome c Induction of cell membrane permeabilization	[163, 171]
			Activation of ROS generation and caspase-3 and caspase-9 activity Reduction of DNA methyltransferases expression	[172]
Lymphoma	Bovine lactoferricin	Raji and Ramos Burkitt's B-lymphoma cells	Induction of apoptosis Stimulation of DNA fragmentation, chromatin condensation, and nuclear disintegration	[114]
		Ramos B-lymphoma cells xenografts in SCID/beige mice	Extension of survival of mice	[114]
		A20 cell lymphomas in syngeneic Balb/c mice	Tumor necrosis and regression of the tumors Induction of long-term specific cellular immunity	[173]
		B16-BL6 melanoma and L5178Y-ML25 lymphoma cells metastasis models in syngeneic mice	Inhibition of tumor metastasis in lung	[117]
Lung cancer	Bovine lactoferrin	A549 cells Transgenic mice overexpressing hVEGF-A165	Downregulation of proinflammatory cytokines Suppression of tumor development	[174]
Melanoma	Bovine lactoferricin	B16F10 cells	Cytotoxic activity	[118]
		Spontaneous B16-BL6 metastasis models in syngeneic mice	Inhibition of tumor metastasis in lung	[117]
Nasopharyngeal carcinoma	Human lactoferrin	5–8F, CNE2, and HONE1 cells Xenograft Balb/c mice	Suppression of tumorigenesis through inhibition of the AKT pathway	[175]
Neuroblastoma	Bovine lactoferricin	Human MYCN-amplified and non-MYCN-amplified neuroblastoma cells	Cytotoxic activity Destabilization of the cytoplasmic membrane Activation of caspase-6, caspase-7, and caspase-9	[119]
		SH-SY-5Y neuroblastoma xenografts in nude rats	Reduction of the tumor growth	[119]
Oral cancer	Bovine lactoferricin	Oral squamous carcinoma SAS cell	Induction of apoptosis Cleavage of caspase-3 and poly-ADP ribose polymerase Phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase/stress activated protein kinase	[176]
Ovarian cancer	Bovine lactoferricin	Skov3 and Caov3	Cytotoxic activity Induction of apoptosis	[163]

oleic acid known as "human alpha-lactalbumin made lethal to tumor cells, HAMLET" or "bovine alpha-lactalbumin made lethal to tumor cells, BAMLET." It has been recognized that both protein and fatty acid are required to show cytotoxic activity against cancer cells [115]. Treatment of cancer cells with HAMLET provokes morphological changes typical of apoptotic cells through caspase activation and causes mitochondrial permeability transition resulting in mitochondrial swelling, loss of mitochondrial membrane potential, and cytochrome c release [120]. These authors also found that this complex induced autophagic cell death and changes in the proteasome structure and function. Similar effects resulting from chromatin condensation and cell shrinkage have been observed after treatment of cancer cells with the complex BAMLET. The efficacy of both complexes has been shown to be influenced by the type of cancer cell line [120]. In the last years, the therapeutic effects against bladder cancer have been studied in animal models as preliminary step for BAMLET use in human trials. It has been demonstrated that intravesical administration of HAMLET delays tumor progression in a murine bladder cancer model although no preventive effects on tumor formation were observed [121].

Intact caseins have not been characterized as chemopreventive proteins but they have been suggested as an important source of peptides with anticancer properties. CPPs are able to bind calcium, to inhibit cell proliferation, and to induce apoptosis of intestinal tumor HT-29 and AZ-97 cells through activation of voltage-activated calcium channels, which mediate the calcium flood according to the depolarization state of the cell [122]. However, in differentiated epithelial intestinal cells, a protective effect from programmed cell death is observed after treatment with these peptides [123]. β-Casomorphin 7 and β-casomorphin 5, two casein-derived sequences with opioid properties, have shown antiproliferative and cell cycle arresting activities on breast and colon cancer cells [115, 124, 125]. It has been suggested that these effects are mediated by interaction with specific opioid and somatostatin receptors although further studies confirming this mode of action are needed.

3. Impact of Milk Oligosaccharides on Human Health

Despite the important role of HMO in infant health, the limited supply of human milk has hindered its use in commercial infant formula [126] and in large-scale clinical trials. Presumably, the health benefits provided by HMO to infants could be extended to humans of all ages if alternative sources of these complex OS are identified [127]. In that view, the need of finding other sources of human-like OS has prompted the identification, characterization, and quantification of unknown OS present in many other types of milk and their respective industrial streams [128, 129].

3.1. Alternative Sources of Oligosaccharides: Major Sources of Nonhuman Milk Oligosaccharides and Their Industrial Effluents. Increasing interest on plant- and lactose-derived OS has been observed in the past decade as an alternative source for complex HMO. Some of these OS include galacto-OS (GOS), fructo-OS (FOS), and lactulose, among others [130]. These indigestible OS are considered prebiotics due to their ability to confer health benefits to the host through the selective growth and activity of commensal bacteria [131]. One such example is inulin, an oligofructan with D-fructofuranosyl β(1-2) links that cannot be broken down by human digestive enzymes, thus exerting several intestinal physiological effects that contribute to the host health. GOS, commonly produced by transgalactosylation of lactose by β-galactosidases, are another example of a current available source of OS for use by the infant formula industry.

Despite the fact that some health promoting effects, such as improved bifidogenic activity, have been attributed to some of those OS [131], little similarity has been observed between commercially available GOS and HMO, except that they are both built on a lactose core [127]. GOS and FOS are composed of a simple linear core, being devoid of structures having high biological activity such as fucose, sialic acid, and *N*-acetyl glucosamine. Because GOS and FOS do not possess the intrinsic structural complexity observed in HMO, it is expected that domestic farm animals and their processing streams, such as whey permeate from cheese manufacturing, can be a source of OS more similar to the ones present in human milk [132].

World milk production is almost entirely derived from cattle (83%), buffaloes (13%), goats (2%), sheep (1%), and camels (0.3%) (http://www.fao.org/agriculture/dairy-gateway/milk-production/dairy-animals/en/#.VA95gvldXXs). Considering that cow milk accounts for 83% of the world milk production, the enormous interest of the scientific community to identify, quantify, and characterize the OS present in cattle milk and their industrial byproducts is not surprising. A comprehensive review by Urashima et al. [132] shows that approximately 25 bovine milk OS (BMO) structures had been characterized before 2011. The development of advanced analytic techniques, such as several mass spectrometric methods and hydrophilic interaction liquid chromatography-high performance liquid chromatography, has enabled significant improvement in the identification of new BMO; as many as 40 BMO have been characterized [133, 134].

The low concentration of BMO makes it challenging to identify and characterize these compounds when compared with HMO. The OS concentration can reach values as high as 0.7–1.0 g/L in bovine colostrum or can be detected as just trace amounts in bovine milk [135], being much lower than the OS concentration in human milk. Caprine milk is another type of milk, which contains complex OS similar to HMO. The discovery of the presence of fucosylated and sialylated OS that are considered as prebiotics and which have the ability to reduce pathogen adherence to the intestine wall has opened up translational opportunities to human health [136]. Approximately 37 caprine milk OS (COS) have been identified, of which nearly half of them have had their structural complexity elucidated. Similar to bovine milk, COS are present in very small concentrations when compared with HMO. However, they have been found in concentrations of 0.25–0.3 g/L, which is 4-5 times higher than BMO [137].

From those two alternative sources of HMO-like OS (BMO and COS), industrial streams arising from cheese manufacturing and production of whey protein concentrates (WPC) and isolates (WPI) have been considered as a more realistic source of OS for future commercialization [129, 138]. Considering the enormous worldwide production of whey (180–190 $\times$ 10^6 tonnes/year; http://www.adpi.org/Portals/0/PDF/09Conference/TAGEAFFERTSHOLT.pdf) and the fact that the major industrial application of whey to produce WPC and WPI generates a new byproduct containing the target OS, the development of economically feasible processes to recover these compounds represents a key step in enabling the large-scale production of OS.

3.2. Biological Activities of Oligosaccharides. While a wide range of biological functions has been attributed to HMO, less information is available regarding the biological activities of BMO and COS. The limited availability of large quantities of OS with high degree of purity can be inferred by the limited number of *in vivo* studies with those compounds, with the majority of milk OS biological activities being described by *in vitro* studies. Recent reports of some of the biological activities of HMO, BMO, and COS are reported in Table 3.

3.2.1. Prebiotic Activity. One of the main features of HMO is that they can only be consumed by very specific bacteria strains that possess the appropriate set of enzymes to cleave their complex structure. This prebiotic effect is associated with improved health outcomes. A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or in the activity in the gastrointestinal microflora, conferring benefits upon host well-being and health" [139]. Because HMO are only partially digested in the small intestine, they can reach the colon intact where they selectively stimulate the development of bifidogenic flora. A recent study has demonstrated the bifidogenic effect of major fucosylated and sialylated HMO when fed as a sole source of carbon to 25 major isolates of the human intestinal microbiota [140]. Most of the *Bifidobacteria* spp. and *Bacteroides* spp. were able to consume those OS and to produce short chain fatty acids, while common pathogenic bacteria were not able to grow on those OS. *In vitro* biological activities of HMO have been supported by *in vivo* studies. One of the newest publications in this topic demonstrated the ability of 2-fucosyllactose and 3-fucosyllactose to selectively increase some intestinal bacteria populations like *Barnesiella*, the major bacterial genus in mice [141], being this effect correlated with reduced level of colitis.

Prebiotic activities of COS, recovered from caprine whey, have been evaluated by *in vitro* studies [142]. The purified COS fraction favored the development of *Bifidobacterium* spp. and produced short chain fatty acids such as lactic and propionic acids but presented no inhibition of *Staphylococcus aureus* and *Escherichia coli* grown in human faeces.

3.2.2. Antipathogenic Activity. A second feature of OS is the ability to reduce pathogen biding to the intestinal mucosa. The intestinal mucosa is heavily glycosylated and covered with complex glycans including glycoproteins, glycolipids, and mucins, among others [143, 144]. Bacteria and viruses are able to recognize certain types of fucosylated and sialylated OS and adhere to them [130], therefore acting as anti-infective agents. Milk OS are also fucosylated and sialylated so bacteria and viruses, in presence of OS, will attach less to intestinal cells. The ability of pathogens to bind to specific OS seems to be intrinsically correlated with their structure. Neutral OS containing HexNAc block adhesion of pathogens that cause diarrhea (*Vibrio cholerae*) and pneumonia (*Streptococcus pneumoniae*) [15, 145], while neutral fucosylated OS have been shown to inhibit adhesion of other pathogens (i.e., *Campylobacter jejuni* and diarrheagenic *E. coli*) that cause gastrointestinal disorders [146]. Acidic OS containing sialic acid are able to block adhesion of *Helicobacter pylori*, which causes peptic ulcers and other gastric diseases [147], *Staphylococcus aureus*, and *Clostridium botulinum* [148].

Recent *in vitro* studies have demonstrated that BMO also possess antibacterial properties as observed for HMO. BMO from colostrum permeate proved to be effective in protecting HEp-2 cells from enteropathogenic *E. coli*, *Cronobacter sakazakii*, and *Salmonella enterica* serovar *typhimurium* [149]. It has also been demonstrated that BMO can inhibit the pili-mediated adhesion of *Neisseria meningitidis in vitro* [150]. Several studies have demonstrated the inhibition of the attachment of enteric pathogens such as *E. coli* and *Campylobacter jejuni* and noroviruses with HMO [151]. This effect has also been demonstrated by *in vivo* studies in which isolated HMO were fed to suckling mice before and after infection with enteropathogenic *E. coli*. Mice that received HMO significantly reduced colonization of this species compared with untreated controls [152].

3.2.3. Anti-Inflammatory Activity. OS have been also considered as anti-inflammatory agents due to their prebiotic activities and their ability to act as receptors of microorganisms. *In vivo* studies have demonstrated that COS possess anti-inflammatory properties towards the development of experimental colitis in rats. Pretreatment of the rats with isolated COS reduced the typical signs of induced colitis, including less anorexia, better body weight gain, and less macroscopic intestinal lesions, among others [153]. Similar results were observed by Lara-Villoslada et al. [154], where COS were shown to play an important role in intestinal protection and repair after a damage caused by DSS in rats.

4. Future Prospects

Milk has long been recognized as a source of macro- and micronutrients. Recent identification of many important biologically active substances on milk and its derivatives has attracted much attention from the scientific community. Not only are many of these bioactive compounds associated with growth, but they also confer many health benefits that might support disease prevention. Milk proteins and peptides are usually well tolerated and demonstrate oral bioavailability. In this view, they have the potential to act as health promoting ingredients and to participate in auxiliary therapies to boost

Table 3: Biological activities of human, bovine, and goat oligosaccharides.

Microorganisms/animals	Molecule used	Dose	Duration/details	Outcome measured	Reference
Bifidobacterium spp., *Bacteroides* spp., *Clostridium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Enterobacter* spp., and *Escherichia coli*	HMO (2′-FL, 3′-FL, LDFT, 3′-SL and 6′-SL)	0.5–2 g/L	48 hrs OS incubation	SCFA quantification, bacterial growth, and OS consumption	[140]
Mice	HMO (2′-FL and 3′-FL)	500 mM, starting with 5 mL, increasing by 2.5 mL every 3 d reaching a daily amount of 25 mL on day 20	From day 1 to day 20 after birth	Bacterial amount, colitis signs	[141]
Bacteria from human feces	Pooled GOS		During incubation	Bacterial amount	[142]
Mice	Pooled HMO	15 mg/day	One day before and after infection with EPEC	Intestinal colonization of EPEC	[152]
HEp-2 cells	Pooled BMO from colostrum	20 mg/L of total carbohydrate in culture	During incubation	Adherence inhibition	[149]
Bovine thyroglobulin and human salivary agglutinin glycoproteins	Pooled HMO and BMO	40 g/L	During incubation	*Neisseria meningitidis* Pili attachment	[150]
Rats	Pooled GOS	500 mg/(kg∗d)	2 days before and 6 days after induced colitis	Colonic damage	[153]

HMO: human milk oligosaccharides; FL: fucosyllactose; LDFT: lacto-difucosyl-tetraose; SL: sialyllactose; GOS: galacto-oligosaccharides; BMO: bovine milk oligosaccharides.

overall success in chronic diseases. However, this research area is only at its beginning and more peptides with physiological effects are to be discovered in the future. Confirming the health benefits of these bioactive compounds requires the design of clinical trials based on metabolomic genomics, proteomics, transcriptomics, and epigenetic data, in order to explore new biomarkers related to the observed health benefits.

While larger data for *in vivo* biological activities of milk and peptides is observed, the same is not observed for OS. To date, few studies have demonstrated the safety and efficacy of OS supplementation [155, 156]. The reduced number of biological activities evaluated for BMO and COS reveals the challenges associated with the production of OS in adequate quantities and purity needed for clinical trials. The development of new synthetic pathways to produce highly purified OS and of large-scale processes to recover those OS from their respective industrial streams will likely improve the elucidation of their biological activities and determine their safety and efficacy in clinical trials with humans. Moreover, the development of more environmentally friendly processes that are also economically feasible not only will enable the production of a new generation of prebiotics but will address environmental issues associated with the disposal of OS-containing waste streams and poor economic viability of our food industry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge funding from the National Institute of Health, the "Bill and Melinda Gates Foundation," and the Peter J. Shields Chair in Dairy Food Science at the University of California at Davis. Chia-Chien Hsieh acknowledges the Ministry of Science and Technology for National Science Council of Taiwan (MOST 103-2320-B-003-003-MY3). Samuel Fernández-Tomé and Blanca Hernández-Ledesma acknowledge the Ministry of Economy and Competitiveness (MINECO) for their FPI fellowship and "Ramón y Cajal" postdoctoral contract, respectively.

References

[1] B. Lamarche, "Review of the effect of dairy products on non-lipid risk factors for cardiovascular disease," *Journal of the American College of Nutrition*, vol. 27, no. 6, pp. 741S–746S, 2008.

[2] C. Jaffiol, "Milk and dairy products in the prevention and therapy of obesity, type 2 diabetes and metabolic syndrome," *Bulletin de l'Academie Nationale de Medecine*, vol. 192, no. 4, pp. 749–758, 2008.

[3] K. Uenishi, "Prevention of osteoporosis by foods and dietary supplements. Prevention of osteoporosis by milk and dairy products," *Clinical Calcium*, vol. 16, no. 10, pp. 1606–1614, 2006.

[4] Y. Shimazaki, T. Shirota, K. Uchida et al., "Intake of dairy products and periodontal disease: the Hisayama study," *Journal of Periodontology*, vol. 79, no. 1, pp. 131–137, 2008.

[5] C. M. Weaver, "Should dairy be recommended as part of a healthy vegetarian diet? Point," *The American Journal of Clinical Nutrition*, vol. 89, no. 5, pp. 1634S–1637S, 2009.

[6] M. Pufulete, "Intake of dairy products and risk of colorectal neoplasia," *Nutrition Research Reviews*, vol. 21, no. 1, pp. 56–67, 2008.

[7] E. Ginter, "Vegetarian diets, chronic diseases and longevity," *Bratislavské Lekárske Listy*, vol. 109, no. 10, pp. 463–466, 2008.

[8] R. Nagpal, P. V. Behare, M. Kumar et al., "Milk, milk products, and disease free health: an updated overview," *Critical Reviews in Food Science and Nutrition*, vol. 52, no. 4, pp. 321–333, 2012.

[9] R. Nagpal, P. Behare, R. Rana et al., "Bioactive peptides derived from milk proteins and their health beneficial potentials: an update," *Food and Function*, vol. 2, no. 1, pp. 18–27, 2011.

[10] D. S. Newburg, "Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans," *Journal of Animal Science*, vol. 87, no. 13, pp. 26–34, 2009.

[11] G. V. Coppa, P. Pierani, L. Zampini, I. Carloni, A. Carlucci, and O. Gabrielli, "Oligosaccharides in human milk during different phases of lactation," *Acta Paediatrica*, vol. 88, no. 430, pp. 89–94, 1999.

[12] O. Ballard and A. L. Morrow, "Human milk composition: nutrients and bioactive factors," *Pediatric Clinics of North America*, vol. 60, no. 1, pp. 49–74, 2013.

[13] D. S. Newburg and S. H. Neubauer, "Carbohydrates in milks: analysis, quantities, and significance," in *Handbook of Milk Composition*, R. G. Jensen, Ed., chapter 4, pp. 273–349, Academic Press, San Diego, Calif, USA, 1995.

[14] M. B. Engfer, B. Stahl, B. Finke, G. Sawatzki, and H. Daniel, "Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract," *American Journal of Clinical Nutrition*, vol. 71, no. 6, pp. 1589–1596, 2000.

[15] G. V. Coppa, L. Zampini, T. Galeazzi et al., "Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli, Vibrio cholerae*, and *Salmonella fyris*," *Pediatric Research*, vol. 59, no. 3, pp. 377–382, 2006.

[16] S. E. Carlson and S. G. House, "Oral and intraperitoneal administration of *N*-acetylneuraminic acid: effect on rat cerebral and cerebellar *N*-acetylneuraminic acid," *Journal of Nutrition*, vol. 116, no. 5, pp. 881–886, 1986.

[17] N. Klein, A. Schwertmann, M. Peters, C. Kunz, and S. Strobe, "Immunomodulatory effects of breast milk oligosaccharides," in *Short and Long Term Effects of Breast Feeding on Child Health*, B. Koletzko, K. F. Michaelsen, and O. Hernell, Eds., pp. 251–259, Springer, New York, NY, USA, 2002.

[18] D. Divajeva, T. Marsh, S. Logstrup et al., "Economics of chronic diseases protocol: cost-effectiveness modelling and the future burden of non-communicable disease in Europe," *BMC Public Health*, vol. 14, no. 1, article 456, 2014.

[19] W. Y. Huang, S. T. Davidge, and J. Wu, "Bioactive natural constituents from food sources-potential use in hypertension prevention and treatment," *Critical Reviews in Food Science and Nutrition*, vol. 53, no. 6, pp. 615–630, 2013.

[20] S. G. Chrysant and G. S. Chrysant, "An update on the cardiovascular pleiotropic effects of milk and milk products," *The Journal of Clinical Hypertension*, vol. 15, no. 7, pp. 503–510, 2013.

[21] M. F. Engberink, M. A. H. Hendriksen, E. G. Schouten et al., "Inverse association between dairy intake and hypertension: the rotterdam study," *The American Journal of Clinical Nutrition*, vol. 89, no. 6, pp. 1877–1883, 2009.

[22] L. A. J. van Mierlo, L. R. Arends, M. T. Streppel et al., "Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials," *Journal of Human Hypertension*, vol. 20, no. 8, pp. 571–580, 2006.

[23] R. J. Fitzgerald, B. A. Murray, and D. J. Walsh, "Hypotensive peptides from milk proteins," *Journal of Nutrition*, vol. 134, no. 4, 2004.

[24] D. Martínez-Maqueda, B. Miralles, I. Recio, and B. Hernández-Ledesma, "Antihypertensive peptides from food proteins: a review," *Food & Function*, vol. 3, no. 4, pp. 350–361, 2012.

[25] B. Hernández-Ledesma, M. J. García-Nebot, S. Fernández-Tomé, L. Amigo, and I. Recio, "Dairy protein hydrolysates: peptides for health benefits," *International Dairy Journal*, vol. 38, no. 2, pp. 82–100, 2014.

[26] M. D. M. Contreras, R. Carrón, M. J. Montero, M. Ramos, and I. Recio, "Novel casein-derived peptides with antihypertensive activity," *International Dairy Journal*, vol. 19, no. 10, pp. 566–573, 2009.

[27] I. Recio, M. M. Contreras, B. Gómez-Sala, C. Vázquez, H. Fernández-Escribano, and R. del Campo, "Effect of a casein hydrolysate containing novel peptides in hypertensive subjects," *Annals of Nutrition and Metabolism*, vol. 58, no. 3, pp. 16–17, 2011.

[28] P. Ruiz-Giménez, M. C. Burguete, M. Castelló-Ruiz et al., "Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction," *International Dairy Journal*, vol. 17, no. 10, pp. 1212–1215, 2007.

[29] P. Ruiz-Giménez, J. B. Salom, J. F. Marcos et al., "Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: identification of novel active peptides," *Food Chemistry*, vol. 131, no. 1, pp. 266–273, 2012.

[30] R. Fernández-Musoles, P. Manzanares, M. C. Burguete, E. Alborch, and J. B. Salom, "*In vivo* angiotensin I-converting enzyme inhibition by long-term intake of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats," *Food Research International*, vol. 54, no. 1, pp. 627–632, 2013.

[31] R. Sugai, U. Murakami, and Y. Yamori, "Angiotensin converting enzyme inhibitors," Japanese Patent, 62270533, 1995.

[32] J. A. Cadée, C.-Y. Chang, C.-W. Chen, C.-N. Huang, S.-L. Chen, and C.-K. Wang, "Bovine casein hydrolysate (c12 Peptide) reduces blood pressure in prehypertensive subjects," *American Journal of Hypertension*, vol. 20, no. 1, pp. 1–5, 2007.

[33] A. Abubakar, T. Saito, H. Kitazawa, Y. Kawai, and T. Itoh, "Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion," *Journal of Dairy Science*, vol. 81, no. 12, pp. 3131–3138, 1998.

[34] G.-W. Chen, J.-S. Tsai, and B. Sun Pan, "Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation," *International Dairy Journal*, vol. 17, no. 6, pp. 641–647, 2007.

[35] B. Hernández-Ledesma, M. Miguel, L. Amigo, M. A. Aleixandre, and I. Recio, "Effect of simulated gastrointestinal digestion on the antihypertensive properties of synthetic β-lactoglobulin peptide sequences," *Journal of Dairy Research*, vol. 74, no. 3, pp. 336–339, 2007.

[36] J.-S. Tsai, T.-J. Chen, B. S. Pan, S.-D. Gong, and M.-Y. Chung, "Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk," *Food Chemistry*, vol. 106, no. 2, pp. 552–558, 2008.

[37] Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, and T. Takano, "Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk," *Journal of Dairy Science*, vol. 78, no. 4, pp. 777–783, 1995.

[38] Y. Nakamura, N. Yamamoto, K. Sakai, and T. Takano, "Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme," *Journal of Dairy Science*, vol. 78, no. 6, pp. 1253–1257, 1995.

[39] A. H. Pripp, "Effect of peptides derived from food proteins on blood pressure: a meta-analysis of randomized controlled trials," *Food and Nutrition Research*, vol. 52, pp. 1–9, 2008.

[40] J.-Y. Xu, L.-Q. Qin, P.-Y. Wang, W. Li, and C. Chang, "Effect of milk tripeptides on blood pressure: a meta-analysis of randomized controlled trials," *Nutrition*, vol. 24, no. 10, pp. 933–940, 2008.

[41] L.-Q. Qin, J.-Y. Xu, J.-Y. Dong, Y. Zhao, P. van Bladeren, and W. Zhang, "Lactotripeptides intake and blood pressure management: a meta-analysis of randomised controlled clinical trials," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 23, no. 5, pp. 395–402, 2013.

[42] M. F. Engberink, E. G. Schouten, F. J. Kok, L. A. J. van Mierlo, I. A. Brouwer, and J. M. Geleijnse, "Lactotripeptides show no effect on human blood pressure: results from a double-blind randomized controlled trial," *Hypertension*, vol. 51, no. 2, pp. 399–405, 2008.

[43] L. Usinger, L. T. Jensen, B. Flambard, A. Linneberg, and H. Ibsen, "The antihypertensive effect of fermented milk in individuals with prehypertension or borderline hypertension," *Journal of Human Hypertension*, vol. 24, no. 10, pp. 678–683, 2010.

[44] A. F. G. Cicero, B. Gerocarni, L. Laghi, and C. Borghi, "Blood pressure lowering effect of lactotripeptides assumed as functional foods: a meta-analysis of current available clinical trials," *Journal of Human Hypertension*, vol. 25, no. 7, pp. 425–436, 2011.

[45] A. F. G. Cicero, F. Aubin, V. Azais-Braesco, and C. Borghi, "Do the lactotripeptides isoleucine-proline-proline and valine-proline-proline reduce systolic blood pressure in european subjects? A meta-analysis of randomized controlled trials," *American Journal of Hypertension*, vol. 26, no. 3, pp. 442–449, 2013.

[46] EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), "Scientific opinion on the substantion of health claims related to isoleucineproline-proline (IPP) and valine-proline-proline (VPP) and maintenance of normal blood pressure (ID 615, 661, 1831, 1832, 2891), and maintenance of the elastic properties of the arteries (ID 1832) pursuant to Article 13(1) of regulation (EC) No 1924/2006 on request from the European Commission," *EFSA Journal*, vol. 7, pp. 1259–1277, 2009.

[47] M. Miguel, I. Recio, M. Ramos, M. A. Delgado, and M. A. Aleixandre, "Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats," *Journal of Dairy Science*, vol. 89, no. 9, pp. 3352–3359, 2006.

[48] J. C. Rodríguez-Figueroa, A. F. González-Córdova, M. J. Torres-Llanez, H. S. Garcia, and B. Vallejo-Cordoba, "Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild *Lactococcus lactis* strains," *Journal of Dairy Science*, vol. 95, no. 10, pp. 5536–5543, 2012.

[49] J. C. Rodríguez-Figueroa, A. F. González-Córdova, H. Astiazaran-García, and B. Vallejo-Cordoba, "Hypotensive and heart rate-lowering effects in rats receiving milk fermented by specific *Lactococcus lactis* strains," *British Journal of Nutrition*, vol. 109, no. 5, pp. 827–833, 2013.

[50] K. Erdmann, B. W. Y. Cheung, and H. Schröder, "The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease," *The Journal of Nutritional Biochemistry*, vol. 19, no. 10, pp. 643–654, 2008.

[51] A. Pihlanto, "Antioxidative peptides derived from milk proteins," *International Dairy Journal*, vol. 16, no. 11, pp. 1306–1314, 2006.

[52] O. Power, P. Jakeman, and R. J. Fitzgerald, "Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides," *Amino Acids*, vol. 44, no. 3, pp. 797–820, 2013.

[53] M. Zommara, H. Toubo, M. Sakono, and K. Imaizumi, "Prevention of peroxidative stress in rats fed on a low vitamin e-containing diet by supplementing with a fermented bovine milk whey preparation: effect of lactic acid and β-lactoglobulin on the antiperoxidative action," *Bioscience, Biotechnology and Biochemistry*, vol. 62, no. 4, pp. 710–717, 1998.

[54] T. Kullisaar, E. Songisepp, M. Mikelsaar, K. Zilmer, T. Vihalemm, and M. Zilmer, "Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects," *British Journal of Nutrition*, vol. 90, no. 2, pp. 449–456, 2003.

[55] G. Tompa, A. Laine, A. Pihlanto, H. Korhonen, I. Rogelj, and P. Marnilab, "Chemiluminescence of non-differentiated THP-1 promonocytes: developing an assay for screening anti-inflammatory milk proteins and peptides," *The Journal of Biological and Chemical Luminiscence*, vol. 26, no. 4, pp. 251–258, 2010.

[56] X.-Y. Mao, X. Cheng, X. Wang, and S.-J. Wu, "Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis," *Food Chemistry*, vol. 126, no. 2, pp. 484–490, 2011.

[57] K. D. Ballard, R. S. Bruno, R. L. Seip et al., "Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: a randomized, placebo controlled trial," *Nutrition Journal*, vol. 8, article 34, 2009.

[58] S. Nagaoka, Y. Kanamaru, and Y. Kuzuya, "Effects of whey protein and casein on the plasma and liver lipids in rats," *Agricultural and Biological Chemistry*, vol. 55, no. 3, pp. 813–818, 1991.

[59] S. Nagaoka, Y. Kanamaru, Y. Kuzuya, T. Kojima, and T. Kuwata, "Comparative studies on the serum cholesterol lowering action of the whey protein and soybean protein in rats," *Bioscience, Biotechnology and Biochemistry*, vol. 56, no. 9, pp. 1484–1485, 1992.

[60] S. Nagaoka, Y. Futamura, K. Miwa et al., "Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin," *Biochemical and Biophysical Research Communications*, vol. 281, no. 1, pp. 11–17, 2001.

[61] R. Yamauchi, K. Ohinata, and M. Yoshikawa, "β-Lactotensin and neurotensin rapidly reduce serum cholesterol via NT2 receptor," *Peptides*, vol. 24, no. 12, pp. 1955–1961, 2003.

[62] M. Shimizu, "Interaction between food substances and the intestinal epithelium," *Bioscience, Biotechnology and Biochemistry*, vol. 74, no. 2, pp. 232–241, 2010.

[63] M. Shimizu, "Functional food in Japan: current status and future of gut-modulating food," *Journal of Food and Drug Analysis*, vol. 20, no. 1, pp. 213–216, 2012.

[64] J. Claustre, F. Toumi, A. Trompette et al., "Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 283, no. 3, pp. G521–G528, 2002.

[65] A. Trompette, J. Claustre, F. Caillon, G. Jourdan, J. A. Chayvialle, and P. Plaisancié, "Milk bioactive peptides and β-casomorphins induce mucus release in rat jejunum," *Journal of Nutrition*, vol. 133, no. 11, pp. 3499–3503, 2003.

[66] S. Zoghbi, A. Trompette, J. Claustre et al., "β-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a μ-opioid pathway," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 290, no. 6, pp. G1105–G1113, 2006.

[67] P. Plaisancié, J. Claustre, M. Estienne et al., "A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine," *Journal of Nutritional Biochemistry*, vol. 24, no. 1, pp. 213–221, 2013.

[68] D. Martínez-Maqueda, B. Miralles, M. Ramos, and I. Recio, "Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells," *Food Research International*, vol. 54, no. 1, pp. 1287–1291, 2013.

[69] K. S. Han, A. Deglaire, R. Sengupta, and P. J. Moughan, "Hydrolyzed casein influences intestinal mucin gene expression in the rat," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 14, pp. 5572–5576, 2008.

[70] R. C. Sprong, A. J. Schonewille, and R. van der Meer, "Dietary cheese whey protein protects rats against mild dextran sulfate sodium-induced colitis: role of mucin and microbiota," *Journal of Dairy Science*, vol. 93, no. 4, pp. 1364–1371, 2010.

[71] M. Faure, C. Mettraux, D. Moennoz et al., "Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats," *Journal of Nutrition*, vol. 136, no. 6, pp. 1558–1564, 2006.

[72] G. A. Castro, J. E. Carvalho, S. V. Tinti, A. Possenti, and V. C. Sgarbieri, "Anti-ulcerogenic effect of a whey protein isolate and collagen hydrolysates against ethanol ulcerative lesions on oral administration to rats," *Journal of Medicinal Food*, vol. 13, no. 1, pp. 83–90, 2010.

[73] L. F. H. Mezzaroba, J. E. Carvalho, A. N. Ponezi et al., "Antiulcerative properties of bovine α-lactalbumin," *International Dairy Journal*, vol. 16, no. 9, pp. 1005–1012, 2006.

[74] S. S. Comstock, E. A. Reznikov, N. Contractor, and S. M. Donovan, "Dietary bovine lactoferrin alters mucosal and systemic immune cell responses in neonatal piglets," *Journal of Nutrition*, vol. 144, no. 4, pp. 525–532, 2014.

[75] Z. Tang, Y. Yin, Y. Zhang et al., "Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin-lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d," *British Journal of Nutrition*, vol. 101, no. 7, pp. 998–1005, 2009.

[76] G. A. Biziulevičius, V. Žukaite, T. Normantiene, G. Biziulevičiene, and I. G. Arestov, "Non-specific immunity-enhancing effects of tryptic casein hydrolysate versus Fermosorb for treatment/prophylaxis of newborn calf colibacillosis," *FEMS Immunology and Medical Microbiology*, vol. 39, no. 2, pp. 155–161, 2003.

[77] H. Otani, K. Nakano, and T. Kawahara, "Stimulatory effect of a dietary casein phosphopeptide preparation on the mucosal IgA response of mice to orally ingested lipopolysaccharide from *Salmonella typhimurium*," *Bioscience, Biotechnology and Biochemistry*, vol. 67, no. 4, pp. 729–735, 2003.

[78] G. Vinderola, C. Matar, and G. Perdigón, "Milk fermentation products of *L. helveticus* R389 activate calcineurin as a signal to promote gut mucosal immunity," *BMC Immunology*, vol. 8, no. 19, pp. 19–28, 2007.

[79] H. Kitamura and H. Otani, "Fecal IgA levels in healthy persons who ingested cakes with or without bovine casein phosphopeptides," *Milchwissenschaft*, vol. 57, no. 11-12, pp. 611–614, 2002.

[80] D. E. W. Chatterton, D. N. Nguyen, S. B. Bering, and P. T. Sangild, "Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 8, pp. 1730–1747, 2013.

[81] F. S. De Medina, A. Daddaoua, P. Requena et al., "New insights into the immunological effects of food bioactive peptides in animal models of intestinal inflammation," *Proceedings of the Nutrition Society*, vol. 69, no. 3, pp. 454–462, 2010.

[82] G. T. Sousa, F. S. Lira, J. C. Rosa et al., "Dietary whey protein lessens several risk factors for metabolic diseases: a review," *Lipids in Health and Disease*, vol. 11, article 67, 2012.

[83] A. S. Gad, Y. A. Khadrawy, A. A. El-Nekeety, S. R. Mohamed, N. S. Hassan, and M. A. Abdel-Wahhab, "Antioxidant activity and hepatoprotective effects of whey protein and *Spirulina* in rats," *Nutrition*, vol. 27, no. 5, pp. 582–589, 2011.

[84] E. M. Hamad, S. H. Taha, A.-G. I. Abou Dawood, M. Z. Sitohy, and M. Abdel-Hamid, "Protective effect of whey proteins against nonalcoholic fatty liver in rats," *Lipids in Health and Disease*, vol. 10, article 57, 2011.

[85] T. Chitapanarux, P. Tienboon, S. Pojchamarnwiputh, and D. Leelarungrayub, "Open-labeled pilot study of cysteine-rich whey protein isolate supplementation for nonalcoholic steatohepatitis patients," *Journal of Gastroenterology and Hepatology*, vol. 24, no. 6, pp. 1045–1050, 2009.

[86] J. E. de Aguilar-Nascimento, B. R. Prado Silveira, and D. B. Dock-Nascimento, "Early enteral nutrition with whey protein or casein in elderly patients with acute ischemic stroke: a double-blind randomized trial," *Nutrition*, vol. 27, no. 4, pp. 440–444, 2011.

[87] R. López-Posadas, P. Requena, R. González et al., "Bovine glycomacropeptide has intestinal antiinflammatory effects in rats with dextran sulfate-induced colitis," *Journal of Nutrition*, vol. 140, no. 11, pp. 2014–2019, 2010.

[88] P. Requena, A. Daddaoua, E. Martínez-Plata et al., "Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17," *British Journal of Pharmacology*, vol. 154, no. 4, pp. 825–832, 2008.

[89] M. B. E. Turbay, A. de Moreno de LeBlanc, G. Perdigón, G. S. de Giori, and E. M. Hebert, "β-Casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice," *Journal of Dairy Science*, vol. 95, no. 3, pp. 1108–1118, 2012.

[90] R. E. Pratley and A. Salsali, "Inhibition of DPP-4: a new therapeutic approach for the treatment of type 2 diabetes," *Current Medical Research and Opinion*, vol. 23, no. 4, pp. 919–931, 2007.

[91] T. Reihner, "Type 2 diabetes in children and adolescents," *World Journal of Diabetes*, vol. 4, no. 6, pp. 270–281, 2013.

[92] K. M. Hirahatake, J. L. Slavin, K. C. Maki, and S. H. Adams, "Associations between dairy foods, diabetes, and metabolic health: potential mechanisms and future directions," *Metabolism: Clinical and Experimental*, vol. 63, no. 5, pp. 618–627, 2014.

[93] D. J. Drucker and M. A. Nauck, "The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes," *The Lancet*, vol. 368, no. 9548, pp. 1696–1705, 2006.

[94] P. V. Bharatam, D. S. Patel, L. Adane, A. Mittal, and S. Sundriyal, "Modeling and informatics and designing anti-diabetic agents," *Current Pharmaceutical Design*, vol. 13, no. 34, pp. 3518–3530, 2007.

[95] M. A. Nauck, N. Kleine, C. Ørskov, J. J. Holst, B. Willms, and W. Creutzfeldt, "Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide 1 (7–36 amide) in Type 2 (non-insulin-dependent) diabetic patients," *Diabetologia*, vol. 36, no. 8, pp. 741–744, 1993.

[96] G. P. Fadini and A. Avogaro, "Cardiovascular effects of DPP-4 inhibition: beyond GLP-1," *Vascular Pharmacology*, vol. 55, no. 1–3, pp. 10–16, 2011.

[97] P. T. Gunnarsson, M. S. Winzell, C. F. Deacon et al., "Glucose-induced incretin hormone release and inactivation are differently modulated by oral fat and protein in mice," *Endocrinology*, vol. 147, no. 7, pp. 3173–3180, 2006.

[98] B. L. Petersen, L. S. Ward, E. D. Bastian, A. L. Jenkins, J. Campbell, and V. Vuksan, "A whey protein supplement decreases post-prandial glycemia," *Nutrition Journal*, vol. 8, article 47, 2009.

[99] G. T. D. Sousa, F. S. Lira, J. C. Rosa et al., "Dietary whey protein lessens several risk factors for metabolic diseases: a review," *Lipids in Health and Disease*, vol. 11, article 67, 2012.

[100] T. Akhavan, B. L. Luhovyy, P. H. Brown, C. E. Cho, and G. H. Anderson, "Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults," *American Journal of Clinical Nutrition*, vol. 91, no. 4, pp. 966–975, 2010.

[101] C. Gaudel, A. B. Nongonierma, S. Maher et al., "A whey protein hydrolysate promotes insulinotropic activity in a clonal pancreatic β-cell line and enhances glycemic function in ob/ob mice," *Journal of Nutrition*, vol. 143, no. 7, pp. 1109–1114, 2013.

[102] A. B. Nongonierma and R. J. Fitzgerald, "Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates," *Peptides*, vol. 39, no. 1, pp. 157–163, 2013.

[103] S. T. Silveira, D. Martínez-Maqueda, I. Recio, and B. Hernández-Ledesma, "Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin," *Food Chemistry*, vol. 141, no. 2, pp. 1072–1077, 2013.

[104] G. Tulipano, V. Sibilia, A. M. Caroli, and D. Cocchi, "Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors," *Peptides*, vol. 32, no. 4, pp. 835–838, 2011.

[105] M. Uchida, Y. Ohshiba, and O. Mogami, "Novel dipeptidyl peptidase-4-inhibiting peptide derived from β-lactoglobulin," *Journal of Pharmacological Sciences*, vol. 117, no. 1, pp. 63–66, 2011.

[106] H. Uenishi, T. Kabuki, Y. Seto, A. Serizawa, and H. Nakajima, "Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-4)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats," *International Dairy Journal*, vol. 22, no. 1, pp. 24–30, 2012.

[107] I. M. E. Lacroix and E. C. Y. Li-Chan, "Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach," *Journal of Functional Foods*, vol. 4, no. 2, pp. 403–422, 2012.

[108] A. B. Nongonierma, C. Mooney, D. C. Shields, and R. J. Fitzgerald, "*In silico* approaches to predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV) inhibitors," *Peptides*, vol. 57, pp. 43–51, 2014.

[109] F. Bray and B. Møller, "Predicting the future burden of cancer," *Nature Reviews Cancer*, vol. 6, no. 1, pp. 63–74, 2006.

[110] M. M. Manson, "Cancer prevention—the potential for diet to modulate molecular signalling," *Trends in Molecular Medicine*, vol. 9, no. 1, pp. 11–18, 2003.

[111] T. M. de Kok, S. G. van Breda, and M. M. Manson, "Mechanisms of combined action of different chemopreventive dietary compounds," *European Journal of Nutrition*, vol. 47, no. 2, supplement, pp. 51–59, 2008.

[112] E. G. De Mejia and V. P. Dia, "The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells," *Cancer and Metastasis Reviews*, vol. 29, no. 3, pp. 511–528, 2010.

[113] R. K. Kanwar and J. R. Kanwar, "Immunomodulatory lactoferrin in the regulation of apoptosis modulatory proteins in cancer," *Protein & Peptide Letters*, vol. 20, no. 4, pp. 450–458, 2013.

[114] S. J. Furlong, J. S. Mader, and D. W. Hoskin, "Bovine lactoferricin induces caspase-independent apoptosis in human B-lymphoma cells and extends the survival of immune-deficient mice bearing B-lymphoma xenografts," *Experimental and Molecular Pathology*, vol. 88, no. 3, pp. 371–375, 2010.

[115] G. Pepe, G. C. Tenore, R. Mastrocinque, P. Stusio, and P. Campiglia, "Potential anticarcinogenic peptides from bovine milk," *Journal of Amino Acids*, vol. 2013, Article ID 939804, 7 pages, 2013.

[116] J. S. Mader, D. Smyth, J. Marshall, and D. W. Hoskin, "Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor165-induced angiogenesis by competing for heparin-like binding sites on endothelial cells," *The American Journal of Pathology*, vol. 169, no. 5, pp. 1753–1766, 2006.

[117] Y. C. Yoo, S. Watanabe, R. Watanabe, K. Hata, K. I. Shimazaki, and I. Azuma, "Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice," *Japanese Journal of Cancer Research*, vol. 88, no. 2, pp. 184–190, 1997.

[118] L. Tone Eliassen, G. Berge, B. Sveinbjørnsson, J. S. Svendsen, L. H. Vorland, and Ø. Rekdal, "Evidence for a direct antitumor mechanism of action of bovine lactoferricin," *Anticancer Research*, vol. 22, no. 5, pp. 2703–2710, 2002.

[119] L. T. Eliassen, G. Berge, A. Leknessund et al., "The antimicrobial peptide, Lactoferricin B, is cytotoxic to neuroblastoma cells *in vitro* and inhibits xenograft growth *in vivo*," *International Journal of Cancer*, vol. 119, no. 3, pp. 493–500, 2006.

[120] C. R. Brinkmann, S. Thiel, and D. E. Otzen, "Protein-fatty acid complexes: biochemistry, biophysics and function," *FEBS Journal*, vol. 280, no. 8, pp. 1733–1749, 2013.

[121] A.-K. Mossberg, Y. Hou, M. Svensson, B. Holmqvist, and C. Svanborg, "HAMLET treatment delays bladder cancer development," *The Journal of Urology*, vol. 183, no. 4, pp. 1590–1597, 2010.

[122] S. Perego, S. Cosentino, A. Fiorilli, G. Tettamanti, and A. Ferraretto, "Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with voltage-operated L-type calcium channels," *Journal of Nutritional Biochemistry*, vol. 23, no. 7, pp. 808–816, 2012.

[123] S. Perego, A. Zabeo, E. Marasco et al., "Casein phosphopeptides modulate calcium uptake and apoptosis in Caco2 cells through their interaction with the TRPV6 calcium channel," *Journal of Functional Foods*, vol. 5, no. 2, pp. 847–857, 2013.

[124] A. Hatzoglou, E. Bakogeorgou, C. Hatzoglou, P.-M. Martin, and E. Castanas, "Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line," *European Journal of Pharmacology*, vol. 310, no. 2-3, pp. 217–223, 1996.

[125] R. Maneckjee, R. Biswas, and B. K. Vonderhaar, "Binding of opioids to human MCF-7 breast cancer cells and their effects on growth," *Cancer Research*, vol. 50, no. 8, pp. 2234–2238, 1990.

[126] V. Packard, *Human Milk and Infant Formula*, Elsevier/Academic Press, New York, NY, USA, 2012.

[127] A. M. Zivkovic and D. Barile, "Bovine milk as a source of functional oligosaccharides for improving human health," *Advances in Nutrition*, vol. 2, no. 3, pp. 284–289, 2011.

[128] D. Barile and R. A. Rastall, "Human milk and related oligosaccharides as prebiotics," *Current Opinion in Biotechnology*, vol. 24, no. 2, pp. 214–219, 2013.

[129] M. Lange, D. C. Dallas, A. le Parc, J. M. L. N. de Moura Bell, and D. Barile, "Human nutrition: determining functional properties ans sources of recently identified food components: oligosaccharides, glycolipids, glycoproteins and peptides," in *Encyclopedia of Agriculture and Food Systems*, N. van Alfen, Ed., 461, p. 441, Elsevier, New York, NY, USA, 2014.

[130] G. Boehm and G. Moro, "Structural and functional aspects of prebiotics used in infant nutrition," *Journal of Nutrition*, vol. 138, no. 9, pp. 1818S–1828S, 2008.

[131] G. R. Gibson and M. B. Roberfroid, "Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics," *Journal of Nutrition*, vol. 125, no. 6, pp. 1401–1412, 1995.

[132] T. Urashima, E. Taufik, K. Fukuda, and S. Asakuma, "Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals," *Bioscience, Biotechnology and Biochemistry*, vol. 77, no. 3, pp. 455–466, 2013.

[133] D. Barile, M. Marotta, C. Chu et al., "Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry," *Journal of Dairy Science*, vol. 93, no. 9, pp. 3940–3949, 2010.

[134] K. Mariño, J. A. Lane, J. L. Abrahams et al., "Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography," *Glycobiology*, vol. 21, no. 10, pp. 1317–1330, 2011.

[135] R. W. Veh, J.-C. Michalski, A. P. Corfield, M. Sander-Wewer, D. Gies, and R. Schauer, "New chromatographic system for the rapid analysis and preparation of colostrum sialyloligosaccharides," *Journal of Chromatography A*, vol. 212, no. 3, pp. 313–322, 1981.

[136] M. Meyrand, D. C. Dallas, H. Caillat, F. Bouvier, P. Martin, and D. Barile, "Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize αs1-casein," *Small Ruminant Research*, vol. 113, no. 2-3, pp. 411–420, 2013.

[137] A. Martinez-Ferez, S. Rudloff, A. Guadix et al., "Goats' milk as a natural source of lactose-derived oligosaccharides: isolation by membrane technology," *International Dairy Journal*, vol. 16, no. 2, pp. 173–181, 2006.

[138] D. Barile, N. Tao, C. B. Lebrilla, J.-D. Coisson, M. Arlorio, and J. B. German, "Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides," *International Dairy Journal*, vol. 19, no. 9, pp. 524–530, 2009.

[139] M. Roberfroid, "Prebiotics: the concept revisited," *The Journal of Nutrition*, vol. 137, supplement 2, no. 3, pp. 830S–837S, 2007.

[140] Z.-T. Yu, C. Chen, and D. S. Newburg, "Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes," *Glycobiology*, vol. 23, no. 11, pp. 1281–1292, 2013.

[141] G. A. Weiss, C. Chassard, and T. Hennet, "Selective proliferation of intestinal *Barnesiella* under fucosyllactose supplementation in mice," *British Journal of Nutrition*, vol. 111, no. 9, pp. 1602–1610, 2014.

[142] D. L. Oliveira, A. Costabile, R. A. Wilbey, A. S. Grandison, L. C. Duarte, and L. B. Roseiro, "*In vitro* evaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey oligosaccharides in batch culture systems," *BioFactors*, vol. 38, no. 6, pp. 440–449, 2012.

[143] C. Nagler-Anderson, "Man the barrier! Strategic defences in the intestinal mucosa," *Nature Reviews Immunology*, vol. 1, no. 1, pp. 59–67, 2001.

[144] D. S. Newburg and W. A. Walker, "Protection of the neonate by the innate immune system of developing gut and of human milk," *Pediatric Research*, vol. 61, no. 1, pp. 2–8, 2007.

[145] H. H. Tong, M. A. McIver, L. M. Fisher, and T. F. DeMaria, "Effect of lacto-N-neotetraose, asialoganglioside-GM1 and neuraminidase on adherence of otitis media-associated serotypes of *Streptococcus pneumoniae* to chinchilla tracheal epithelium," *Microbial Pathogenesis*, vol. 26, no. 2, pp. 111–119, 1999.

[146] A. L. Morrow, G. M. Ruiz-Palacios, M. Altaye et al., "Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants," *Journal of Pediatrics*, vol. 145, no. 3, pp. 297–303, 2004.

[147] P. M. Simon, P. L. Goode, A. Mobasseri, and D. Zopf, "Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides," *Infection and Immunity*, vol. 65, no. 2, pp. 750–757, 1997.

[148] A. Imberty, Y. M. Chabre, and R. Roy, "Glycomimetics and glycodendrimers as high affinity microbial anti-adhesins," *Chemistry*, vol. 14, no. 25, pp. 7490–7499, 2008.

[149] M. X. Maldonado-Gómez, H. Lee, D. Barile, M. Lu, and R. W. Hutkins, "Adherence inhibition of enteric pathogens to epithelial cells by bovine colostrum fractions," *International Dairy Journal*, vol. 40, pp. 24–32, 2015.

[150] J. Hakkarainen, M. Toivanen, A. Leinonen et al., "Human and bovine milk oligossaccharides inhibit *Neisseria meningitidis* pili attachment *in vitro*," *Journal of Nutrition*, vol. 135, no. 10, pp. 2445–2448, 2005.

[151] D. S. Newburg, G. M. Ruiz-Palacios, and A. L. Morrow, "Human milk glycans protect infants against enteric pathogens," *Annual Review of Nutrition*, vol. 25, no. 1, pp. 37–58, 2005.

[152] C. F. Manthey, C. A. Autran, L. Eckmann, and L. Bode, "Human milk oligosaccharides protect against enteropathogenic *Escherichia coli* attachment *in vitro* and EPEC colonization in suckling mice," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 58, no. 2, pp. 165–168, 2014.

[153] A. Daddaoua, V. Puerta, P. Requena et al., "Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis," *Journal of Nutrition*, vol. 136, no. 3, pp. 672–676, 2006.

[154] F. Lara-Villoslada, E. Debras, A. Nieto et al., "Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis," *Clinical Nutrition*, vol. 25, no. 3, pp. 477–488, 2006.

[155] C. Ashley, W. H. Johnston, C. L. Harris, S. I. Stolz, J. L. Wampler, and C. L. Berseth, "Growth and tolerance of infants fed formula supplemented with polydextrose (PDX) and/or galactooligosaccharides (GOS): double-blind, randomized, controlled trial," *Nutrition Journal*, vol. 11, no. 1, article 38, 2012.

[156] S. Fanaro, B. Marten, R. Bagna et al., "Galacto-oligosaccharides are bifidogenic and safe at weaning: a double-blind randomized multicenter study," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 48, no. 1, pp. 82–88, 2009.

[157] M. E. Davis, A. Rao, S. Gauthier, Y. Pouliot, L. Gourley, and A. Allain, "Preparing an aqueous solution of whey protein isolate and a proteolytic enzyme, holding solution under conditions effective to partially hydrolyze whey protein isolate to provide a hydrolysate having increased ACE-suppressing activity in mammals," US Patent, 6998259, 2006.

[158] I. Recio, I. López-Expósito, A. Quirós et al., "Bioactive peptides identified in enzymatic hydrolyzates of milk caseins and method of obtaining same," WO Patent, 131586, 2006.

[159] O. Tossavainen, T. Suomalainen, J. Sahlstein, and A. M. Makinen, "Process for producing a product containing antihypertensive tripeptides," US Patent, 6972282 B1, 2005.

[160] E. Damiens, I. Yazidi, J. Mazurier, I. Duthile, G. Spik, and Y. Boilly-Marer, "Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells," *Journal of Cellular Biochemistry*, vol. 74, no. 3, pp. 486–498, 1999.

[161] X. Sun, R. Jiang, A. Przepiorski, S. Reddy, K. P. Palmano, and G. W. Krissansen, "'Iron-saturated' bovine lactoferrin improves the chemotherapeutic effects of tamoxifen in the treatment of basal-like breast cancer in mice," *BMC Cancer*, vol. 12, article 591, 2012.

[162] B. Fang, M. Zhang, M. Tian, L. Jiang, H. Y. Guo, and F. Z. Ren, "Bovine lactoferrin binds oleic acid to form an anti-tumor complex similar to HAMLET," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1841, no. 4, pp. 535–543, 2014.

[163] J. S. Mader, J. Salsman, D. M. Conrad, and D. W. Hoskin, "Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines," *Molecular Cancer Therapeutics*, vol. 4, no. 4, pp. 612–624, 2005.

[164] H. M. Habib, W. H. Ibrahim, R. Schneider-Stock, and H. M. Hassan, "Camel milk lactoferrin reduces the proliferation of colorectal cancer cells and exerts antioxidant and DNA damage inhibitory activities," *Food Chemistry*, vol. 141, no. 1, pp. 148–152, 2013.

[165] J. R. Kanwar, G. Mahidhara, and R. K. Kanwar, "Novel alginate-enclosed chitosan-calcium phosphate-loaded iron-saturated bovine lactoferrin nanocarriers for oral delivery in colon cancer therapy," *Nanomedicine*, vol. 7, no. 10, pp. 1521–1550, 2012.

[166] C. Freiburghaus, B. Janicke, H. Lindmark-Månsson, S. M. Oredsson, and M. A. Paulsson, "Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line," *Journal of Dairy Science*, vol. 92, no. 6, pp. 2477–2484, 2009.

[167] C. Freiburghaus, H. Lindmark-Månsson, M. Paulsson, and S. Oredsson, "Reduction of ultraviolet light-induced DNA damage in human colon cancer cells treated with a lactoferrin-derived peptide," *Journal of Dairy Science*, vol. 95, no. 10, pp. 5552–5560, 2012.

[168] N. Zemann, P. Klein, E. Wetzel, F. Huettinger, and M. Huettinger, "Lactoferrin induces growth arrest and nuclear accumulation of Smad-2 in HeLa cells," *Biochimie*, vol. 92, no. 7, pp. 880–884, 2010.

[169] A. Varadhachary, J. S. Wolf, K. Petrak et al., "Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy," *International Journal of Cancer*, vol. 111, no. 3, pp. 398–403, 2004.

[170] Y.-C. Yoo, R. Watanabe, Y. Koike et al., "Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species," *Biochemical and Biophysical Research Communications*, vol. 237, no. 3, pp. 624–628, 1997.

[171] J. S. Mader, A. Richardson, J. Salsman et al., "Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria," *Experimental Cell Research*, vol. 313, no. 12, pp. 2634–2650, 2007.

[172] T.-N. Zhang and N. Liu, "Effect of bovine lactoferricin on DNA methyltransferase 1 levels in Jurkat T-leukemia cells," *Journal of Dairy Science*, vol. 93, no. 9, pp. 3925–3930, 2010.

[173] G. Berge, L. T. Eliassen, K. A. Camilio, K. Bartnes, B. Sveinbjørnsson, and Ø. Rekdal, "Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide," *Cancer Immunology, Immunotherapy*, vol. 59, no. 8, pp. 1285–1294, 2010.

[174] Y. T. Tung, H. L. Chen, C. C. Yen et al., "Bovine lactoferrin inhibits lung cancer growth through suppression of both inflammation and expression of vascular endothelial growth factor," *Journal of Dairy Science*, vol. 96, no. 4, pp. 2095–2106, 2013.

[175] M. Deng, W. Zhang, H. Tang et al., "Lactotransferrin acts as a tumor suppressor in nasopharyngeal carcinoma by repressing AKT through multiple mechanisms," *Oncogene*, vol. 32, no. 36, pp. 4273–4283, 2013.

[176] T. Sakai, Y. Banno, Y. Kato, Y. Nozawa, and M. Kawaguchi, "Pepsin-digested bovine lactoferrin induces apoptotic cell death with JNK/SAPK activation in oral cancer cells," *Journal of Pharmacological Sciences*, vol. 98, no. 1, pp. 41–48, 2005.

Rizzello *et al. Microb Cell Fact (2015) 14:168*
DOI 10.1186/s12934-015-0358-6

MICROBIAL CELL FACTORIES

RESEARCH Open Access

Italian legumes: effect of sourdough fermentation on lunasin-like polypeptides

Carlo Giuseppe Rizzello[1*], Blanca Hernández-Ledesma[2], Samuel Fernández-Tomé[2], José Antonio Curiel[1], Daniela Pinto[3], Barbara Marzani[3], Rossana Coda[4] and Marco Gobbetti[1]

Abstract

Background: There is an increasing interest toward the use of legumes in food industry, mainly due to the quality of their protein fraction. Many legumes are cultivated and consumed around the world, but few data is available regarding the chemical or technological characteristics, and especially on their suitability to be fermented. Nevertheless, sourdough fermentation with selected lactic acid bacteria has been recognized as the most efficient tool to improve some nutritional and functional properties. This study investigated the presence of lunasin-like polypeptides in nineteen traditional Italian legumes, exploiting the potential of the fermentation with selected lactic acid bacteria to increase the native concentration. An integrated approach based on chemical, immunological and ex vivo (human adenocarcinoma Caco-2 cell cultures) analyses was used to show the physiological potential of the lunasin-like polypeptides.

Results: Italian legume varieties, belonging to *Phaseulus vulgaris*, *Cicer arietinum*, *Lathyrus sativus*, *Lens culinaris* and *Pisum sativum* species, were milled and flours were chemically characterized and subjected to sourdough fermentation with selected *Lactobacillus plantarum* C48 and *Lactobacillus brevis* AM7, expressing different peptidase activities. Extracts from legume doughs (unfermented) and sourdoughs were subjected to western blot analysis, using an anti-lunasin primary antibody. Despite the absence of lunasin, different immunoreactive polypeptide bands were found. The number and the intensity of lunasin-like polypeptides increased during sourdough fermentation, as the consequence of the proteolysis of the native proteins carried out by the selected lactic acid bacteria. A marked inhibitory effect on the proliferation of human adenocarcinoma Caco-2 cells was observed using extracts from legume sourdoughs. In particular, sourdoughs from Fagiolo di Lamon, Cece dell'Alta Valle di Misa, and Pisello riccio di Sannicola flours were the most active, showing a decrease of Caco-2 cells viability up to 70 %. The over-expression of Caco-2 filaggrin and involucrin genes was also induced. Nine lunasin-like polypeptides, having similarity to lunasin, were identified.

Conclusions: The features of the sourdough fermented legume flours suggested the use for the manufacture of novel functional foods and/or pharmaceuticals preparations.

Keywords: Legumes, Sourdough, Lactic acid bacteria, Lunasin

Background

According to the Food and Agricultural Organization (FAO) pulses are dry seeds of annual legume plants, belonging to the *Fabaceae* (also known as *Leguminosae*) family. FAO classifies the large number of legume species and varieties employed as food or feed into eleven main groups (dry beans, dry broad beans, dry peas, chickpeas, dry cowpeas, pigeon peas, lentils, bambara beans, vetches and lupins) and minor pulses [1]. Nutritionally, pulses are an important source of proteins, which, in spite of being deficient in sulfur-containing amino acids and tryptophan, possess higher amounts of lysine, arginine, glutamic, and aspartic acid compared to cereal grains [2]. Beyond the nutritional benefits, consumption

*Correspondence: carlogiuseppe.rizzello@uniba.it
[1] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy
Full list of author information is available at the end of the article

of pulses is recently associated with protective or therapeutic effects on chronic health conditions, such as cardiovascular diseases, diabetes, cancer, overweight, and obesity [2]. Owing to the low cost and easy adaptation to grow under poor conditions, pulses are used as staple foods in several low-income countries, serving as main source of both protein and calories [2, 3]. Otherwise, in high-income countries of America and Europe, pulses consumption is low and efforts are done to promote their healthy intake.

Several health organizations recommend pulse consumption as a part of a healthy diet and initiatives are addressed to increase the cultivation, intake, and food processing uses [2, 4]. The protein content of pulses ranges from 20 to 40 % of dry weight. Within this interval, the most abundant are the seed storage proteins. The remaining part are minor or housekeeping proteins, which include enzymes, protease, amylase inhibitors, lectins, lipoxygenases, defense proteins, and others [3, 5]. Legume seeds also contain proteins, which are considered as anti-nutritional compounds due to the effect on the quality of the diet. Nevertheless, the harmful effects of such compounds is easily inactivated after cooking or processes like fermentation, germination and dehulling [6]. Once inactivated, lectins or protease inhibitors may present potential health benefits. Protease inhibitors are potential anti-inflammatory and anticancer agents, whereas lectins have demonstrated to play a key role in preventing certain cancers and activating innate defense mechanisms. Besides, lectins are also proposed as therapeutic agents to prevent or control obesity [5].

Many of the physiological and functional properties of proteins are attributed to biologically active peptides which are often encrypted in the native sequence [7, 8]. Biogenic or bioactive peptides are released from their precursor proteins either by enzymes during gastrointestinal digestion or through proteolysis (e.g., microbial fermentation), which occurs during food processing [8]. In particular, legume hydrolysates and bioactive peptides had in vitro activities towards cancer and cardiovascular diseases or their physiological manifestations like oxidative damage, inflammation, hypertension, and high cholesterol [2]. Lunasin is a 43-amino acid peptide with anticancer, anti-inflammatory, antioxidant and cholesterol lowering activities [9]. It is purified from soybean and commercialized as an ingredient or dietary supplement. In the quest for readily available natural sources of lunasin, the identification and purification of lunasin from different vegetable sources deserve a marked interest [10, 11]. Moreover, the potential of sourdough fermentation for increasing the concentration of lunasin in food matrices was recently investigated [12]. Sourdough is the natural starter traditionally used for making leavened baked goods, harboring a rich lactic acid bacteria and yeast microbiota. A large number of studies [13] showed that the fermentative and proteolytic activities of sourdough lactic acid bacteria not only determined optimal sensory, technology and nutritional characteristics, but also increased the functional value of leavened baked goods. Compared to unfermented soybean, amaranth, barley and wheat flours, the concentration of lunasin and related fragments increased up to four times during fermentation with lactic acid bacteria, which were selected based on proteolytic activities [12].

This study reported the presence of lunasin-like polypeptides in nineteen traditional Italian legumes, and exploited the potential of the fermentation with selected lactic acid bacteria to increase the native concentration. An integrated approach based on chemical, immunological and ex vivo (human adenocarcinoma Caco-2 cell cultures) analyses was used to show the physiological potential of the lunasin-like polypeptides.

Results

Chemical and microbiological characteristics of the flours

All the traditional Italian legumes used in this study have specific certifications (names, abbreviations, geographical origin and certification are listed in Table 1). Legume grains were milled to obtain the corresponding flours. The proximate composition of the flours is reported in Table 2. Moisture ranged from 7.2 ± 0.4 to 11.1 ± 0.7 %. The protein concentration of all the flours was higher than 15 % of dry matter (d.m.). In particular, grass pea flours (CS and CC) showed the highest values (>24.0 %). The lipid concentration varied from 1.4 ± 0.2 to 3.5 ± 0.1 % of d.m., with the exception of chickpea flours (CM and CV), showing a significantly ($P < 0.05$) higher concentration. All flours had an amount of carbohydrates higher than 60 % of d.m. The highest concentration was found for FCo and LA flours, produced by milling Fagiolo di Controne kidney beans and Lenticchia di Altamura lentils, respectively. Legume flours had concentrations of total dietary fiber higher than 17 % of d.m. PS and CC flours had the highest values (35.5 ± 2.9 and 32.1 ± 2.0 %, respectively), whereas the lentils group contained the lowest value of total dietary fiber. Ash ranged from 2.1 ± 0.2 to 4.6 ± 0.3 %, being the lowest values for lentil flours.

Total mesophilic aerobic bacteria ranged from 1.61 ± 0.32 to 4.31 ± 0.11 log cfu/g (Table 3). Enterobacteria were below 2.0 log cfu/g in all the samples. The number of presumptive lactic acid bacteria varied from 1.0 ± 0.08 to 2.54 ± 0.12 log cfu/g. Except for grass pea flour CC (4.23 ± 0.22 log cfu/g), yeasts were 1.11 ± 0.05–2.77 ± 0.20 log cfu/g. Yeasts were not found in 10 g of LP, FV, FSa, and FBb flours. Molds were found

Table 1 List and abbreviations of the Italian legumes

Legume	Name	Abbreviation	Product certification[a]	Origin
Kidney bean (*Phaseolus vulgaris*)	Fagiolo di Lamon	FL	IGP	Veneto
	Fagiolo di Controne	FCo	DOP	Campania
	Fagiolo di Cuneo	FCu	PAT	Piedmont
	Fagiolo Stregoni	FSt	PAT	Piedmont
	Fagiolo Vellutina	FV	PAT	Sicily
	Fagiolo di Saluggia	FSa	PAT	Piedmont
	Fagiolo Badda di Polizzi (white)	FBw	SFP	Sicily
	Fagiolo Badda di Polizzi (black)	FBb	SFP	Sicily
Chickpea (*Cicer arietinum*)	Cece di Merella	CM	PAT	Piedmont
	Cece dell'Alta Valle del Misa	CV	SFP	Marche
Grass pea (*Lathyrus sativus*)	Cicerchia di Serra de Conti	CS	SFP	Marche
	Cicerchia di Campodimele	CC	PAT	Lazio
Lentil (*Lens culinaris*)	Lenticchia di Castelluccio di Norcia	LN	IGP	Umbria
	Lenticchia di Ustica	LU	SFP	Sicily
	Lenticchia di Santo Stefano di Sessanio	LS	SFP	Abruzzo
	Lenticchia rossa di Pantelleria	LP	PAT	Sicily
	Lenticchia di Altamura	LA	PAT	Apulia
	Lenticchia di Villalba	LV	PAT	Sicily
Pea (*Pisum sativum*)	Pisello riccio di Sannicola	PS	PAT	Apulia

Product certifications and origin are also reported

[a] IGP (Indicazione Geografica Protetta, Protected Geographical Indication) and DOP (Denominazione d'Origine Protetta, Designation of Protected Origin) are regulated by Reg. (CE) N. 510/2006 (20.03.2006); PAT (Prodotti Agroalimentari Tradizionali, Traditional Food Products) are included in the list of the Italian Ministry of Agriculture, Food and Forestry (D.M. 07/06/2012); SFP (Slow Food Presidia) are listed at http://www.slowfoodfoundation.org

at 2.0 ± 0.21–3.51 ± 0.13 log cfu/g in most of the flours and were absent in 10 g of LS, FCo, CC, and PS flours.

Lactic acid bacteria fermentation

After incubation for 24 h at 30 °C, control doughs (D), without bacterial inoculum, showed values of pH that ranged from 5.8 ± 0.2 to 6.5 ± 0.3, corresponding to values of TTA of 3.8 ± 0.2 to 8.5 ± 0.5 ml 0.1 M NaOH/10 g of dough (Table 4). When *L. plantarum* C48 and *L. brevis* AM7 were used as starters for sourdough (S) fermentation, the cell density of presumptive lactic acid bacteria was 9.8–10.2 log cfu/g. All S had values of pH significantly ($P < 0.05$) lower (3.9 ± 0.1–4.5 ± 0.3) than the corresponding D. TTA ranged from 20.2 ± 1.3 to 27.2 ± 1.5 ml NaOH/10 g of dough.

The concentration of total free amino acids (FAA) of D and S is reported in Table 4. Before incubation, doughs had concentrations of total FAA varying from 2429 ± 20 (D made with LU flour) to 4547 ± 18 mg/kg (D made with FBw flour). In several cases, the concentration of total free amino acids (FAA) of S was significantly ($P < 0.05$) higher than that of the corresponding D. The average increase was ca. 28 % for kidney bean and pea S. Almost the same average increases (23–26 %) were found for grass pea, chickpea, and lentil S. Compared to D, slight increases of total FAA were shown by FSa, CM, CC, LS, and PS sourdoughs.

Western blot

The water/salt soluble extracts (WSE) of D and S were analyzed by SDS PAGE, and the proteins were electroblotted and detected after incubation with lunasin polyclonal primary antibody (Fig. 1). A polyclonal primary antibody was already used for identification and quantification of lunasin and its use proposed for optimized methods of detection [14]. Lunasin peptide was not found in any of the samples analyzed. Nevertheless, different immunoreactive bands, having molecular masses higher than lunasin, were found. Among D, grass pea and chickpea doughs did not shown any immunoreactive bands, while pea and all the lentil doughs (with the exception of LV) showed very weak signals, mainly distributed below 15 kDa. A large variability was found among the doughs made with bean flours. Very weak bands were found for Fl, FCo, FCu, and Fst, while a large protein band (molecular mass of ca. 17 kDa) was found for LV, FV, FSa, FBw, and FBb.

Table 2 Proximate composition of the Italian legume flours

		Moisture (%)	Proteins (% of d.m.)	Lipids (% of d.m.)	Carbohydrates (% of d.m.)	Dietary fiber (% of d.m.)	Ash (% of d.m.)
FL	Fagiolo di Lamon	7.2 ± 0.4^{e}	20.2 ± 1.5^{b}	3.4 ± 0.2^{b}	65.0 ± 3.5^{c}	21.2 ± 1.5^{d}	4.0 ± 0.4^{b}
FCo	Fagiolo di Controne	7.2 ± 0.5^{e}	17.3 ± 1.5^{d}	2.7 ± 0.3^{c}	69.2 ± 5.0^{a}	26.0 ± 1.3^{b}	3.5 ± 0.2^{c}
FCu	Fagiolo di Cuneo	8.0 ± 0.8^{d}	20.5 ± 2.0^{b}	2.0 ± 0.4^{d}	65.2 ± 3.0^{c}	24.3 ± 2.3^{c}	3.5 ± 0.3^{b}
FSt	Fagiolo Stregoni	10.2 ± 0.7^{b}	17.2 ± 1.5^{e}	2.3 ± 0.2^{c}	67.3 ± 2.5^{b}	22.6 ± 2.2^{c}	3.3 ± 0.2^{c}
FV	Fagiolo Vellutina	11.1 ± 0.7^{a}	18.6 ± 1.4^{d}	2.5 ± 0.3^{c}	64.5 ± 5.0^{c}	26.5 ± 1.9^{b}	3.8 ± 0.1^{b}
FSa	Fagiolo di Saluggia	10.9 ± 0.8^{a}	18.4 ± 0.6^{d}	2.6 ± 0.4^{c}	65.8 ± 4.0^{c}	22.7 ± 1.7^{c}	3.0 ± 0.1^{c}
FBw	Fagiolo Badda di Polizzi (white)	7.3 ± 0.2^{e}	21.0 ± 1.4^{b}	2.6 ± 0.3^{c}	65.4 ± 3.6^{c}	19.6 ± 2.3^{d}	3.6 ± 0.2^{b}
FBb	Fagiolo Badda di Polizzi (black)	8.5 ± 0.5^{d}	19.0 ± 1.8^{c}	3.5 ± 0.1^{b}	65.2 ± 3.4^{c}	19.4 ± 0.9^{d}	3.6 ± 0.2^{b}
CM	Cece di Merella	8.8 ± 0.5^{d}	15.7 ± 1.0^{e}	6.2 ± 0.4^{a}	66.5 ± 3.6^{b}	27.5 ± 1.1^{b}	3.5 ± 0.3^{c}
CV	Cece Alta Valle di Misa	8.9 ± 0.3^{d}	20.0 ± 0.8^{c}	6.3 ± 0.1^{a}	61.2 ± 3.5^{e}	26.8 ± 2.3^{b}	4.0 ± 0.2^{b}
CS	Cicerchia di Serra de Conti	9.2 ± 0.5^{c}	24.3 ± 1.5^{a}	1.4 ± 0.2^{e}	60.8 ± 3.5^{e}	25.1 ± 1.2^{c}	3.7 ± 0.4^{b}
CC	Cicerchia di Campodimele	9.3 ± 0.6^{c}	24.1 ± 2.0^{a}	2.2 ± 0.4^{d}	61.5 ± 3.1^{e}	32.1 ± 2.0^{a}	3.8 ± 0.2^{b}
LN	Lenticchia di Castelluccio di Norcia	8.2 ± 0.7^{d}	23.5 ± 1.2^{a}	3.0 ± 0.5^{b}	62.8 ± 2.5^{d}	18.2 ± 1.9^{e}	2.6 ± 0.1^{d}
LU	Lenticchia di Ustica	8.8 ± 0.4^{d}	23.0 ± 1.0^{a}	3.4 ± 0.2^{b}	62.6 ± 3.7^{d}	17.6 ± 2.1^{e}	2.3 ± 0.2^{e}
LS	Lenticchia di Santo Stefano di Sessanio	8.7 ± 0.8^{d}	22.0 ± 1.3^{b}	3.2 ± 0.2^{b}	64.1 ± 3.0^{d}	25.2 ± 1.9^{c}	3.0 ± 0.3^{d}
LP	Lenticchia rossa di Pantelleria	8.0 ± 0.5^{d}	21.2 ± 1.5^{b}	2.7 ± 0.1^{c}	65.9 ± 2.9^{c}	20.5 ± 1.1^{d}	2.2 ± 0.1^{e}
LA	Lenticchia di Altamura	7.4 ± 0.2^{e}	18.7 ± 0.9^{d}	2.4 ± 0.2^{d}	69.1 ± 2.2^{a}	22.8 ± 1.8^{c}	2.5 ± 0.2^{d}
LV	Lenticchia di Villalba	8.6 ± 0.5^{d}	20.8 ± 1.5^{b}	2.1 ± 0.2^{d}	65.9 ± 3.0^{b}	17.5 ± 0.4^{e}	2.1 ± 0.2^{e}
PS	Pisello riccio di Sannicola	9.2 ± 0.7^{c}	19.6 ± 1.0^{c}	2.2 ± 0.2^{d}	65.3 ± 2.0^{c}	35.5 ± 2.9^{a}	4.6 ± 0.3^{a}

The data are the means of three independent experiments ± standard deviations (n = 3)

d.m. dry matter

[a–e] Values in the same column with different superscript letters differ significantly ($P < 0.05$)

Lactic acid bacteria fermentation induced a large modification of the profiles of immunoreactive protein of all the legume flours. A band of ca. 30 kDa, which was absent in the corresponding D, was found for LN, LU, LS, LP, and LA sourdoughs. The intensity of the 17 kDa band of fermented LV increased compared to D. The same signal became evident in all bean sourdoughs, especially for FL. Two protein bands were detected at ca. 30 kDa in FV, FSa, FBw and FBb. The same was found for CS and PS. Moreover, FV, FSa, FBw and FBb showed reactive bands also having molecular masses of 14–17 kDa.

Effect on proliferation of Caco-2 cells

Aiming at determining the cytotoxicity effect of the freeze-dried WSE from D and S towards human colon adenocarcinoma cells (Caco-2), five samples (FL, CV, CC, LN, and PS) with different immunoreactive protein profiles, were chosen as representatives of the legume species considered in this study, and were subjected to further characterization. On the basis of the results obtained from the western blot analysis, WSE were partially purified by ultra-filtration, collecting the fractions containing the molecules with molecular mass lower than 30 kDa. In particular, the MTT assay was performed after treatment of Caco-2 cells with 0.1, 1, and 10 mg/ml of proteins for 24, 48, or 72 h.

Overall, all the WSE from D and S allowed a significant ($P < 0.05$) decrease of the cell proliferation compared to control (Fig. 2). Compared to the results obtained after 24 h-treatment, the inhibitory effect increased after prolonging the incubation to 48 h, and in many cases, to 72 h. Exceptions were the treatments with 10 mg/ml of proteins, which did not cause a further significant decrease of proliferation. The cytotoxic effect of WSE from S was in all the cases higher than the corresponding D. Besides the effect of the treatment duration, cytotoxicity increased proportionally to the concentration of proteins tested.

After 24 h of treatment (Fig. 2a), a weak anti-proliferative effect was found for WSE from D and S at 0.1 mg/ml of proteins. The strongest effect corresponded to LN: (72.25 ± 2.12 and 69.54 ± 1.88 % of vitality for D and S, respectively. When WSE from S were used at concentration of 1 mg/ml, the vitality of Caco-2 cells decreased by

Table 3 Microbiological analyses of the Italian legume flours

Legume	Total mesophilic aerobic bacteria	Lactic acid bacteria	Yeasts	Molds	Total entero-bacteria
FL	3.52 ± 0.12^{b}	1.93 ± 0.12^{b}	2.77 ± 0.20^{c}	3.51 ± 0.13^{a}	0.82 ± 0.11^{b}
FCo	3.60 ± 0.20^{b}	2.49 ± 0.22^{a}	1.84 ± 0.20^{d}	nf	0.53 ± 0.09^{c}
FCu	3.23 ± 0.22^{b}	2.54 ± 0.12^{a}	1.11 ± 0.05^{e}	3.33 ± 0.21^{a}	0.82 ± 0.11^{b}
FSt	4.21 ± 0.18^{a}	2.25 ± 0.23^{a}	2.14 ± 0.21^{c}	3.31 ± 0.22^{a}	0.91 ± 0.07^{b}
FV	2.92 ± 0.13^{c}	1.14 ± 0.24^{c}	nf	2.81 ± 0.15^{b}	0.51 ± 0.09^{c}
FSa	2.55 ± 0.25^{c}	1.16 ± 0.11^{c}	nf	2.47 ± 0.15^{c}	0.62 ± 0.13^{c}
FBw	3.34 ± 0.19^{b}	1.47 ± 0.15^{c}	1.30 ± 0.21^{e}	3.23 ± 0.16^{a}	0.73 ± 0.09^{b}
FBb	1.61 ± 0.32^{d}	1.00 ± 0.16^{c}	nf	2.45 ± 0.15^{c}	0.82 ± 0.09^{b}
CM	2.84 ± 0.22^{c}	1.31 ± 0.18^{c}	1.69 ± 0.23^{d}	2.47 ± 0.22^{c}	0.91 ± 0.14^{b}
CV	2.47 ± 0.14^{c}	1.47 ± 0.21^{c}	1.95 ± 0.09^{c}	2.30 ± 0.24^{c}	0.9 ± 0.09^{b}
CS	2.25 ± 0.15^{c}	1.69 ± 0.23^{b}	nf	2.00 ± 0.21^{c}	0.8 ± 0.10^{b}
CC	2.32 ± 0.23^{c}	1.84 ± 0.22^{b}	4.23 ± 0.22^{a}	nf	0.53 ± 0.05^{c}
LN	2.69 ± 0.21^{c}	1.77 ± 0.24^{b}	1.47 ± 0.13^{e}	2.04 ± 0.23^{c}	1.32 ± 0.10^{a}
LU	2.36 ± 0.24^{c}	1.84 ± 0.25^{b}	2.08 ± 0.15^{c}	2.30 ± 0.20^{c}	0.64 ± 0.11^{c}
LS	2.95 ± 0.09^{c}	1.47 ± 0.24^{c}	2.11 ± 0.15^{c}	nf	0.75 ± 0.13^{b}
LP	4.31 ± 0.11^{a}	1.30 ± 0.25^{c}	nf	3.23 ± 0.20^{a}	0.52 ± 0.22^{c}
LA	3.69 ± 0.16^{b}	1.30 ± 0.08^{c}	3.50 ± 0.15^{b}	2.11 ± 0.20^{c}	0.72 ± 0.14^{b}
LV	2.61 ± 0.17^{c}	1.00 ± 0.08^{c}	2.47 ± 0.12^{c}	2.23 ± 0.15^{c}	0.82 ± 0.12^{b}
PS	2.47 ± 0.23^{c}	1.30 ± 0.10^{c}	1.30 ± 0.21^{e}	nf	0.92 ± 0.13^{b}

Total mesophilic aerobic bacteria were estimated on Plate Count Agar (PCA), lactic acid bacteria on agar MRS; yeasts and molds on Yeast extract Peptone Dextrose Agar (YPD-y and YPD-m, respectively); and total enterobacteria on Violet Red Bile Glucose Agar (VRBGA). Details are reported in "Methods" section

The data are the means of three independent experiments ± standard deviations (n = 3)

[a–e] Values in the same column with different superscript letters differ significantly ($P < 0.05$)

ca. 10 %. In particular, WSE from CC sourdough caused the highest decrease of vitality (58.47 ± 2.08 %). At the highest concentration assayed, the most remarkable cytotoxicity was found for CC, LN, and PS sourdoughs (30.49 ± 1.13, 36.97 ± 1.01, and 35.13 ± 0.95 %, respectively). The same trend was found after 48 h of treatment (Fig. 2b), although lower values of vitality were observed. The lowest proliferation was found for CC sourdough, when treatments were done with 1 mg/ml (39.59 ± 1.05 %), and for CC, LN, and PS sourdoughs, when 10 mg/ml of proteins (30.67 ± 0.87, 29.32 ± 1.11, and 37.75 ± 1.23 %, respectively) were used.

After 72 h of treatment (Fig. 2c), the vitality of Caco-2 cells was lower than that found after 24 or 48 h for treatments performed with 0.1 and 1 mg/ml of proteins, while the use of 10 mg/ml of proteins was less effective than 48 h-treatments, with the only exception of FL sourdough.

Transcriptional regulation of filaggrin (FLG) and involucrin (IVL)

Human colon adenocarcinoma cells (Caco-2) were treated with the partially purified and freeze dried WSE from legume D and S aiming at investigating filaggrin (*FLG*) and involucrin (*IVL*) gene expressions through RT-PCR. Treatments were lasting 4, 8, and 24 h, After 4 h of exposure (Fig. 3A), a significant ($P < 0.05$ %) over-expression of FLG was found only for fermented PS. All the other WSE, both from D and S, did not cause significant ($P < 0.05$) variations compared to LPS (positive control). After 8 h of exposure, a significant ($P < 0.05$) up-regulation of the *FLG* gene was also found for FL dough and FL, CV, and PS sourdoughs (Fig. 3B). The same trend was found for treatment lasting 24 h (Fig. 3C) with FL, CV, CC, and PS sourdoughs.

Regarding *IVL* gene, none of the extracts, except for PS sourdough, induced an expression after 4 h of treatment higher than that of LPS after 4 h of treatment (Fig. 4A). After 8 h of treatment, WSE extracted from S caused significant ($P < 0.05$) increases of the expression compared to the corresponding D, especially FL, CV, and PS (Fig. 4B). A longer treatment with the WSE (24 h) did not favour a further increase of the gene expression. Only PS sourdough caused the same expression of IVL gene under all the conditions assayed (Fig. 4C).

Identification of lunasin-like polypeptides

Based on the results from the MTT assay on Caco-2 cell, the immunoreactive protein bands of FL, CV, and PS sourdoughs were recovered from Tris-Tricine gels, and subjected to tryptic digestion, and HPLC coupled to nanoESI-MS/MS analysis.

Table 4 Biochemical characteristics of doughs

Legume	pH		TTA (ml of 0.1 M NAOH)		Free amino acids (mg/kg)	
	D	S	D	S	D	S
FL	6.5 ± 0.2^{a}	4.4 ± 0.1^{a}	5.7 ± 0.5^{c}	26.6 ± 1.3^{a}	4272 ± 10^{a}	5900 ± 18^{b}
FCo	6.2 ± 0.1^{b}	4.2 ± 0.2^{c}	6.3 ± 0.2^{b}	23.0 ± 0.9^{c}	$3348 \pm 14c$	5414 ± 17^{c}
FCu	6.5 ± 0.3^{a}	4.4 ± 0.2^{a}	5.5 ± 0.3^{c}	26.8 ± 2.1^{a}	4018 ± 9^{a}	5605 ± 15^{b}
FSt	6.4 ± 0.5^{a}	4.5 ± 0.3^{a}	3.1 ± 0.4^{e}	22.7 ± 0.9^{d}	3025 ± 11^{c}	3982 ± 16^{e}
FV	6.5 ± 0.2^{a}	4.2 ± 0.2^{c}	4.7 ± 0.3^{d}	23.5 ± 1.8^{c}	3991 ± 13^{b}	5904 ± 12^{b}
FSa	6.2 ± 0.2^{b}	4.3 ± 0.1^{b}	5.2 ± 0.4^{c}	20.2 ± 1.3^{e}	3512 ± 12^{b}	3634 ± 18^{e}
FBw	6.4 ± 0.3^{a}	4.2 ± 0.1^{c}	6.1 ± 0.6^{b}	25.5 ± 1.8^{b}	4547 ± 18^{a}	6620 ± 12^{a}
FBb	6.2 ± 0.6^{b}	4.4 ± 0.2^{a}	7.1 ± 0.5^{a}	25.4 ± 1.5^{b}	3901 ± 15^{b}	6269 ± 14^{a}
CM	6.0 ± 0.1^{c}	3.9 ± 0.1^{e}	3.8 ± 0.2^{e}	22.5 ± 1.0^{d}	2550 ± 9^{d}	2573 ± 16^{f}
CV	6.2 ± 0.3^{b}	4.1 ± 0.4^{d}	5.1 ± 0.5^{c}	22.3 ± 0.7^{d}	2961 ± 13^{c}	4524 ± 20^{d}
CS	6.3 ± 0.2^{b}	4.2 ± 0.1^{c}	6.2 ± 0.3^{b}	23.2 ± 1.2^{c}	2652 ± 11^{d}	4183 ± 17^{d}
CC	6.0 ± 0.5^{c}	4.2 ± 0.1^{c}	7.4 ± 0.3^{a}	27.2 ± 1.5^{a}	2611 ± 13^{d}	2730 ± 15^{f}
LN	6.3 ± 0.5^{b}	4.2 ± 0.2^{c}	4.6 ± 0.4^{d}	23.1 ± 1.4^{c}	2825 ± 18^{d}	3634 ± 18^{e}
LU	6.0 ± 0.3^{c}	4.0 ± 0.1^{d}	4.2 ± 0.6^{d}	22.2 ± 1.5^{d}	2429 ± 20^{d}	3274 ± 16^{e}
LS	5.8 ± 0.2^{d}	4.0 ± 0.1^{d}	8.5 ± 0.2^{a}	22.8 ± 2.0^{d}	4324 ± 11^{a}	4743 ± 21^{d}
LP	6.3 ± 0.4^{b}	4.1 ± 0.4^{d}	4.1 ± 0.2^{d}	23.1 ± 1.5^{c}	2631 ± 9^{d}	3173 ± 11^{e}
LA	6.3 ± 0.2^{b}	4.1 ± 0.2^{d}	3.9 ± 0.3^{e}	22.5 ± 0.9^{d}	3207 ± 13^{c}	5104 ± 17^{c}
LV	6.0 ± 0.2^{c}	4.1 ± 0.3^{d}	5.3 ± 0.2^{c}	22.2 ± 1.1^{d}	3196 ± 15^{c}	5173 ± 22^{c}
PS	6.3 ± 0.5^{b}	4.4 ± 0.5^{a}	4.3 ± 0.4^{d}	20.4 ± 1.5^{e}	3629 ± 16^{b}	3686 ± 19^{e}

Control doughs (D), without bacterial inoculation, and sourdoughs (S), started with selected lactic acid bacteria, made with the Italian legume flours were incubated at 30 °C for 24 h

The data are the means of three independent experiments ± standard deviations (n = 3)

[a–f] Values in the same column with different superscript letters differ significantly ($P < 0.05$)

Ten different proteins were identified through the Mascot research on the NCBI database (Table 5). It can be hypothesized that lunasin-like polypeptides were released from native legume proteins via proteolysis during sourdough fermentation. This should explain the lack of correspondence between the molecular masses of the proteins identified and the polypeptide bands revealed by western blot analyses. The immunodetected polypeptide bands from FL sourdough corresponded to the following matches: Subtilisin inhibitor 1 (P16064), Legumin A2 (P15838) and Phaseolin (P02853). The protein bands from CV sourdough matched with: leucoagglutinating phytohemagglutinin (P05087), phatogenesis related protein (CAA56142) and seed linoleate 9S-lipoxygenase-3 (P09918). The proteins from PS sourdough were identified as: Provicilin (P02855), seed linoleate 9S-lipoxygenase-2 (P14856), seed biotin-containing protein SBP65 (Q41060) and Albumin-1 C (P62928). All the database matches corresponded to proteins previously identified from legumes. For five proteins, the exact correspondence between the species analyzed and the database matches was not found, especially for CV. Probably, this was due to the limited number of legume protein sequences previously identified and included in the NCBI database.

The protein sequences were compared to soy lunasin sequence (deposited at the National Center for Biotechnology Information, NCBI database with the accession number AAP62458) using BLAST. Except for Provicilin identified that was identified in PS sourdough, all the sequences might be aligned with different lunasin epitopes (Fig. 5). The longest alignments were found for Legumin A2, corresponding to fragments (f) 24–38 and 33–41 of the soy lunasin sequence, and Phaseolin (f13–28) from FL sourdough; pathogenesis related protein and seed-linoleate 9S lipoxygenate-3 (f9–32 and f1–18, respectively) from CV, and seed-linoleate 9S lipoxygenate-2 and seed biotin-containing protein SBP65 (f1–22 and f11–36, respectively) from PS. The alignments showed identities from 26 to 67 % (pathogenesis related protein and subtilisin inhibitor1/leucoagglutinating phytoemagglutinin, respectively) and positives from 35 to 93 % (Albumin-1C and Legumin A2, respectively).

Peptidase activities

The peptidase activities of the two lactic acid bacteria strains used as starters were assayed using relatively specific synthetic substrates. Significant ($P < 0.05$) differences were found between strains. Compared to that

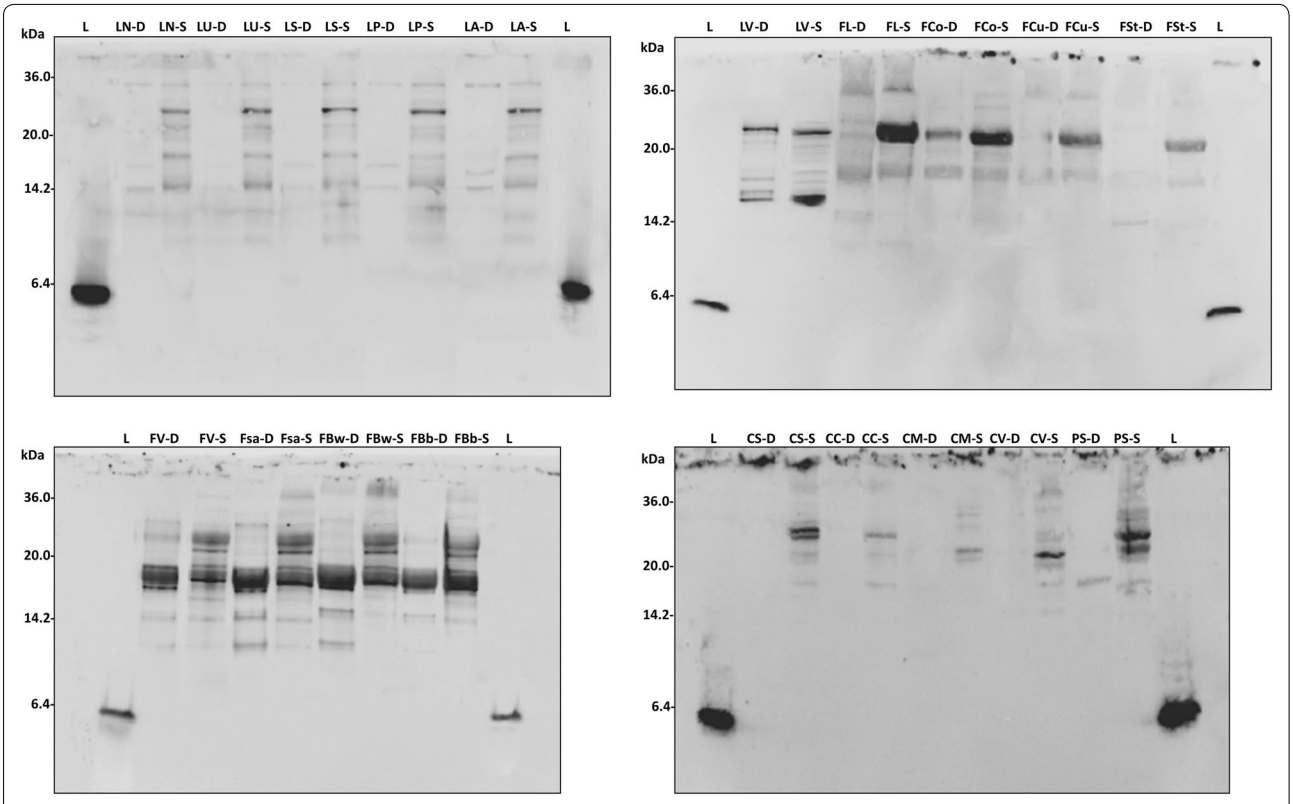


Fig. 1 Western blot analysis. Water/salt soluble extracts obtained from control doughs (D), without bacterial inoculum, and sourdoughs (S), started with selected lactic acid bacteria, made with Italian legume flours, were used. A lunasin polyclonal primary antibody was used. Before electroblotting, the electrophoretic separation was obtained by Tris-Tricine SDS-PAGE. Image was performed using the VersaDoc Imaging System (Bio-Rad). Synthetic lunasin (L) was included in the analysis. The correspondence of the legume flour abbreviations is reported in Table 1

of *L. plantarum* C48 (5.87 ± 0.02 and 3.52 ± 0.02 U), PepN activity on Leu-*p*-NA and endopeptidase activity (PepO) of *L. brevis* AM7 were higher (6.83 ± 0.08 U and 4.31 ± 0.03). Also PepA activity was higher in *L. brevis* AM7 (14.02 ± 0.04 U). That of *L. plantarum* C48 resulted 35 % lower. This latter strain showed higher tripeptidase (PepT) activity (9.12 ± 0.02 vs. 5.61 ± 0.05 U). No significant differences ($P > 0.05$) were found between the two strains for PepT (7.20 ± 0.04 and 7.12 ± 0.05 U) and PepX (0.95 ± 0.03 and 1.01 ± 0.02 U) activities.

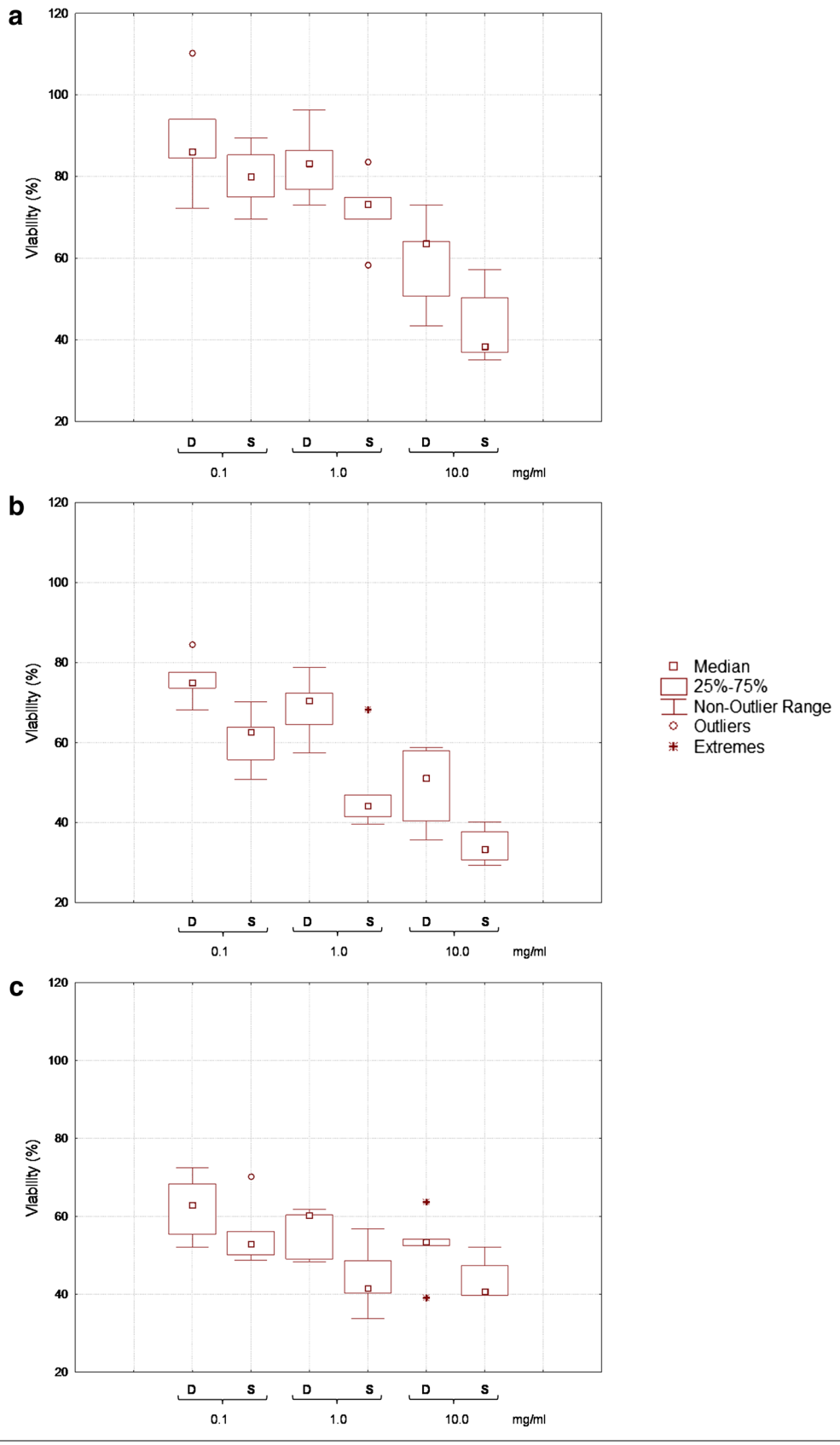
Discussion

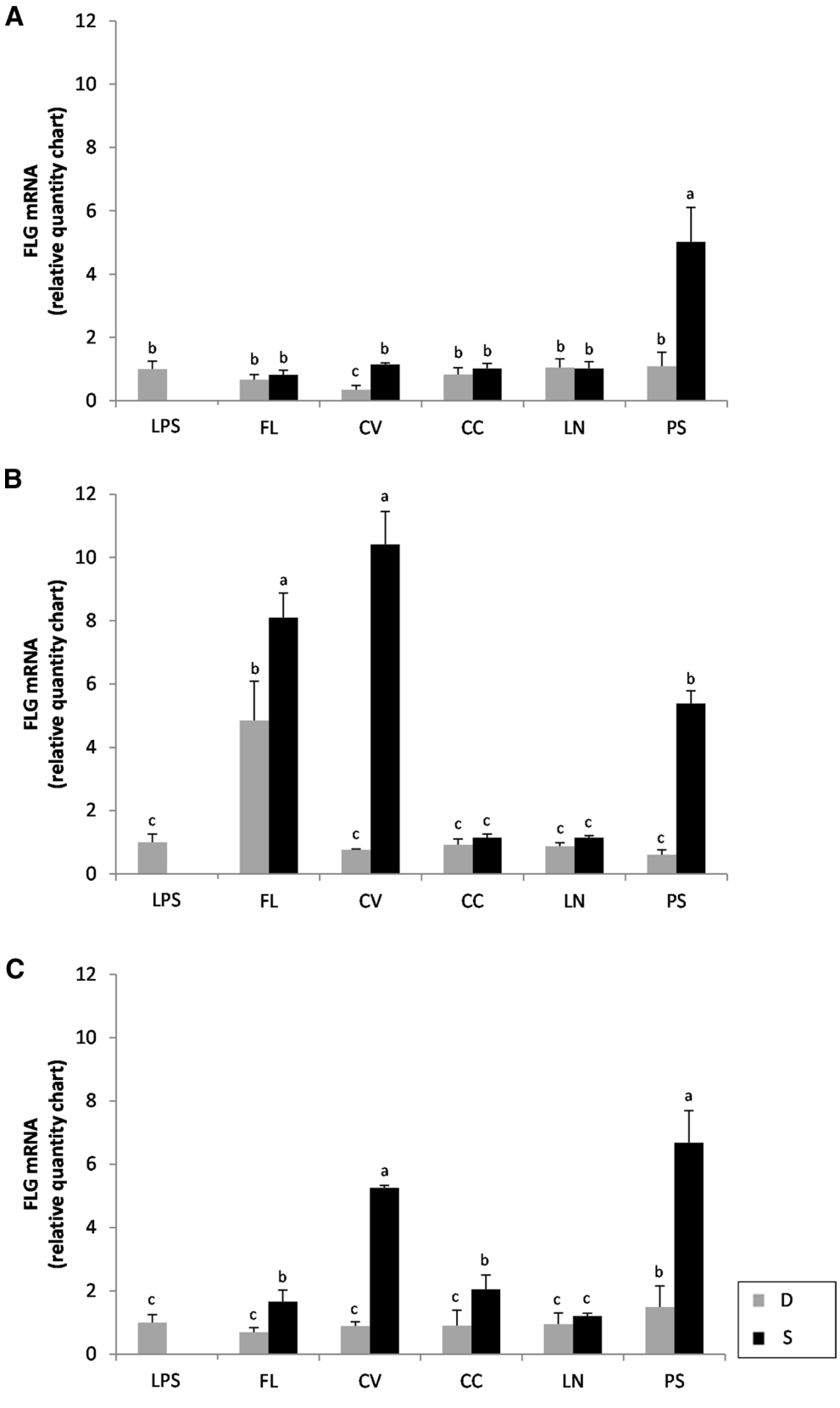
Despite the beneficial effects on the human diet, the worldwide consumption of legumes is declining [15] and below the recommended dose [16]. One potential way to increase the consumption of legumes could be the rediscovery of traditional and local varieties, and, especially, the use of legumes into novel and healthy foods, also exploiting the potential of non-conventional processing [17]. The complementation between cereal and legume flours into new formulas may deserve an interest either to increase the levels of biogenic compounds or to fulfill nutritional deficiencies of cereal-based diets [18].

Traditional Italian legumes, all with product certifications and belonging to *Phaseulus vulgaris*, *Cicer arietinum*, *Lathyrus sativus*, *Lens culinaris* and *Pisum sativum* species, were used in this study. Seeds were milled, and flours were subjected to sourdough fermentation, using

(See figure on next page.)

Fig. 2 Effect of lunasin-like polypeptides on the Caco-2 cells proliferation. *Box-plot* showing aggregate data for human colon adenocarcinoma (Caco-2) cells proliferation after treatments of 24 (*panel* **a**), 48 (*panel* **b**), and 72 h (*panel* **c**) with water/salt soluble extracts obtained from control doughs (D), without bacterial inoculum, and sourdoughs (S), started with selected lactic acid bacteria, made with Italian legume flours. Protein concentration of 0.1, 1.0, and 10 mg/ml were assayed. Data were expressed as the mean percentage of viable cells compared to the control culture, grown in basal media without the addition of the WSE. The *centre line* of the *box* represents the median (*open square*), the *top* and *bottom* of the *box* represent the 75th and 25th percentile of the data, respectively. The *top* and *bottom* of the *bars* represent the 5th and 95th percentile of the data, respectively. Outliers (*open circle*) and extremes (*aterisk*) are represented


a
b
c
Viability (%)
120
100
80
60
40
20
D
S
0.1
1.0
10.0
mg/ml
Median
25%-75%
Non-Outlier Range
Outliers
Extremes


A
B
C
FLG mRNA
(relative quantity chart)
12
10
8
6
4
2
0
LPS
FL
CV
CC
LN
PS
D
S

(See figure on previous page.)
Fig. 3 Expression of the filaggrin (*FLG*) gene in Caco-2 cells. The expression of the *FLG* gene in human colon adenocarcinoma cells (Caco-2) was determined using RT-PCR. Caco-2 cells were treated at 37 °C for 4 (**A**), 8 (**B**) and 24 h (**C**) with basal medium containing 1.0 mg/ml of the freeze-dried water/salt soluble extracts obtained from control doughs (D), without bacterial inoculum, and sourdoughs (S), started with selected lactic acid bacteria, made with FL, CV, CC, LN, and PS legume flours. Data are the mean ± SD of three separate experiments, performed in triplicate. *a–c* Columns with different *superscript letters* differ significantly ($P < 0.05$)

the selected *Lactobacillus plantarum* C48 and *Lactobacillus brevis* AM7.

Compared to cereals, all legume flours showed elevated amount of protein (mainly grass pea and several lentil flours), FAA (>2 g/kg), dietary fibers, and ash. In particular, grass pea varieties had the highest concentration of proteins, while pea, chickpea, and grass pea flours contained the highest levels of dietary fibre. The microbiota of legume flours was poorly represented by lactic acid bacteria and yeasts. All the data agreed with previous findings [19].

Recently, the potential of sourdough fermentation was exploited to enhance the nutritional and functional features of legume flours [19]. Apart from the legume species and variety, sourdough fermentation with selected starters is a suitable biotechnology option either to increase the nutritional and functional value or to decrease the levels of anti-nutritional factors [19]. Selected sourdough lactic acid bacteria were able to decrease the concentration of raffinose up to ca. 64 % and a similar trend was found for the concentration of condensed tannins [19]. The sourdough fermentation also increased the concentration of GABA, and promoted antioxidant and phytase activities compared to the raw flours [19].

According to protocols used for cereal sourdough fermentation [20], legume flours were started with *Lactobacillus brevis* AM7 and *Lactobacillus plantarum* C48 [21]. As previously shown [19], the conditions of incubation (24 h at 30 °C) allowed the optimal growth and metabolism of selected lactic acid bacteria [19, 22]. As expected, fermentation caused a marked decrease of the values of pH as well as an increase of TTA and of the concentration of peptides and FAA, especially when kidney bean flours were used.

Based on their proteolytic activity towards vegetable proteins [20], the use of sourdough lactic acid bacteria for synthesizing bioactive peptides deserves a marked interest [23]. Bioactive peptides derived from food proteins may possess physiological properties beyond the role in nutrition. These properties are influenced by the protein source, enzyme and processing conditions used [24]. Most of the research related to bioactive peptides and cancer was focused on lunasin [25] and, recently, on pulse hydrolysates [2].

To the best of our knowledge, no literature data dealt with the presence of lunasin or lunasin-like polypeptides from native protein sequences of Italian pulses, and with the effect of sourdough fermentation on bioactivity and bioavailability.

Proteinase activity and a large portfolio of peptidases are the pre-requisites to liberate bioactive peptides from native oligopeptides [7, 26]. Although with some differences, the two starters used showed different peptidase activities.

Western blot analyses showed that the sequence of lunasin sequence was absent in all the legume flours, and, therefore, in the corresponding sourdoughs. Nevertheless, immunoreactive polypeptides with molecular masses lower than 30 kDa were detectable in all the samples. In some cases, immunoreactive polypeptides appeared as multiple bands in western blot, probably due to the presence of multiple fragments differing for few aminoacid residues [12]. Regarding unfermented doughs, proteins with high intensity signals were found for all the bean, followed by lentil varieties. After lactic acid bacteria fermentation, the number and the intensity of the proteins reacting with the anti-lunasin antibody increased for all the legume flours. This was probably due to an acid activation of endogenous proteinases, responsible for primary proteolysis, and peptidase activities by lactic acid bacteria, which completed the hydrolysis (secondary proteolysis). All the reactive protein bands had molecular masses higher than that of lunasin. Although some differences were found among varieties, one representative was chosen for each legume species to assay the effect on Caco-2 cells proliferation. Caco-2 cells derived from a colonic tumor and have a cancerous phenotype, and can be cultivated to become confluent. In this case, they differentiate into enterocyte-like cells [27].

(See figure on next page.)
Fig. 4 Expression of the involucrin (*IVL*) gene in Caco-2 cells. The expression of the *IVL* gene in human colon adenocarcinoma cells (Caco-2) was determined using RT-PCR. Caco-2 cells were treated at 37 °C for 4 (**A**), 8 (**B**) and 24 h (**C**) with basal medium containing 1.0 mg/ml of the freeze-dried water/salt soluble extracts obtained from control doughs (D), without bacterial inoculum, and sourdoughs (S), started with selected lactic acid bacteria, made with FL, CV, CC, LN, and PS legume flours. Data are the mean ± SD of three separate experiments, performed in triplicate. *a–c* Columns with different *superscript letters* differ significantly ($P < 0.05$)

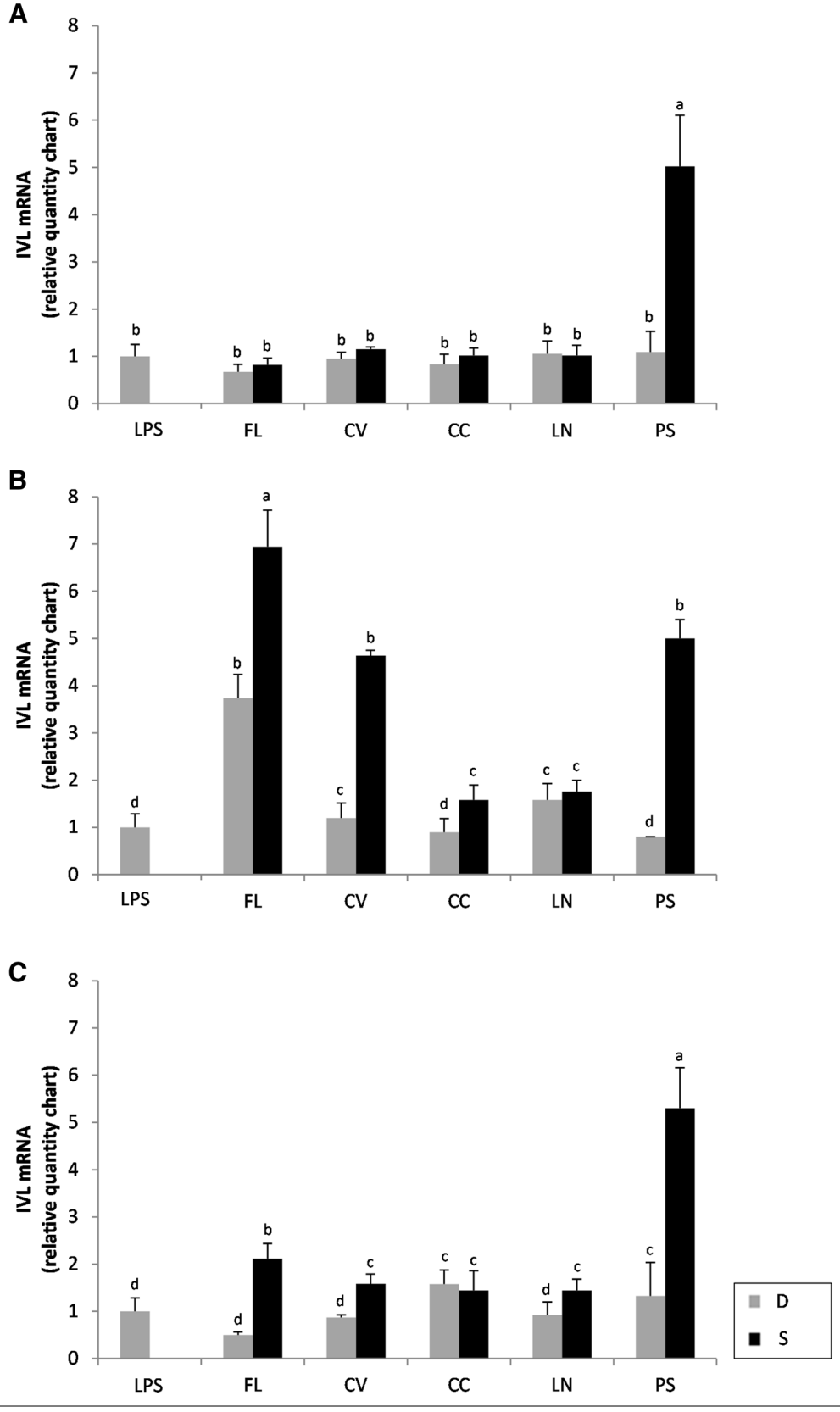

A
IVL mRNA
(relative quantity chart)
LPS
FL
CV
CC
LN
PS
B
IVL mRNA
(relative quantity chart)
LPS
FL
CV
CC
LN
PS
C
IVL mRNA
(relative quantity chart)
LPS
FL
CV
CC
LN
PS
D
S

Table 5 Lunasin-like polypeptides sequences

WSE	Protein	Sequence[a]	NCBI accession number	Theoretical mass (kDa)	Sequence coverage	Mascot score
FL-S	Subtilisin inhibitor 1 (*Vigna angularis*)	QEQGTNPSQEQNVPLPRNYKQALETNTPTKTSWPELVGVTAEQAETKI KEEMVDVQIQVSPHDSFVTADYNPKRVRLYVDESNKVTRTPSIG	P16064	10.38	82	114
	Legumin A2 (*Pisum sativum*)	MATKLLALSLSFCFLLLGGCFALREQPEQNECQLERLNALEPD NRIESEGGLIETWNPNNKQFRCAGVALSRATLQHNALRRPY YSNAPQEIFIQQGNGYFGMVFPGCPETFEEPQESEQGEGR RYRDRHQKVNRFREGDIIAVPTGIVFWMYNDQDTPVIAVSLTDIRSSN NQLDQMPRRFYLAGNHEQEFLRYQHQQGGKQEQENEGNNIF SGFKRDFLEDAFNVNRHIVDRLQGRNEDEEKGAIVKVKGGLSIISP PEKQARHQRGSRQEEDEDEDEERQPRHQRGSRQEEEEDEDEER QPRHQRRRGEEEEEDKKERRGSQKGKSRRQGDNGLEETVCTAKLRL NIGPSSSPDIYNPEAGRIKTVTSLDLPVLRWLKLSAEHGSLHKNAMFV PHYNLNANSIIYALKGRARLQVVNCNGNTVFDGELEAGRALTVPQN YAVAAKSLSDRFSYVAFKTNDRAGIARLAGTSSVINNLPLDVVAATFN LQRNEARQLKSNNPFKFLVPARQSENRASA	P15838	59.63	62	410
	Phaseolin (*Phaseolus vulgaris*)	MMRARVPLLLLGILFLASLSASFATSLREEEESQDNPFYFNSDN SWNTLFKNQYGHIRVLQRFDQQSKRLQNLEDYRLVEFRSKPETLLL PQQADAELLLVVRSGSAILVLVKPDDRREYFFLTSDNPIFSDHQKIPAGI FYLVNPDPKEDLRIIQLAMPVNNPQIHEFFLSSTEAQQSYLQEFSKH ILEASFNSKFEEINRVLFEEEGQQEGVIVNIDSEQIKELSKHAKSSSRK SLSKQDNTIGNEFGNLTERTDNSLNVLISSIEMEEGALFV PHYYSKAIVILVVNEGEAHVELVGPKGNKETLEYESYRAELSKD DVFVIPAAYPVAIKATSNVNFTGFGINANNNNRNLLAGKTDNVISSI GRALDGKDVLGLTFSGSGDEVMKLINKQSGSYFVDAHHHQQEQQ KGRKGAFVY	P02853	47.540	75	515
CV-S	Leucoagglutinating phytohemagglutinin (*Phaseolus vulgaris*)	MASSKFFTVLFLVLLTHANSSNDIYFNFQRFNETNLILQRDASVSSS GQLRLTNLNGNGEPRVGSLGRAFYSAPIQIWDNTTGTVASFATSFT FNIQVPNNAGPADGLAFALVPVGSQPKDKGGFLGLFDGSNSNFHT VAVEFDTLYNKDWDPTERHIGIDVNSIRSIKTTRWDFVNGENAEV LITYDSSTNLLVASLVYPSQKTSFIVSDTVDLKSVLPEWVSVGFSATT GINKGNVETNDVLSWSFASKLSDGTTSEGLNLANLVLNKIL	P05087	29.54	61	506
	Pathogenesis related protein (*Cicer arietinum*)	MGVFTFEQETASTVPPAKLYKAMVKDADVIIPKAVDAIKTVETVEGNGG PGTIKKLTFVEGGQTLYVLHKIEAIDEANLGYNYSIVGGAGLSETVERY HFEAKLCEGPNGGSIGKVSVKYQTKGDAKPNEKEVQEGKAKGDA LFKAIEGYVLANPNYN	CAA56142	16.93	83	408

Table 5 continued

WSE	Protein	Sequence[a]	NCBI accession number	Theoretical mass (kDa)	Sequence coverage	Mascot score
	Seed linoleate 9S-lipoxygenase-3 (*Pisum sativum*)	MFSGVTGILNRGHKIKGTVVLMRKNVLDINSLTTVGGVIGQGFDILG STVDNLTAFLGRSVSLQLISATKPDATGKGKLGKATFLEGIISSLPTL GAGQSAFKIHFEWDDDMGIPGAFYIKNFMQTEFFLVSLTLDDIPN HGSIYFVCNSWIYNAKHHKIDRIFFANQTYLPSETPAPLVHYREEELNN LRGDGTGERKEWERIYDYDVYNDLGNPDSGENHARPVLGGSE TYPYPRRGRTGRKPTRKDPNSESRSDYVYLPRDEAFGHLKSSDFL TYGLKAVSQNVVPALESVFFDLNFTPNEFDSFDEVHGLYEGGIKLPT NILSQISPLPVLKEIFRTDGENTLKYPPPKVIQVSRSGWMTDEEFAREM LAGVNPNVICCLQEFPPRSKLDSQIYGDHTSKISKEHLEPNLEGLT VEEAIQNKKLFLLDHHDSIMPYLRRINSTSTKAYATRTILFLNNNQN LKPLAIELSLPHPQGDEHGAVSYVYQPALEGVESSIWLLAKAYVIVND SCYHQLVSHWLNTHAVVEPFVIATNRHLSCLHPIYKLLYPHYRDT MNINSLARLSLVNDGGIIEKTFLWGRYSMEMSSKVYKNWVFTEQAL PADLIKRGMAIEDPSSPCGVKLVVEDYPYAVDGLEIWAIIKTWVQDY VSLYYTSDEKLRQDSELQAWWKELVEVGHGDKKNEPWWPKMQ TREDLIEVCSIVIWTASALHAAVNFGQYSYGGLILNRPTLSRRFMPEK GSAEFEELVKSPQKAYLKTITPKFQTLIDLSVIEILSRHASDELYLGERD NPNWTSDKRALEAFKKFGNKLAEIEKKLTQRNNDEKLRNRHGPVEM PYTLLYPSSKEGLTFRGIPNSISI	P09918	97.57	43	207
PS-S	Provicilin (*Pisum sativum*)	DNAEIEKILLEEHEKETHHRRGLRDKRQQSQEKNVIVKVSKK QIEELSKNAKSSSKKSVSSRSEPFNLKSSDPIYSNQYGKFFEITPK KNPQLQDLDIFVNYVEIKEGSLWLPHYNSRAIVIVTVNEGKGD FELVGQRNENQQGLREEDDEEEEQREEETKNQVQSYKAKLTPG DVFVIPAGHPVAVRASSNLNLLGFGINAENNQRNFLAGEEDNVIS QIQKQVKDLTFPGSAQEVDRLLENQKQSYFANAQPQQRETRSQEIKE HLYSILGAF	P02855	31.52	53	589

Table 5 continued

WSE	Protein	Sequence[a]	NCBI accession number	Theoretical mass (kDa)	Sequence coverage	Mascot score
	Seed linoleate 9S-lipoxygenase-2 (*Pisum sativum*)	MFPNVTGLLNKGHKIRGTVVLMRKNVLDFNTIVSIGGGNVHGVIDSGI NIIGSTLDGLTAFLGRSVSLQLISATKSDANGKGKVGKDTFLEGV LASLPTLGAGESAFNIHFEWDHEMGIP121GAFYIKNYMQVEF FLKSLTLEDVPNHGTIRFVCNSWVYNSKLYKSPRIFFANKSYLPSET PSPLVKYREEELQTLRGDGTGERKLHERIYDYDVYNDLGNPDHGE HLARPILGGSSTHPYPRRGRTGRYPTRKDPNSEKPATETYVPRDEN FGHLKSSDFLAYGIKSVSQCVVPAFESAFDLNFTPNEFDSFQDVRN LFEGGIKLPLDVISTLSPLPVVKEIFRTDGEQVLKFTPPHVIRVSKSAW MTDEEFAREMLAGVNPCMIRGLQEFPPKSNLDPAEYGDHTSKISVD VLNLDGCTIDEALASGRLFILDYHDTFIPFLRRINETSAKAYATRTILFLK ENGTLKPVAIELSLPHPDGDKSGFVSKVILPADEGVESTIWLLAKAYV VVNDSCYHQLMSHWLNTHAVIEPFVIATNRQLSVVHPINKLLAPHY RDTMMNINALARDSLINANGLIERSFLPSKYAVEMSSAVYKYWVFT DQALPNDLIKRNMAVKDSSSPYGLRLLIEDYPYAVDGLEIWTAIKT WVQDYVSLYYATDNDIKNDSELQHWWKEVVEKGHGDLKDKPWWP KLQTFDELVEVCTIIIWTASALHAAVNFGQYPYGGLILNRPTLSRRLL PEEGTAEYDEMVKSSQKAYLRTITPKFQTLIDLSVIEILSRHASDEVYL GQRENPHWTSDSKALQAFQKFGNKLAEIEAKLTNKNNDPSLYHRVG PVQLPYTLLHPSSKEGLTFRGIPNSISI	P14856	97.07	36	178
	Seed biotin-containing protein SBP65 (*Pisum sativum*)	MASEQLSRRENITTERKIQNAEDSVPQRTTHFELRETHELGPN FQSLPRNENQAYLDRGARAPLSANVSESYLDRARVPLNANIP EHRVREKEDFGGVRDMGKFQMESKGGNKSLAEDRETLDTRSRM VTGTPHIKEASGKGQVVEERERARERAMEEEEKRLTMEEISKYRN QAQQSALEALSAAQEKYERAKQATNETLRNTTQAAQEKGEAAQAK DATFEKTQQGYEMTGDTVSNSARTASEKAAQAKNTTLGKTQQGYE ATRDTVSNAARTAAEYATPAAEKARCVAVQAKDVTLETGKTAAEKAK CAAEIAAKVAVDLKEKATVAGWTASHYATQLTVDGTRAAANAVE GAVGYVAPKASELAAKSVETVKGLAASAGETAKEFTARKKEESWRE YEAKRASQLQEGEEILPSTGGIGKVLPSGERTQAQGTNLQEKVQGK GSDILGAVTETVSDIGSSMIKPIDNANTKVKEHGGTTITPKGQDAG GVLDAIGETIAEIAHTTKVIVVGEDDEVEKSMQKNIGSDSHSLDRAKH EGYRAPKNNVS	Q41060	59.52	46	783
	Albumin-1 C (*Pisum sativum*)	MASVKLASLIVLFATLGMFLTKNVGAISCNGVCSPFDIPPCGSPLCRCI PAGLVIGNCRNPYGVFLRTNDEHPNLCESDADCRKKGSGTFCGHYP NPDIEYGWCFASKSEAEDVFSKITPKDLLKSVSTA	P62928	13.90	55	333

Identification was carried out by nano-LC–ESI–MS–MS. Polypeptides were purified from water/salt-soluble extracts obtained from sourdoughs (-S) made with Fagiolo di Lamon (FL), Cece dell'Alta Valle del Misa (CV), and Pisello riccio di Sannicola (PS)

[a] Single-letter amino acid code is used

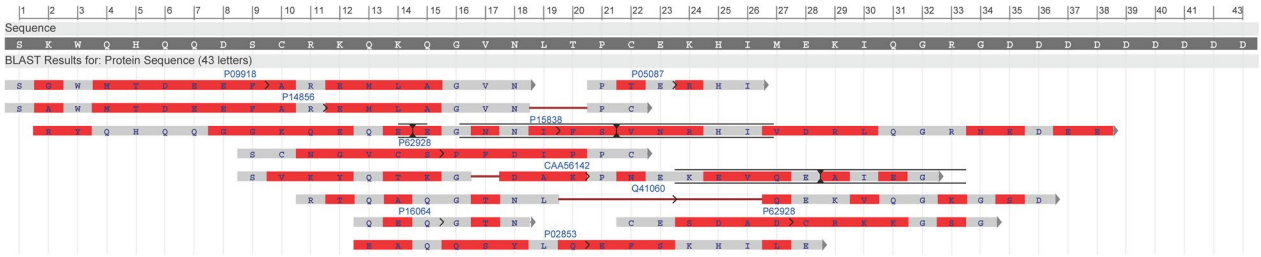


Fig. 5 Alignments with lunasin. Alignments of the lunasin-like polypeptides identified from FL, CV, and PS sourdoughs with the soy lunasin sequence, as obtained through the BLAST alignment on-line tool (http://blast.ncbi.nlm.nih.gov/)

Polypeptides able to regulate cell proliferation and survival were already liberated by enzyme hydrolysis of plant and animal proteins [28]. Such peptides, inhibiting cell growth or promoting apoptosis, could have protective effects on tumor growth at the digestive tract level [29, 30]. In particular, it was found that the antitumoral mechanism of soybean lunasin is related to the capacity of the peptide to bind the deacetylated histones, causing the acetylation inhibition [31, 32]. Girón-Calle et al. [28] assessed the cancer cell proliferation after treatment with chickpea hydrolysates made with pepsin/pancreatin. These hydrolysates inhibited the proliferation of human epithelial colorectal adenocarcinoma cells (Caco-2) and monocytics leukemia cells (THP-1) up to 48 and 78 %, respectively. Hydrolysates from the common bean (*P. vulgaris*) varieties Negro 8025 and Pinto Durango inhibited the inflammation in lipopolysaccharide-induced macrophages through suppression of NF-κB pathways [2]. After hydrolysis with Alcalase, proteins extracted from both the above varieties inhibited various markers of inflammation (cyclooxygenase-2 expression, prostaglandin E2 production, inducible nitric oxide synthase expression, and nitric oxide production) [2]. Inflammation and cancer are linked, chronic inflammation predisposes individuals to various types of cancer and inflammatory mediators and cells are involved in the migration, invasion, and metastasis of malignant cells [33]. The suppression of pro-inflammatory pathways may provide opportunities for both prevention and treatment of cancer [34].

In this study, extracts from legume doughs regulated the proliferation of Caco-2 cells, including those obtained from control doughs, in which very weak signals of immunoreactive bands were found with the western blot analysis. It can be hypothesized that the effect could be related to the contribution of some legume proteins or peptides able to act as anticancer compounds in their native form, causing cytotoxicity and apoptosis of tumoral cells [2, 5]. Nevertheless, a strong inhibition of the Caco-2 cell proliferation was found only after lactic acid fermentation. A marked inhibition was found when cells were treated for 24 h with the fermented legume extracts at concentrations from 1 to 10 mg/ml. Sourdoughs from FL, CV, and PS flours were the most active, showing a decrease of Caco-2 cells vitality up to 70 %. A correlation between the anti-proliferative effect of protein hydrolysates towards Caco-2 cells and the in vivo growth inhibition of tumors in the digestive tract was demonstrated [29, 30].

The nanoESI-MS/MS spectrometer analysis of the immunoreactive protein bands from water/salt-soluble extracts of FL, CV and PS sourdoughs allowed the identification of ten legume proteins. According to the molecular masses of the identified proteins, it can be hypothesized that immunoreactive polypeptides are encrypted into the native sequences and released as fragments during lactic acid bacteria fermentation. Nine of them showed similarities to soy lunasin sequence, probably related to their recognition by the anti-lunasin antibody.

Using Caco-2 cells, the capacity to induce the expression of human *FLG* and *IVL* genes by sourdough fermented legumes was investigated. FLG and IVL are important proteins for the formation of the epidermal skin barrier [35, 36]. FLG aggregates keratin filaments and provides a cytoskeleton for the cornified envelope [35]. The expression of FLG was markedly induced by PS sourdough, and under some of the assayed conditions (e.g., 8 h of treatment), also by CV and FL sourdoughs. Compared to the unfermented dough, the level of IVL gene expression markedly increased when Caco-2 cells were subjected to 24-h treatment with FL, CV, and PS sourdoughs. In particular, the sourdough made with PS was effective under all the conditions likely it was observed for FLG gene expression. IVL serves as a substrate for the covalent attachment of ceramides to the cornified envelope [35, 36]. An improvement of the IVL activity may result in an increase of the ceramide binding activity, which, in turn, positively affects the barrier function [37, 38].

Conclusions

Nowadays, an unexpected and considerable number of small proteins and peptides are available as drugs [39]. They show high bioactivity, target specificity and wide spectrum of therapeutic actions, with low levels of toxicity, structural diversity, and absence or low levels of accumulation in body tissues. However, the manufacturing protocols (e.g. chemical synthesis and recombinant transgenic approach) are very expensive, representing a hindrance for the use as therapeutic peptides [40]. The alternative option is the exploitation of the potential of bioactive peptides derived from food proteins. Legume flours were already proposed as adjuvant ingredients into a range of baked products and snacks for increasing the nutritional value [41–44], as well as sourdough fermentation was shown to have a functional potential [19, 45, 46]. Despite the need to have an in vivo confirmation, the presence of lunasin-like polypeptides allows to hypothesize the use of sourdough fermented legumes in pharmaceuticals preparations (e.g., capsules and powders), protein hydrolysates or as purified peptide mixtures to be incorporated as health-enhancing ingredients in novel functional foods (e.g., leavened baked goods).

Methods

Legumes

Nineteen Italian legume varieties, belonging to the species *Phaseulus vulgaris* (Fagiolo di Lamon, Fagiolo di Controne, Fagiolo di Cuneo, Fagiolo Stregoni, Fagiolo Vellutina, Fagiolo di Saluggia, Fagiolo Badda di Polizzi—white, and Fagiolo Badda di Polizzi—black), *Cicer arietinum* (Cece di Merella and Cece dell'Alta Valle del Misa), *Lathyrus sativus* (Cicerchia di Serra de Conti and Cicerchia di Campodimele), *Lens culinaris* (Lenticchia di Castelluccio di Norcia, Lenticchia di Ustica, Lenticchia di Santo Stefano di Sessanio, Lenticchia rossa di Pantelleria, Lenticchia di Altamura and Lenticchia di Villalba), and *Pisum sativum* (Pisello riccio di Sannicola), were used in this study. All legumes were chosen among the Italian pulses that have specific product certification (Table 1). Flours were obtained from whole legume seeds through the laboratory mill Ika-Werke M20 (GMBH, and Co. KG, Staufen, Germany). Protein (total nitrogen ×5.7), ash and moisture contents were determined according to AACC approved methods 46-11A, 08-01 and 44-15A, respectively [47]. Lipids were determined by Soxhlet method. Total carbohydrates were calculated as the difference, using the following formula: [100 − (proteins + lipids + ash + moisture)]. Proteins, lipids, carbohydrates and ash were expressed as % of dry matter (d.m.). The determination of total dietary fiber was carried out by the enzymatic–gravimetric procedure approved by the Association of Official Analytical Chemists [48], as described by Lee et al. [49].

Microbiological analyses of the flours

Ten grams of flour were homogenized with 90 ml of sterile peptone water [1 % (wt/vol) of peptone and 0.9 % (wt/vol) of NaCl] solution. Lactic acid bacteria were enumerated using MRS (Oxoid, Basingstoke, Hampshire, UK) agar medium, supplemented with cycloheximide (0.1 g/l). Plates were incubated, under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid), at 30 °C for 48 h. Cell density of yeasts and molds were estimated on Yeast Extract Peptone Dextrose Agar (YPD) (Oxoid) medium (pour and spread plate enumeration, respectively), supplemented with chloramphenicol (0.15 g/l), at 30 °C for 72 h. The attribution (yeasts/molds) was confirmed by microscope observation. Total mesophilic aerobic bacteria were determined on Plate Count Agar (PCA, Oxoid) at 30 °C for 48 h, and total enterobacteria were determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid) at 37 °C for 24 h.

Fermentation

Sourdough lactic acid bacteria, belonging to the Culture Collection of the Department of Soil, Plant, and Food Sciences (University of Bari, IT) and previously selected for some biotechnological features, were used. *Lactobacillus plantarum* C48 showed the capacity of synthesizing relevant amount of γ-aminobutyric acid (GABA) [21], and *Lactobacillus brevis* AM7 was characterized by high proteolytic activities towards cereal and legume flours [12]. Starters were cultivated separately on MRS broth at 30 °C for 24 h. Cells were harvested by centrifugation (10,000×*g*, 10 min, 4 °C) until the late exponential phase of growth was reached (ca. 10 h), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0) and re-suspended in tap water at the cell density of ca. 8.0 log cfu/ml. Each legume flour was mixed with tap water, containing the bacterial suspension (initial cell density of 7.0 log cfu/g of sourdough for each strain). Doughs, having dough yield (DY, dough weight × 100/flour weight) of 160 (corresponding to 62.5 and 37.5 % of flour and water, respectively), were mixed at 60 *x g* for 5 min with a IM 58 high-speed mixer (Mecnosud, Flumeri, Italy) and incubated at 30 °C for 24 h. The most common DY value for cereal-based sourdoughs (160) was applied [20, 22, 46, 50]. After fermentation, legume sourdoughs (S) were compared to control doughs (D), without bacterial inoculum, prepared as described above (DY 160) and incubated under the same conditions. S and D were stored at −20 °C before the chemical analyses, while microbiological analysis was carried out before freezing. All the

doughs were obtained in triplicate and each of them was analyzed twice.

Characterization of fermented flours

The values of pH were determined on-line by a pHmeter (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined after homogenization of 10 g of dough with 90 ml of distilled water, and expressed as the amount (ml) of 0.1 M NaOH needed to reach the value of pH of 8.3.

The water/salt-soluble extract (WSE) of D and S was prepared according to Weiss et al. [51], and used to analyze free amino acids (FAA) by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK) with a Na-cation-exchange column (20 by 0.46 cm internal diameter), as described by Rizzello et al. [50].

Peptidase activities of the starters

General aminopeptidase type N (EC 3.4.11.11; PepN), specific aminopeptidase type A (EC 3.4.11.7; PepA) and endopeptidase (EC 3.4.23; PepO) activities were determined as described by Gobbetti et al. [13], using, respectively, Leu-*p*-nitroanilides (*p*-NA), Glu-*p*-NA and NCBZ-Gly-Gly-Leu-*p*-NA (Sigma Aldrich Co.) as relatively specific substrates. The assay mixture contained 900 µl of 2.0 mM substrate in 0.05 M potassium phosphate buffer, pH 7.0, and 100 µl of cell suspension (9 log cfu/ml). The mixture was incubated at 30 °C for 1 h and the absorbance was measured at 410 nm. The data obtained were compared to standard curves set up by using *p*-nitroanilide [52]. One unit (U) of activity was defined as the amount of enzyme required to liberate 1 µmol/min of *p*-nitroanilide under the assay conditions. Tripeptidase (EC3.4.11.4; PepT), and X-prolyl dipeptidyl aminopeptidase (EC 3.4.14.5; PepX) activities were determined using Leu-Leu-Leu and Gly-Pro-Ala substrates (Sigma Aldrich Co.), respectively. Activities on tripeptides were determined by the Cd-ninhydrin method [52], [53]. The assay conditions were the same as those described for *p*-nitroanilide substrates. One unit (U) of activity was defined as the amount of enzyme required to liberate 1 µmol amino acid released per min under the assay conditions. The data obtained were compared to the standard curve set up by using leucine [52].

Western blot analysis

Protein concentration of samples was determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA), using bovine serum albumin (BSA) as standard protein. SDS-PAGE was performed with samples and synthetic lunasin diluted in tricine sample buffer (Bio-Rad, Richmond, CA, USA), containing 2 % (v/v) β-mercaptoethanol, and heated at 100 °C for 5 min. Equal amounts of proteins (20–40 µg) were analyzed on Precast Criterion 16.5 % Tris-Tricine gels (Bio-Rad), and electrophoretic separations were carried out at 100 V, using Tris-Tricine-SDS as running buffer in the Criterion cell (Bio-Rad). For the attribution of the molecular masses, a Precision Plus Protein Standards mix (BioRad) was used. After SDS-PAGE separation, gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20 % methanol, pH 9.2) for 30 min. Proteins were electroblotted into nitrocellulose membranes by semidry transfer in a Trans-Blot SD (Bio-Rad) for 30 min at 18 V. Then, the membranes were blocked for 3 h in Tris buffered saline with 0.05 % (v/v) Tween 20 (TBST), containing 1 % (w/v) bovine serum albumin, (TBST-1 % BSA). Afterwards, the membrane was washed three times with TBST, and incubated overnight at 4 °C with lunasin polyclonal primary antibody (diluted 1:2000 in TBST-0.1 % BSA). After washing with TBST, the membrane was incubated overnight at 4 °C with horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:3000 in TBST-0.1 % BSA). Finally, the membranes were washed six times with TBST, and visualized by chemioluminescence using the detection agent Amersham TM ECL Prime (GE Healthcare, Chalfont St Giles, UK), according to the manufacturer´s recommendations. Image acquisition (exposure time 1–4 min) was performed using the VersaDoc Imaging System (Bio-Rad).

MTT assay in Caco-2 cells

In order to assess the effect of legume D and S on cell proliferation, the viability of colon adenocarcinoma Caco-2 cells was measured using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [54].

Colon adenocarcinoma Caco-2 cells (ICLC HTL97023), provided by the National Institute for Cancer Research of Genoa (Italy), were cultured in RPMI medium, supplemented with 10 % Fetal Bovine Serum (FBS), 1 % 2 mM L-glutamine, 1 % penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml) mixture, 0.1 % gentamicin and 0.1 % β-mercaptoethanol, and maintained in 25 cm^2 culture flasks at 37 °C, 5 % CO_2. Every 2 days, confluent cultures were washed with PBS 1× (without Ca^{2+} and Mg^{2+}), split 1:3–1:6 using Trypsin/EDTA, and seeded at 2–5 × 10^4 cell/cm^2, 37 °C, and 5 % CO_2. For the assay, Caco-2 cells were harvested at 80 % confluence with trypsin/EDTA, seeded at the density of 5 × 10^4 cells/ml into 96-well plates, and then incubated at 37 °C, 5 % CO_2 for 24 h. Before analysis, WSE obtained from legume D and S were partially purified by ultra-filtration, using Vivaspin (GE Healthcare) centrifugal filter units (cut-off 30 kDa), following the manufacturer's instructions.

Cells were exposed to partially purified and freeze dried WSE at the following concentrations: 0.1, 1.0, and 10.0 mg/ml of proteins. Each WSE was tested in duplicate. A control in basal medium, without addition of WSE, was used. Incubation was carried out for 24, 48 and 72 h. After incubation, the medium was removed and replaced by 100 µl/well of MTT solution. Then, plates were incubated in the dark for 3 h (37 °C, 5 % CO_2). MTT salt was dissolved in PBS (5 mg/ml) and added (1:10) to RPMI, containing 10 % FBS, 1 % 2 mM L-glutamine, 1 % penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml) mixture, 0.1 % gentamicin and 0.1 % β-mercaptoethanol. Then, the medium was removed and 100 µl/well of dimethyl sulphoxide (DMSO) were added to dissolve the purple formazan product. Plates were shacked for 15 min at room temperature and the absorbance was read at a wavelength of 570 nm, with a Biotek microplate reader (BioTek Instruments Inc., Bad Friedrichshall, Germany), and elaborated with the ELX808 software (BioTek Instruments Inc., Bad Friedrichshall, Germany). Data were expressed as the mean percentage of viable cells compared to control culture, grown in basal media without addition of WSE.

Identification of the lunasin-like polypeptides

After Western blot analysis, the immunoreactive bands, separated by Tris-Tricine SDS PAGE, were cut and stored in 20 % ethanol before identification. Protein identification was performed by Proteome Factory (Proteome Factory AG, Berlin, Germany). Protein bands were in-gel digested by trypsin (Promega, Mannheim, Germany) and analyzed by Nano-Liquid Cromatography-Electrospray Ionisation-Mass Spectrometry (nanoLC-ESI–MS/MS). The LC–MS system consisted of an Agilent 1100 nanoHPLC system (Agilent, Waldbronn, Germany), PicoTip electrospray emitter (New Objective, Woburn, MA, USA) and an Orbitrap XL or LTQFT Ultra mass spectrometer (ThermoFisher Scientific, Bremen, Germany). Peptides were first trapped and desalted on the enrichment column (Zorbax 300SB-C18, 0.3 × 5 mm, Agilent) for 5 min (solvent: 2.5 % acetonitrile/0.5 % formic acid), then separated on a Zorbax 300SB-C18, 75 µm × 150 mm column (Agilent), using a linear gradient from 10 to 32 % B (solvent A: 5 % acetonitrile in water, solvent B: acetonitrile, both with 0.1 % formic acid). Ions of interest were data-dependently subjected to MS/MS, according to the expected charge state distribution of peptide ions. Proteins were identified by database search against the plant sequences of the National Center for Biotechnology Information, (NCBInr, Bethesda, USA) protein database, using MS/MS ion search of the Mascot search engine (Matrix Science, London, UK). Only peptide matches with a score of 20 or above were accepted. The sequences of the protein identified were aligned using the BLAST on-line tools (http://blast.ncbi.nlm.nih.gov).

Transcriptional regulation of filaggrin (*FLG*) and involucrin (*IVL*) genes

Colon adenocarcinoma Caco-2 cells (ICLC HTL97023) were cultured in RPMI medium supplemented with 10 % FBS, 1 % 2 mM L-glutamine, 1 % penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml) mixture, 0.1 % gentamicin and 0.1 % β-mercaptoethanol and maintained in 25 cm^2 culture flasks at 37 °C, 5 % CO_2. Caco-2 cells were incubated in 25-cm^2 culture flasks at 37 °C, under 5 % CO_2 atmosphere [55].

Subconfluent monolayers of Caco-2 cells were subjected to treatment with basal medium, containing 2.5 % FBS. The freeze-dried WSE, partially purified by ultra-filtration using Vivaspin (GE Healthcare) centrifugal filter units (cut-off 30 kDa), were dissolved in RPMI medium, at the concentration of 10 mg/ml and added to the culture media at the final concentration of 1 mg/ml. The control was the basal medium, containing 2.5 % FBS. Plates were incubated at 37 °C for 24 h, under 5 % CO_2. Samples were taken after 4, 8, and 24 h of treatment. Each experiment was carried out at least twice in triplicate. For quantitative real-time PCR (RT-PCR), total RNA was extracted from Caco-2 cells using the Ribospin Minikit-GeneAll kit. cDNA was synthesized from 2 µg RNA template in a 20-µl reaction volume, using the Prime Script RT reagent kit (perfect Real time) (Takara). Total RNA solution (10 µl) was added to the Master Mix and subjected to reverse transcription in a thermal cycler (Stratagene Mx3000P Real-time PCR System, Agilent Technologies Italia Spa, Milan, Italy). The conditions were 37 °C for 15 min, 85 °C for 5 s, holding the samples at 25 °C.

The cDNA was amplified and detected using the same instrument and the TaqMan assay (Applied Biosystems). The following Taqman gene expression assays were used: FLG Hs00863478_g1 (FLG), IVL Hs00846307_s1 (IVL), and GAPDH Hs99999905_m1 (human glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Human GAPDH was used as the housekeeping gene. PCR amplifications were carried out using 40 ng cDNA in a total volume of 20 µl. The reaction mixture contained 10 µL of Premix Ex Taq, 0.4 µl of RoxTM reference dye II, 1 µL of 20X TaqMan Gene Expression assay and 4 µl of cDNA.

PCR conditions were 95 °C for 30 s (for AmpliTaq activation), followed by 40 amplification cycles (95 °C for 5 s, 60 °C for 20 s). Analyses were carried out in triplicate. Based on preliminary results, the expression of FLG and IVL genes was also assayed by treating Caco-2 cells with lipopolysaccharide (LPS; Sigma Aldrich Co.) at 10 µg/ml in basal medium, containing 2.5 % FBS. Analyses were

carried out in triplicate. The average value of target gene was normalized using *GAPDH* gene and the values were elaborated automatically by the MXPro v.4.01 Stratagene software [56].

Statistical analysis

Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software, Statistica 7.0 for Windows. Student's *t* test was used for MTT assay (GraphPAD 6.0 for Windows).

Abbreviations

BSA: bovine serum albumin; D: dough; DY: dough yield; FBS: fetal bovine serum; FLG: filaggrin; GABA: γ-aminobutyric acid; IVL: involucrin; MTT: 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; S: sourdough; TBS: tris buffered saline; TTA: total titratable acidity; WSE: water/salt-soluble extract.

Authors' contributions

CGR was responsible for the experimental design of the work, the identification of polypeptides, and the elaboration of all the data; BHL and SFT performed immunological analyses and purification of polypeptides; JAC carried out fermentations and microbiological analyses; DP and BM performed all the analyses on Caco-2 cells; RC performed the biochemical characterization of samples; MG was the supervisor and the coordinator of the research unit. All authors read and approved the final manuscript.

Author details

[1] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy. [2] Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM CEI UAM + CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain. [3] Giuliani S.p.a., Milano, Italy. [4] Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland.

Competing interests

The authors declare that they have no competing interests.

Received: 2 September 2015 Accepted: 9 October 2015
Published online: 22 October 2015

References

1. Food and Agriculture Organization of the United Nations (Rome) (FAO) Cereals, pulses, legumes and vegetable proteins. CODEX Alimentarius. Italy: FAO Corporate Document Repository; 2007. p. 1–96.
2. López-Barrios L, Gutiérrez-Uribe JA, Serna-Saldívar SO. Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. J Food Sci. 2014;79:R273–83.
3. Duranti M. Grain legume proteins and nutraceutical properties. Fitoterapia. 2006;77:67–82.
4. Leterme P. Recommendations by health organizations for pulse consumption. Br J Nutr. 2002;88:239–42.
5. Roy F, Boye J, Simpson B. Bioactive proteins and peptides in pulse crops: pea, chickpea and lentil. Food Res Int. 2010;43:432–42.
6. Champ MMJ. Non-nutrient bioactive substances of pulses. Br J Nutr. 2002;88:307–19.
7. Gobbetti M, Di Cagno R, De Angelis M. Functional microorganisms for functional food quality. Crit Rev Food Sci Nutr. 2010;50:716–27.
8. Shahidi F, Zhong Y. Bioactive peptides. J AOAC Int. 2008;91:914–31.
9. Hernández-Ledesma B, Lumen BO, De Hsieh C. 1997–2012: fifteen years of research on peptide lunasin. In: Hernández-Ledesma B, Chia-Chien H, editors. Bioactive food peptides in health and disease. Rijeka: InTech; 2013. p. 3–22.
10. Hernández-Ledesma B, Lumen BO. Lunasin: a novel cancer preventive seed peptide. Perspect Medicin Chem. 2008;2:75.
11. Jeong HJ, Jeong JB, Kim DS, Park JH, Lee JB, Kweon DH, Chung GY, Seo EW, Ben O. The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. Cancer Lett. 2007;255:42–8.
12. Rizzello CG, Nionelli L, Coda R, Gobbetti M. Synthesis of the cancer preventive peptide lunasin by lactic acid bacteria during sourdough fermentation. Nutr Cancer. 2012;64:111–20.
13. Gobbetti M, De Angelis M, Corsetti A, Di Cagno R. Biochemistry and physiology of sourdough lactic acid bacteria. Trends Food Sci Tech. 2005;16:57–69.
14. Dia VP, Frankland-Searby S, Laso del Hierro F, Garcia G, de Mejia EG. Structural property of soybean lunasin and development of a method to quantify lunasin in plasma using an optimized immunoassay protocol. Food Chem. 2013;138:333–41.
15. Kohajdová Z, Karovičová J, Magala M. Effect of lentil and bean flours on rheological and baking properties of wheat dough. Chem Pap. 2013;67:398–407.
16. McCrory MA, Hamaker BR, Lovejoy JC, Eichelsdoerfer PE. Pulse consumption, satiety, and weight management. Adv Nutr Int Rev J. 2010;1:17–30.
17. Gómez M, Oliete B, Rosell CM, Pando V, Fernández E. Studies on cake quality made of wheat—chickpea flour blends. LWT Food Sci Technol. 2008;41:1701–9.
18. Angioloni A, Collar C. High legume-wheat matrices: an alternative to promote bread nutritional value meeting dough viscoelastic restrictions. Eur Food Res Technol. 2012;234:273–84.
19. Curiel JA, Coda R, Centomani I, Summo C, Gobbetti M, Rizzello CG. Exploitation of the nutritional and functional characteristics of traditional Italian legumes: the potential of sourdough fermentation. Int J Food Microbiol. 2015;196:51–61.
20. Rizzello CG, Cassone A, Di Cagno R, Gobbetti M. Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. J Agric Food Chem. 2008;56:6936–43.
21. Siragusa S, De Angelis M, Di Cagno R, Rizzello C, Coda R, Gobbetti M. Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Envir Microbiol. 2007;73:7283–90.
22. Coda R, Rizzello CG, Gobbetti M. Use of sourdough fermentation and pseudo-cereals and leguminous flours for the making of a functional bread enriched of γ-aminobutyric acid (GABA). Int J Food Microbiol. 2010;137(2):236–45.
23. Coda R, Rizzello CG, Pinto D, Gobbetti M. Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. Appl Environ Microbiol. 2012;78:1087–96.
24. Mine Y, Li-Chan E, Jiang B. Biologically active food proteins and peptides in health: an overview. In: Mine Y, Li-Chan E, Jiang B, editors. Bioactive proteins and peptides as functional foods and nutraceuticals. Hoboken: Wiley-Blackwell; 2010. p. 5–11.
25. Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: production, processing, and potential health benefits. J Food Sci. 2012;77:R11–24.
26. De Angelis M, Rizzello CG, Fasano A, Clemente MG, De Simone C, Silano M, De Vincenzi M, Losito I, Gobbetti M. VSL# 3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for celiac sprue probiotics and gluten intolerance. BBA Mol Basis Dis. 2006;1762:80–93.
27. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev. 2012;64:280–9.
28. Girón-Calle J, Alaiz M, Vioque J. Effect of chickpea protein hydrolysates on cell proliferation and in vitro bioavailability. Food Res Int. 2010;43:1365–70.
29. Ganjam L, Thornton W, Marshall R, MacDonald R. Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. J Dairy Sci. 1997;80:2325–9.
30. Kannan A, Hettiarachchy N, Johnson MG, Nannapaneni R. Human colon and liver cancer cell proliferation inhibition by peptide hydrolysates derived from heat-stabilized defatted rice bran. J Agr Food Chem. 2008;56:11643–7.

31. Galvez AF, Chen N, Macasieb J, Ben O. Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. Cancer Res. 2001;61:7473–8.
32. Seber LE, Barnett BW, McConnell EJ, Hume SD, Cai J, Boles K, Davis KR. Scalable purification and characterization of the anticancer lunasin peptide from soybean. PLoS One. 2012;7:e35409.
33. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436–44.
34. Aggarwal BB, Vijayalekshmi R, Sung B. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res. 2009;15:425–30.
35. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Bio. 2005;6:328–40.
36. Marekov LN, Steinert PM. Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. J Biol Chem. 1998;273:17763–70.
37. Macheleidt O, Kaiser HW, Sandhoff K. Deficiency of epidermal protein-bound &ohgr;-hydroxyceramides in atopic dermatitis. J Invest Dermatol. 2002;119:166–73.
38. Jensen JM, Fölster-Holst R, Baranowsky A, Schunck M, Winoto-Morbach S, Neumann C, Schütze S, Proksch E. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. J Invest Dermatol. 2004;122:1423–31.
39. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug Discov Today. 2010;15:40–56.
40. Agyei D, Danquah MK. Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. Biotech Adv. 2011;29:272–7.
41. Anton AA, Fulcher RG, Arntfield SD. Physical and nutritional impact of fortification of corn starch-based extruded snacks with common bean (*Phaseolus vulgaris* L.) flour: effects of bean addition and extrusion cooking. Food Chem. 2009;113:989–96.
42. Anton AA, Ross KA, Lukow OM, Fulcher RG, Arntfield SD. Influence of added bean flour (*Phaseolus vulgaris* L.) on some physical and nutritional properties of wheat flour tortillas. Food Chem. 2008;109:33–41.
43. Betancur-Ancona D, Martínez-Rosado R, Corona-Cruz A, Castellanos-Ruelas A, Jaramillo-Flores ME, Chel-Guerrero L. Functional properties of hydrolysates from *Phaseolus lunatus* seeds. Int J Food Sci Tech. 2009;44:128–37.
44. Borsuk Y. Incorporation of pulse flours with coarse and fine particle size milled from green lentils (*Lens culinaris*), yellow peas (*Pisum sativum* L.), navy beans (*Pisum sativum* L.), and pinto beans (*Phaseolus vulgaris* L.) into baked products. Masters thesis. Winnipeg, Canada University of Manitoba. 2011.
45. Coda R, Melama L, Rizzello CG, Curiel JA, Sibakov J, Holopainen U, Pulkkinen M, Sozer N. Effect of air classification and fermentation by *Lactobacillus plantarum* VTT E-133328 on faba bean (*Vicia faba* L.) flour nutritional properties. Int J Food Microbiol. 2015;193:34–42.
46. Rizzello CG, Calasso M, Campanella D, De Angelis M, Gobbetti M. Use of sourdough fermentation and mixture of wheat, chickpea, lentil and bean flours for enhancing the nutritional, texture and sensory characteristics of white bread. Int J Food Microbiol. 2014;180:78–87.
47. AACC. Approved methods of the American Association of Cereal Chemistry. 10th ed. St. Paul: AACC; 2003.
48. AOAC. Official methods of analysis of AOAC International, vol 1. 16th ed. Arlington: AOAC; 1995.
49. Lee S, Prosky L, De Vries J. Determination of total, soluble, and insoluble, dietary fiber in foods - enzymaticgravimetric-enzymatic-gravimetric method, MES-TRIS buffer: collaborative study. J Assoc Off Anal Chem. 1992;75:395–416.
50. Rizzello CG, Nionelli L, Coda R, De Angelis M, Gobbetti M. Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem. 2010;119:1079–89.
51. Weiss W, Vogelmeier C, Görg A. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. Electrophoresis. 1993;14:805–16.
52. Gobbetti M, Lanciotti R, De Angelis M, Corbo MR, Massini R, Fox PF. Study of the effects of temperature, pH and NaCl on the peptidase activities of non-starter lactic acid bacteria (NSLAB) by quadratic response surface methodology. Int Dairy J. 1999;9:865–75.
53. Rizzello CG, De Angelis M, Di Cagno R, Camarca A, Silano M, Losito I, De Vincenzi M, De Bari MD, Palmisano F, Maurano F. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. Appl Environ Microbiol. 2007;73:4499–507.
54. Curiel JA, Pinto D, Marzani B, Filannino P, Farris GA, Gobbetti M, Rizzello CG. Lactic acid fermentation as a tool to enhance the antioxidant properties of *Myrtus communis* berries. Microb Cell Fact. 2015;14:67.
55. Sanchez L, Mitjans M, Infante MR, Vinardell MP. Assessment of the potential skin irritation of lysine-derivative anionic surfactants using mouse fibroblasts and human keratinocytes as an alternative to animal testing. Pharm Res. 2004;21:1637–41.
56. Rizzello CG, Coda R, Macías DS, Pinto D, Marzani B, Filannino P, Giuliani G, Paradiso VM, Di Cagno R, Gobbetti M. Lactic acid fermentation as a tool to enhance the functional features of *Echinacea* spp. Microb Cell Fact. 2013;12:44.

16

An Update on Lunasin Research, a Bioactive Seed Peptide for Health Promotion

SAMUEL FERNÁNDEZ-TOMÉ AND BLANCA HERNÁNDEZ-LEDESMA*

ABSTRACT

Among the most prevalent and costly health conditions, chronic diseases have become the medical challenge of the 21st century, being the management of lifestyle habits defined as the most strategic tool for the prevention of this public issue. Termed as nutraceuticals, several dietary compounds have revealed desirable health-promoting benefits beyond basic nutrition. Firstly identified in soybean and in other seeds and plants afterwards, the bioactive peptide lunasin has proven properties that allow to point to this 43-amino acids peptide as a potential and promising chemo-preventive candidate against chronic diseases. The purpose of this chapter is to summarize the current evidence on presence of lunasin in foods, bioavailability and biological activity. The protective benefits demonstrated against cancer, oxidative stress, inflammation and cardiovascular disorders are described, and the physiological relevance as well as the molecular mechanisms involved in these effects have also been discussed.

***Key words*:** Lunasin, Bioactive peptide, Chemopreventive properties, Cancer, Chronic diseases

Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain.

**Corresponding author*: E-mail: b.hernandez@csic.es

INTRODUCTION

Chronic diseases including cardiovascular and metabolic syndrome-associated disorders and cancer are the most common diseases leading to death worldwide. Approximately half of the total death occurring from these chronic disorders can be attributed to modificable risks, and thus, changes in nutritional and lifestyle habits are considered the best preventive strategy. A large number of compounds naturally present in the diet have been demonstrated to exert beneficial effects on the health. Bioactive peptides, naturally present in foods or released from the source protein by processing conditions or *in vivo* gastrointestinal digestion, may act on different body systems through their diverse biological activities. Lunasin is one of the naturally occurring plant peptides which demonstrated antioxidant, anti-proliferative and anti-inflammatory properties have made it to be considered one of the most promising food-derived chemopreventive agents. Different research institutions worldwide have conducted or are currently conducting studies on this peptide to evaluate its bioavailability and biological activity, and to elucidate its complete mechanism of action. This chapter reviews the current data of the beneficial properties of lunasin against cancer and other chronic diseases as evidenced by cell culture experiments, animal models and clinical trials.

LUNASIN IN PLANTS AND FOOD: FROM DISCOVERY TO CHARACTERIZATION IN COMMERCIAL PRODUCTS

Soybean lunasin is a 43-amino acids peptide corresponding to the small subunit of the 2S albumin (Galvez and de Lumen, 1999). It is characterized by a high content of charged amino acids (19 in total), including a continuous sequence of nine aspartate residues at the C-terminus (sequence accession AAB71140). Studies carried out in the last decade have purified lunasin from different soybean species quantifying its concentration that is ranged from 500–8130 µg lunasin/g seed or 1100–14000 µg lunasin/g extracted protein (Table 1). The soybean genotype has been demonstrated to be the main affecting factor on quantity of lunasin in soybean seeds, indicating the possibility of selecting and breeding varieties of soybean with higher content of this peptide (de Mejia *et al*., 2004). Other factors, such as the stage of seed development and maturation, environmental factors, and processing conditions have been also reported to influence on concentration of lunasin in both soybean seeds and derived foods (Wang *et al*., 2008; Hernández-Ledesma *et al*., 2009b; Paucar-Menacho *et al*., 2010a, b; Cavazos *et al.*, 2012).

Once the chemopreventive properties of lunasin were suggested, researchers focused their interest on identifying this peptide in other cereals, legumes and medicinal plants. As shown in Table 1, lunasin has been found in different cereals such as wheat, barley, rye, oat and triticale at different

Table 1: Lunasin´s presence in seeds, grains and plants: characterization techniques and determination of its content

Source	***Isolation/ Purification***	***Identification***	***Quantification***[a]	***Ref.(s)***
Soybean	Ion exchange column chromatography	Commassie Blue staining and Western Blot	N.R.	Jeong *et al*. (2003)
	Immunoaffinity column chromatography	MALDI peptide mass mapping (in-gel tryptic digest)		
	Proteins extraction procedures	Indirect ELISA	1100–14000[b]	de Mejia *et al*. (2004)
		Commassie Blue staining and Western Blot		
	Ion exchange column chromatography	Western Blot	500–8130	Jeong *et al*. (2007a)
	HPLC reverse phase	HPLC (Comparison with standard synthetic lunasin)		
	Ultrafiltration	Indirect ELISA	N.R.	Dia *et al*.
	Ion exchange column chromatography	Commassie Blue staining and Western Blot		(2009b)
	Size exclusion column chromatography	HPLC and MALDI-TOF (comparison with standard synthetic lunasin)		
		LC/MS-MS peptide mass mapping (in-gel tryptic digest)		
	Ion exchange column chromatography	Direct ELISA	N.R.	Seber *et al*. (2012)
	Reduction and ultrafiltration	Commassie Blue staining and Western Blot		
	HPLC reverse phase	ESI-MS LC-MS/MS peptide mass mapping (in-gel tryptic digest)		
Barley	Ion exchange column chromatography	Commassie Blue staining and Western Blot	13.6–21.5	Jeong *et al*. (2002)
	Immunoaffinity column chromatography	MALDI peptide mass mapping (in-gel tryptic digest)		
	HPLC reverse phase	Western Blot HPLC (comparison with standard synthetic lunasin)	12.7–99.0	Jeong *et al*. (2010b)
Wheat	Ion exchange column chromatography	Western Blot HPLC (comparison with standard synthetic lunasin)	211.1–290.2	Jeong *et al*. (2007b)

Table 1: (*Contd...*)

Table 1: (*Contd...*)

Source	***Isolation/ Purification***	***Identification***	***Quanti-fication***[a]	***Ref.(s)***
		LC-ESI-MS peptide mass mapping (in-gel tryptic digest)		
	HPLC reverse phase - ESI-MS/MS (comparison with standard synthetic lunasin)		10.0–233.0	Nakurte *et al*. (2012)
Rye	HPLC reverse phase	Commassie Blue staining and Western Blot	45.4 – 150.1	Jeong *et al*. (2009)
		HPLC (comparison with standard synthetic lunasin)		
	HPLC reverse phase - ESI-MS/MS (comparison with standard synthetic lunasin)		733.0–1510.0	Nakurte *et al*. (2012)
Oat	HPLC reverse phase - ESI-MS/MS (comparison with standard synthetic lunasin)		34.0–197.0	Nakurte *et al*. (2013)
Triticale	HPLC reverse phase - ESI-MS/MS (comparison with standard synthetic lunasin)		429.0–6458.0	Nakurte *et al*. (2012)
Amaranth	Fractionation of seed proteins	Indirect ELISA	9.5–12.1[c]	Silva-Sanchez
	Two-dimensional electrophoresis	Western Blot		*et al*. (2007)
	Immuno-precipitation	MALDI-TOF peptide mass mapping (in-gel tryptic digest)		
	Fractionation of seed proteins	Commassie Blue staining and Western Blot	N.R.	Maldonado-Cervantes *et al*. (2010)
	Two-dimensional electrophoresis	Cloning and characterization of Open Reading Frame		
		LC-MS peptide mass mapping (in-gel tryptic digest)		
Solanaceae family	N.R.	Western Blot	132.0–1810.0	Jeong *et al*. (2007c)
*Solanum nigrum*L.	Ion exchange column chromatography	Commassie Blue staining and Western Blot	1810.0	Jeong *et al*. (2007c)
	HPLC reverse phase	HPLC (comparison with standard synthetic lunasin)		Jeong *et al*. (2010a)

[a] Quantification range between the different assessed varieties given in µg lunasin/g seed, except where otherwise indicated
[b] µg lunasin/g extracted protein
[c] µg lunasin equivalent/g total extracted protein
N.R.: non-reported

concentrations. Because of the absence of 2S albumins in cereals, Mitchell *et al*. (2013) brought into question the origin of lunasin. These authors carried out extensive searches of proteomic, transcriptome, and DNA sequences databases for wheat and other cereals that failed to identify sequences with similarity to those encoding the lunasin peptide in soybean. Similar results have been recently reported by Dinelli *et al*. (2014) using chemical (liquid chromatography coupled to mass spectrometry, LC-ESI/MS) and molecular (polymerase chain reaction, PCR) analysis that pointed out the absence of lunasin in the investigated wheat genotypes. Until now, a definitive explanation for these results has not been found. Rizzello *et al*. (2011) have suggested a possible microbial origin of lunasin. These authors indicated that lunasin is synthesized and secreted by microorganisms, either as a mature peptide or a precursor which is then processed by a microbial or endogenous plant proteinase. Further studies are thus needed to elucidate the real origin of this peptide.

LUNASIN´S BIOAVAILABILITY

Food-derived compounds are expected to exert their activities after being taken orally, thus bioavailability is a crucial property for any preventive agent, and studies demonstrating its ability to survive degradation by digestive enzymes and to reach the target tissues in an intact and active form after passing the gastrointestinal tract are crucial. *In vitro* bioavailability studies simulating gastrointestinal digestion have demonstrated that lunasin, both synthetic as well as purified from food is markedly hydrolyzed by pepsin and pancreatin; however, lunasin in foods has been shown to resist the action of these enzymes. Naturally present protease inhibitors in foods, such as Bowman-Birk protease inhibitor (BBI) and Kunitz trypsin inhibitor (KTI), have been found to be major responsible for the lunasin's protection against gastrointestinal enzymes (Jeong *et al*., 2007a,b,c; Park *et al*., 2007; Hsieh *et al*., 2010c). Presence of BBI in soybean products and its role protecting lunasin from *in vitro* digestion has been reported (Hernández-Ledesma *et al*., 2009b).

In vivo bioavailability studies carried out with animals and humans have also confirmed above *in vitro* findings. Hsieh *et al*. (2010c) demonstrated not only that lunasin was bioavailable when orally administered to mice and rats, being absorbed through gastrointestinal barrier and extensively distributed reaching different tissues such as liver, kidney, lung, mammary gland, prostate, and brain, but also that lunasin extracted from the liver and blood of rats fed lunasin-enriched soy flour was bioactive and able to suppress foci formation as effectively as equimolar amount of synthetic lunasin. In this regard, several studies have found an intact and bioactive form of this peptide in the liver and blood of rats fed lunasin-enriched soybean (Jeong *et al*., 2007a), wheat (Jeong *et al.*, 2007b), rye (Jeong *et al*., 2009), and barley (Jeong *et al.*, 2010b) diets. Interestingly,

determination and quantification of lunasin in human plasma after consumption of soy protein has been reported (Dia *et al*., 2009a). These authors estimated an average of 4.5% absorption of lunasin from the total lunasin ingested from 50 g of soy protein. Moreover, it has been recently developed an optimized method for the direct isolation and quantification of lunasin from plasma using an ion-exchange microspin column and a validated immunoassay protocol (Dia *et al.,* 2013).

Peptide bioavailability after oral administration is one of the main reasons for the inconsistency between *in vitro* and *in vivo* and clinical studies, and it must be one of the primary aspects to be studied before food health claims and implementation of food-based therapies (Bhutia and Maiti, 2008; Segura-Campos *et al*., 2011). All these results indicate that lunasin is bioavailable after ingestion due to the protective role of naturally occurring protease inhibitors, and are relevant in supporting future clinical trials demonstrating the potential chemo-preventive properties of this peptide.

CHEMOPREVENTIVE PROPERTIES OF LUNASIN

Lunasin´s Roles in Cancer Prevention

First results in the chemopreventive role of lunasin were casually found in a project to enhance the nutritional quality of soy protein through bioengineering (Galvez *et al*., 1997). Lunasin peptide was identified in a gen (GM2S-1) coding not only for the methione-rich protein that was sought but also for other three proteins, a signal peptide, a linker peptide, and a 43-amino acids small subunit (SKWQHQQDSCRKQLQG VNLTPCEKHIMEKIQGRGDDDDDDDDD) termed lunasin. Transfection and constitutive expression of the lunasin cDNA into *Escherichia coli* and mammalian cells (murine hepatoma and fibroblast cells, and human breast cancer cells) led to mitotic arrest and cell death, by lunasin's binding to the kinetochore of the centromere, hampering its attachment with microtubules and subsequent cellular events involved in the mitotic replication process (Galvez and de Lumen, 1999). Elucidation of lunasin's sequence contributed on suggesting these effects. It contains at its carboxyl end: (i) a poly-D carboxyl end with 9 D-residues, with a high negatively charge, suggested to preferentially bind to positively charged deacetylated histones and cellular regions, such as the hypoacetylated chromatin found in telomeres and centromeres; (ii) a RGD motif, known to allow attachment to the extracellular matrix; and (iii) a predicted helix with structural homology to a conserved region of chromatin-binding proteins Galvez *et al*., 2001; de Lumen, 2005).

These first analyses prompted lunasin as a potential and promising chemopreventive candidate against cancer, and since then, several studies

focused on verifying this role have been carried out. Tables 2 and 3 summarize evidence on the chemo-preventive properties of this peptide proven in different cancer cell lines and animal models, respectively. Moreover, discussion of the cellular mechanisms involved in its antiproliferative activity is also included below.

First studies demonstrated that lunasin, in absence of carcinogens, seems not to affect neither cell morphology nor cell proliferation. However, when a transformation event occurs lunasin gets into action within the cell preventing induced-carcinogenic processes. As shown in Table 2 lunasin has been found to suppress transformation of both chemical and viral and *ras*-oncogenes-induced carcinogenesis in mammalian cells. Besides, subsequent studies have proved that lunasin peptide also exerts several anti-neoplastic effects in established cancer breast, colon, prostate, leukemia, and lymphoma cells lines. Moreover, efforts demonstrating the *in vivo* chemo-preventive role of this peptide have been designed in chemical carcinogen-induced and xenograft animal models (Table 3).

Table 2: Chemopreventive effects and mechanisms of action of peptide lunasin demonstrated by cell experiments

Type of cancer	***Cell line***	***Evidence***	***Reference(s)***
Breast cancer	MDA-MB-231	Inhibition of cell proliferation	Hsieh *et al.* (2010a, 2011a)
		Arrest cell cycle at S-phase	Hernández-Ledesma *et al.* (2011)
		Inhibition of H3 and H4 acetylation	
		Up-regulation of Rb gene expression	
		Down-regulation of cyclins D1, D3, CDK4 and CDK6 and CDC25A, caspase-8, Ets2, Myc, Erbb2, PIK3R1 and JUN signaling genes expression	
		Synergistic effect with aspirin and anacardic acid	
	MCF-7	Up-regulation of tumor suppressor PTEN expression, protein level, and nuclear localization	Pabona *et al.* (2013)
		Induction of apoptosis *via* PTEN	
		Promotion of E-cadherin and β-catenin non-nuclear levels	
Colon cancer	HT-29	Inhibition of cell proliferation	Dia and de Mejia (2010)
		Arrest cell cycle at G2/M phase	
		Cyclin-dependent kinase p21 increased expression	

Table 2: (*Contd...*)

Table 2: (*Contd...*)

Type of cancer	***Cell line***	***Evidence***	***Reference(s)***
		Induction of the apoptotic mitochondrial pathway by modulating expression of Bcl-2, Bax, nCLU, and caspase-3	
		Synergistic effect with cysplatin	
	KM12L4	Cytotoxicity to the cell line, and its oxaliplatin-resistant variant	Dia and de Mejia (2011b, c)
		Arrest cell cycle at G2/M phase	
		Cyclin-dependent kinase p21 and p27 increased expression	
		Induction of the apoptotic mitochondrial pathway by modulating expression of Bcl-2, Bax, nCLU, cytochrome c and caspase-2, -3 and -9	
		Nuclear condensation and DNA fragmentation	
		Inhibition of FAK/ERK and NF–κB signaling pathways	
		Binding to $\alpha_5\beta_1$ integrin, and down-regulation of its expression	
		Modulation on the expression of angiogenesis and metastasis-related genes	
		Synergistic effect with cysplatin	
	HCT-116	Cytotoxicity to the cell line, and its oxaliplatin-resistant variant	Dia and de Mejia (2011b, c)
		Inhibition of FAK/ERK and NF–κB signaling pathways	
	RKO	Cytotoxicity to the cell line, and its oxaliplatin-resistant variant	Dia and de Mejia (2011b, c)
		Inhibition of FAK/ERK and NF–κB signaling pathways	
		Inhibition of cell migration	
Prostate cancer	RWPE-2	Up-regulation of expression of chemo-preventive genes involved in tumor suppression, apoptosis, mitotic check-point, protein degradation, and cellular communication pathways	Galvez *et al*. (2011)
		Inhibition of H4K8, and induction of H4K16 acetylation	
Leukemia	L1210	Inhibition of cell proliferation	de Mejia *et al*. (2010)
		Arrest cell cycle at G2 phase, and increased percentage of cells in sub-G1 phase	

Table 2: (*Contd...*)

Table 2: (*Contd...*)

Type of cancer	***Cell line***	***Evidence***	***Reference(s)***
		Induction of apoptosis through activation of caspase-8, -9 and -3	
Lymphoma	Human peripheral blood mononuclear; Human B Raji	Synergistic effect with IL-12 and IL-2 cytokines in: restoration of IFNγ production, up-regulation of GZMB and CSF2, and down-regulation of TGFB1 and TGFB2 expression	Chan *et al*. (2014)
		Inhibition of H3 acetylation	
		Induction of NK cells-mediated anti-tumor activity	
DMBA and MCA -induced cancer	C3H10T1/2; NIH/3T3	Inhibition of cell proliferation and foci formation	Galvez *et al*. (2001)
		Induction of tumor suppressors p21 and p15	Hsieh *et al*. (2010b, 2011b)
		Inhibition of Rb phosphorylation and cyclin D1 expression	Jeong *et al*. (2010b)
		Synergistic effect with aspirin and anacardic acid	
E1A oncogen-induced cancer	C3H10T1/2; NIH/3T3	Inhibition of foci formation	Galvez *et al*. (2001)
		Enhance p21 protein level	
		Induction of non-adherent and apoptotic cells	Lam *et al*. (2003)
Viral *ras* oncogen-induced cancer	NIH/3T3	Inhibition of colony formation	Jeong *et al*. (2002, 2003)
		Inhibition of H3 acetylation	

Lunasin exerts its anti-neoplasic effects by modulating a number of different cellular pathways involved in the stages of initiation, promotion and progression of cancer. First studies highlighted the ability of lunasin added exogenously to internalize into mammalian cells and to sit within the nuclear compartment (Galvez *et al*., 2001; Lam *et al.,* 2003). These authors hypothesized whether RGD motif was responsible for the cellular internalization of lunasin. Whereas Galvez *et al.* (2001), demonstrated that an RGD-deletion mutant led to an inability of lunasin to be internalized, and thus to carry its bioactivity in murine fibroblast C3H 10T1/2 cells, Lam *et al.* (2003), reported that RGD-deletion mutants were slightly less effective against neoplasic transformations compared with full length lunasin peptide, but were able to be internalized into the nucleus of murine fibroblast NIH/3T3 cells. This disagreement was thought to be due to differences in the internalization mechanisms between both cell lines. Moreover, lunasin´s ability to internalize into the cell and surround the nucleus has been also demonstrated in cancer cell lines, such as colon

Table 3: Chemopreventive effects and mechanisms of action of peptide lunasin demonstrated by animal models

Type of cancer	*Animal model*	*Evidence*	*Reference(s)*
Skin cancer	DMBA-induced cancer in	Reduction in tumor incidence and yield/mouse	Galvez *et al.* (2001)
	SENCAR mice	Delay in the tumor appearance	
Breast cancer	DMBA-induced cancer in SENCAR mice	Decrease in tumor incidence and generation	Hsieh *et al.* (2010b)
	Xenograft MDA-MB-231 nude mice	Decrease in tumor incidence, generation, time-appearance, weight and volume	Hsieh *et al.* (2010c)
		Inhibition of cell proliferation	
		Induction of cell apoptosis	
Colorectal cancer	Liver metastasis mice model	Decrease in the liver metastasis recount, tumor burden and tumor weight	Dia and de Mejia (2011c, 2013)
		Increase in the cell death recount, and reduction in the PCNA expression	
		Modulation on Bcl-2/Bax proteins	
		Inhibition of IKK-α and p-p65 signals related to NF–κB pathway	
		Synergistic effect with oxaliplatin	
Lymphoma	Xenograft Raji mice model	Synergistic effect with IL-2 cytokine in reducing tumor volume	Chan *et al.* (2014)

KM12L4 cells (Dia and de Mejia, 2011b), and prostate epithelial RWPE-2 cells (Galvez *et al.,* 2011). However, it remains unknown how lunasin exerts this effect, thus, more studies elucidating the interactive behavior of lunasin with and within the cell are required.

Beyond its internalization into the cell, biochemical evidence has shown that lunasin tends to bind to deacetylated core histones and inhibits histone acetylation. Under steady-state conditions in the cell, core H3 and H4 histones are mostly in a deacetylated (repressed) state, being the balance between histone acetylation-deacetylation a key cellular process linked to signal transduction pathways including those involved in cancer development (Archer and Hodin, 1999). Lunasin either synthetic (Galvez *et al*., 2001; Hernandez-Ledesma *et al*., 2011) or extracted from natural soybean (Jeong *et al*., 2007a), barley (Jeong *et al.,* 2002), wheat (Jeong *et al*., 2007b), *Solanum nigrum* L. (Jeong *et al*., 2007c), and amaranth

(Maldonado-Cervantes *et al.*, 2010) seeds has been shown to inhibit acetylation of core histone H3 and H4. Moreover, lunasin has been found to inhibit the acetylation process in cells exposed to sodium butyrate, a known histone deacetylase inhibitor, and to compete with different histone acetyltransferases enzymes (HAT) such as PCAF, CBP/p300 and yGCN5 (Galvez *et al.*, 2001; Jeong *et al.*, 2002, 2003, 2007c). The affinity of this peptide for hypoacetylated chromatin and its activity inhibiting histone acetylation point out lunasin as a cancer-preventive food compound. These evidence markedly imply a role in chromatin remodeling, process intimately related to cell cycle progress and control of tumor progression signals (Esteller and Herman, 2002; Lund and van Lohuizen, 2004), and suggest an epigenetic nature for this chemopreventive mechanism of action (Stefanska *et al.*, 2012). The proposed epigenetic model indicates that lunasin acts against cells that are being transformed or newly transformed. Thus, when a transformation event occurs in the cell, this peptide acts as a surrogate tumor suppressor, competing with HAT in binding to histones (exposed as a consequence of the transformation episode), turning off the transcription of genes encoding for the cell cycle progress, and disrupting the dynamics of histone acetylation-deacetylation, which is perceived as abnormal by the cell and ultimately leads to cell cycle arrest, apoptosis and cellular death (de Lumen, 2005). The current importance of epigenetic effects carried by food-derived compounds and their proven role in the regulation of cellular pathways involved in aging-related diseases clearly suggest further research in order to deeply demonstrate the lunasin's mechanisms of action at the genetic, epigenetic and molecular levels.

Evidence on the molecular processes underlying cancer pathogenesis propose that cancer cells are able to display six essential alterations that collectively lead to the development of the malignant growth. Cancer cells are provided with their own growth signals are capable to ignore growth inhibitor signals, present an elevated apoptotic threshold evading programmed cell death, are potentially able to replicate without limit, sustain angiogenesis, and can invade tissue and capillary walls supporting the metastasis event (Hanahan and Weinberg, 2000). Food proteins and products thereof, such as lunasin peptide, have become one group of nutraceuticals with demonstrated effects preventing the different stages of cancer, including initiation, promotion, and progression (de Mejia and Dia, 2010). In this line, several studies on the lunasin´s cancer-preventive effects have reported that this peptide is able to modulate expression of different genes and proteins involved in former mentioned characteristics of tumor cells by control of cell cycle, apoptosis, and signaling transduction (Tables 2 and 3).

Disruption of the normal regulation of cell cycle progression and division are important events in the development of cancer. This process is highly regulated by internal checkpoints that ensure the proper transition of the

cell to the next phase, such as the protein kinases cyclin-dependent complexes (CDKs), catalytically competent after binding to their related cyclin regulatory subunits (Malumbres and Barbacid, 2009). Over-expression of cyclins and CDKs, low levels of the two families of CDK inhibitors (Cip1/p21 and INK4), and subsequent cellular events such as phosphorylation of the retinoblastoma protein (Rb) to E2F transcription factors, are all hallmarks of transformed cells Qu *et al*., 2003; Massague, 2004). *In vitro* studies carried out in transfected fibroblasts, and breast and colon cancer cells have shown lunasin´s capability to arrest cell cycle at the S- and G2-M phases by increasing the levels of CDK inhibitors p15, p21 and p27, and down-regulating the cyclins D1, D3, CDK4 and CDK6, and the Rb phosphorylation (Table 2). The *in vivo* role of lunasin on suppression of the cell cycle progression has been suggested by the analysis of the proliferation molecular markers Ki-67 and proliferating cell nuclear antigen (PCNA) in the xenograft breast MDA-MB-231 and colorectal KM12L4 cancer liver metastasis mice models, respectively. The expression of the human Ki-67 protein is present during all active phases of the cell cycle while is absent in resting cells (Scholzen and Gerdes, 2000), and PCNA is known to form a homotrimer required for DNA replication being its expression elevated during the S-phase of the cell cycle (Wang, 2014). Moreover, higher levels of these factors have been found in a variety of malignant cells lines and have been markedly linked with the aggressiveness and growth capacities of the tumor (Tan *et al.*, 2012). Mammary-tumor sections from intraperitoneally lunasin-treated mice have been found to have significantly lower rate of the Ki-67 index (Hsieh *et al*., 2010c). Dia and de Mejia, (2011b) showed the capability of the lunasin´s intraperitoneally-administration to reduce the PCNA expression of liver-tumor tissues derived from KM12L4 cells. However, these authors reported that orally administered lunasin was able to reduce the expression of PCNA, but lacking of statistical difference with the control group (Dia and de Mejia, 2013). The disagreement between these results might be explained by the different route of administration and also attributed to the fact that the remained amount of the bioavailable lunasin after digestion was not enough, being needed more studies that determine the proper oral dose of this peptide for cancer-preventive therapies.

Another mechanism by which lunasin has shown its anti-neoplasic effect is through its apoptosis-inducing activity. Tumor cell populations expand in number not only by their ability for limitlessly replicate but also by their acquired resistance toward apoptotic signals (Hanahan and Weinberg, 2000). Cancer cells present an elevated apoptotic threshold, thus strategies overcoming tumor resistance to either extrinsic or intrinsic apoptotic pathways and ultimately inducing apoptosis are increasingly described as one of the most desirable goals for chemopreventive agents (de Mejia and Dia, 2010). In colon cancer HT-29 and KM12L4 cells lunasin has been demonstrated to activate the apoptotic mitochondrial pathway as evidenced

by the changes in the expression of Bcl-2:Bax ratio, pro-apoptotic nuclear clusterin, cytochrome c, and caspase-3 activity (Table 2). Human breast cancer MCF-7 cells treated with lunasin have been found to increase their expression and protein levels of the tumor suppressor PTEN, and to enhance PTEN nuclear localization resulting in the induction of PTEN-mediated cellular apoptosis, but unlike that for genistein, p53-independent (Pabona *et al.*, 2013). Moreover, lunasin has been discovered to up-regulate the caspase -8, -9, and -3 activity in leukemia L1210 cells, and to possess a synergistic effect in combination with both aspirin and anacardic acid increasing the percentage of cells undergoing both early and late apoptosis in breast cancer MDA-MB-231 cells (Table 2). A few animal studies evaluating the *in vivo* apoptosis-inducing effects of this peptide have been also carried out. Histological analysis of stained tumor sections of xenograft MDA-MB-231 mice showed that lunasin-treated animals presented tumor destruction areas replaced by apoptotic and necrotic cells, and significantly higher levels of TUNEL-positive apoptotic cells were further found in these tumors (Hsieh *et al.*, 2010c). In the colorectal cancer liver metastasis mouse model, whereas Dia and de Mejia (2011b) reported that intraperitoneal injection of lunasin increased the expression of the pro-apoptotic Bax marker, non-significantly reduced the expression of the anti-apoptotic Bcl-2 marker, and exerted a synergistic effect with oxaliplatin inducing apoptosis by the modulation of the Bcl-2:Bax ratio. Dia and de Mejia, (2013) found that orally-administered lunasin yielded an increase in the expression of both pro-apoptotic Bax and anti-apoptotic Bcl-2 markers, prompting the design of more *in vivo* studies that clearly and deeply evaluate the lunasin's role in preventing colon cancer malignancies.

The abilities of cancer cells to induce the formation of new blood vessels, and to infiltrate into lymphatic and blood vessels, reaching bloodstream and disseminating to other parts of the body are known as angiogenesis and metastasis. These processes are often linked to the deregulation and degradation of the extracellular matrix (ECM) and disorders in the integrin family of cell adhesion receptors (Westermarck and Kahari, 1999; Parise *et al.*, 2000). In this line, elevated levels of $\alpha_5\beta_1$ integrin have been associated with enhanced invasiveness of colorectal cancer cells as well as lack of $\alpha_5\beta_1$ function has been related with reduction of metastasis in these cells (Gong *et al.,* 1997; Stoeltzing *et al.,* 2003). Moreover, peptides containing RGD-motif have been demonstrated to alter integrin-ECM interactions by binding to these complexes and ultimately blocking the integrin signals (Ruoslahti and Pierschbacher, 1987). *In vitro* studies in colorectal cancer cell lines have shown that lunasin is able to down-regulate the expression of $\alpha_5\beta_1$ integrin and, interestingly, it is worthy to mention that this peptide had the most potent cytotoxic effect in the cell line that most highly expressed this integrin (Dia and de Mejia, 2011b). Furthermore, these authors also reported that lunasin shows capability to modulate the expression of ECM and adhesion genes, by down-regulating the expression of transcripts

integrin α_5, SELE, MMP10, integrin β_2 and COL6A1, and up-regulating COL12A1, mechanisms that suggest lunasin as an anti-metastatic agent and by which this peptide might play its chemo-preventive action (Dia and de Mejia, 2011b).

Antioxidant and Anti-inflammatory Activities

Oxygen may be partially reduced during normal metabolism to yield reactive molecules termed reactive oxygen species (ROS). At physiological concentrations, ROS are beneficial supporting the immune system and acting as second messengers in a wide range of cellular processes, including proliferation, cell cycle arrest, cell death, and signal transduction (Rosen *et al*., 1995; Martindale and Holbrook, 2002; Owuor and Kong, 2002). However, at high levels, ROS are harmful and they can oxidatively attack nucleophilic centers in the cell causing lipid peroxidation, protein oxidation and most importantly, genetic alterations, including DNA damage, mutations, epigenetic changes, and genomic instability (Khan *et al.,* 2008).

Inflammation is a complex biological response of vascular tissues against harmful agents, such as microbial infections, damaged cells, or irritants, however, chronic or uncontrolled inflammatory states lead to tissue damage and disorders in cell signaling pathways. Accumulating evidence point out repeated ROS generation, persistent inflammatory cells recruitment, and continued proliferation of genomically unstable cells as critical triggers for the etiology of several human degenerative diseases, including cardiovascular and neurodegenerative disorders, and cancer (Allavena *et al*., 2008; Khan *et al*., 2008).

In vitro, peptide lunasin has been found to exert potent antioxidant properties through different mechanisms of action. It is able to act as a potent scavenger of superoxide and peroxil radicals and inhibitor of linoleic acid oxidation (Hernández-Ledesma *et al*., 2009a; García-Nebot *et al*., 20014). Moreover, lunasin purified from *Solanum nigrum* L. has been found to block the generation of hydroxyl radical by chelating iron ferrous ions, protecting DNA from induced-oxidative damage (Jeong *et al*., 2010a). The antioxidant properties of lunasin have been confirmed by cell culture. ROS production in lipopolysaccharide (LPS)-induced RAW264.7 macrophages was inhibited by lunasin in a dose-dependent manner (Hernández-Ledesma *et al*., 2009a). Also, lunasin´s ability to improve cell viability and decrease ROS levels has been proven in intestinal Caco-2 cells challenged by hydrogen peroxide and *tert*-butylhydroperoxide (*t*-BOOH) (García-Nebot *et al*., 2014). The protective mechanisms carried out by lunasin on several biomarkers of redox status have been recently reported by Fernández-Tomé *et al.* (2014). Pre-treatment of *t*-BOOH-induced liver HepG2 cells with this peptide prevented the increased ROS generation as well as the depletion of reduced glutathione, modulated the glutathione peroxidase and catalase activities,

and evoked a decline in carbonyl groups and a recovery from cell death by restraining the caspase-3 apoptotic pathway.

The anti-inflammatory properties of lunasin have been evidenced through some *in vitro* studies on its effects on key mediators of the inflammatory response. Lunasin and lunasin-like peptides purified from defatted soybean flour have been found to inhibit pro-inflammatory markers, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α by suppression of cyclooxygenase (COX)-2/prostaglandin (PG) E_2, inducible nitric oxide synthase (iNOS)/nitric oxide (NO) and nuclear factor-kappa B (NF–κB) pathways, in the LPS-induced RAW 264.7 murine macrophage cellular model (de Mejia and Dia, 2009; Dia *et al.,* 2009b; Hernández-Ledesma *et al.*, 2009a). The molecular mechanisms involved on these effects and its potential significance in the pathogenesis of inflammation-related diseases have been recently reported (Cam and de Mejia, 2012; Cam *et al*., 2013). Lunasin treatment in LPS-induced THP-1 human macrophages was shown to inhibit the expression of NF–κB-dependent markers of inflammation through down-regulation of Akt phosphorylation and NF–κB subunit p65 protein expression (Cam and de Mejia, 2012). Evidence on inflammatory cellular signaling has linked activation of αVβ3 integrin to the Akt pathway. In this work, lunasin was demonstrated to internalize into macrophages and physically interact with αVβ3 integrin, suggesting that its biological activity could be exerted by an antagonistic effect on αVβ3 receptors (Cam and de Mejia, 2012). Further experiments showed that while αVβ3 integrin expression is enhanced in inflammatory conditions, lunasin is able to attenuate this up-regulation in LPS-induced THP-1 macrophages, and to internalize into these cells by integrin signaling, clathrin-coated structures and macropinosomes-mediated mechanisms (Cam *et al*., 2013). Dia and de Mejia (2011a) reported the effect of lunasin on the gene expression profile of RAW 264.7 macrophages in a normal and LPS-challenged state. Interestingly, lunasin was found to affect the expression of genes involved in cellular growth and proliferation, cellular function, cell to cell signaling, cell cycle, cell death, and apoptosis.

Above-mentioned findings might contribute on lunasin's potential chemo-preventive activity against cancer and other oxidative and inflammatory-related disorders.

OTHER EFFECTS OF LUNASIN

Lunasin Against Cardiovascular Diseases

Cardiovascular diseases are a significant public health problem worldwide, ranking as the leading cause of human death in western countries. Diet and other lifestyle habits have been remarkably demonstrated to impact the development of the major risk factors for cardiovascular diseases, such

as hypertension, lipid abnormalities, diabetes, obesity, inflammation and atherosclerosis. Food-derived bioactive peptides have become one group of nutraceuticals with proven effects in cardiovascular health (Erdmann *et al.*, 2008). Daily consumption of 25 g of soy protein has an approved Food and Drug Administration (FDA) health claim for reducing the risk of coronary heart disease (FDA, 1999). Beyond, lunasin has been reported as one of the soy protein active nutrients responsible for reducing the risk of cardiovascular diseases by decreasing the hepatic cholesterol biosynthesis (Galvez, 2012). In this study, lunasin was found to down-regulate low density lipoprotein (LDL) cholesterol content by directly inhibiting 3-hydroxy-3-methyl-glutaryl (HMG) Co-A reductase gene expression, and enhancing its clearance from plasma through increasing the LDL receptor levels of HepG2 liver cells. Moreover, this effect was also tested *in vivo* in LDL-receptor mutant pigs, whose LDL cholesterol levels were found to be decreased after supplementation of their casein diets with a lunasin-enriched soy extract (Galvez, 2012). A human trial for the evaluation of soy-based food supplements in the management of patients with lipid abnormalities was designed by Sison and co-workers. In this study, lower cholesterol, LDL, triglycerides, and blood uric acid levels were shown after 60 days consumption of a lunasin-enriched food supplement. Interestingly, these effects were even greater among the diabetic and overweight individuals, demonstrating lunasin´s ability for the control of the lipid imbalances associated with the cardiovascular disease.

A recent work carried out with the previously reported dietary supplement in combination with a lunasin-enriched soy extract has shown the lunasin's ability to reduce free fatty acids by increasing leptin and adiponectin levels in the plasma of LDL-receptor mutant pigs (Galvez *et al.,* 2013). Results of this study suggest the use of that combination as an effective, low-cost and natural alternative to therapeutic drugs against obesity, metabolic syndrome and associated cardiovascular diseases.

Lunasin´s Influence on the Central Nervous System

In vivo studies have shown that orally-administered lunasin is absorbed and distributed in various target tissues, being even able to cross the blood-brain barrier, reaching the brain (Dia *et al.*, 2009a; Hsieh *et al.*, 2010c). From this point of view, the study of Dzirkale *et al.* (2013) has recently assessed the central effects exerted by this peptide. Central (intracisternally)-administered synthetic lunasin was demonstrated to decrease the locomotor activity, amphetamine-induced hyperlocomotion, and apomorphine-induced climbing effect of mice. Binding assays showed that lunasin possesses a modest affinity for dopamine D_1 receptors. However, no binding effect was detected for dopamine D_2 receptors. Also, lunasin was able to inhibit the apomorphine-initiated cAMP formation in

HEK293 cells expressing dopamine D_1 receptor activation. The findings of this study also suggested that glutamate and GABAergic systems do not play an essential role in lunasin´s central effects (Dzirkale *et al.,* 2013). These authors showed for lunasin for the first time a motionless/neuroleptic/ cataleptic effect that at least in part might be provided *via* dopaminergic pathways, and set up the basis for further doubtlessly needed investigations in this novel research focus for this peptide.

FUTURE PROSPECTS

Since its discovery in 1997, lunasin has become one of the natural peptides present in plant and derived-foods with higher potential against chronic disorders such as cancer and cardiovascular diseases. Its notable bioavailability and demonstrated efficacy against different types of cancer are the two main aspects making this peptide a promising chemopreventive agent. Animal models carried out in the last years have confirmed the effectiveness of lunasin against skin, breast, colorectal cancer and lymphoma, and have suggested an additional role for this peptide against obesity, metabolic syndrome and associated cardiovascular diseases. Nowadays, the major challenge on the use of lunasin would be the conversion of existing results into clinical outcomes, being needed to design clinical trials to confirm the already demonstrated properties. Moreover, genomics, proteomics and biochemical tools should be applied to elucidate its molecular mechanism of action. Other aspects, such as searching for lunasin in other seeds, optimization of techniques to enrich products with this peptide and studying lunasin's interactions with other food constituents affecting its activity should also be conducted.

ACKNOWLEDGMENTS

This work has received financial support from projects AGL2011-24643, FEDER-INNTERCONECTA-GALICIA (ENVELLEFUN and LACTMETABOL) and FP7-SME-2012-315349 (FOFIND). S. F. -T. and B. H. -L. acknowledge Ministry of Economy and Competitiveness (MINECO) for their FPI fellowship and "Ramón y Cajal" post-doctoral contract, respectively.

REFERENCES

Allavena, P., Garlanda, C., Borrello, M.G., Sica, A. and Mantovani, A. (2008). Pathways connecting inflammation and cancer. *Curr. Opin. Gen. Develop.,* 18: 3–10.

Archer, S.Y. and Hodin, R.A. (1999). Histone acetylation and cancer. *Curr. Opin. Gen. Develop.,* 9: 171–74.

Bhutia, S.K. and Maiti, T.K. (2008). Targerting tumors with peptides from natural sources. *Trends Biotechnol.,* 26: 210–17.

Cam, A. and de Mejia, E. (2012). RGD-peptide lunasin inhibits Akt-mediated NF–κB activation in human macrophages through interaction with the αVβ3 integrin. *Mol. Nutr. Food Res.,* 56: 1569–81.

Cam, A., Sivaguru, M. and de Mejia, E. (2013). Endocytic mechanism of internalization of dietary peptide lunasin into macrophages in inflammatory condition associated with cardiovascular disease. *PLoS ONE,* 8: e72115.

Chang, H.C., Lewis, D., Tung, C.Y., Han, L., Henriquez, S.M.P., Voiles, L. *et al*. (2014). Soypeptide lunasin in cytokine immunotherapy for lymphoma. *Cancer Immunol. Immun.,* 63: 283–95.

Cavazos, A., Morales, E.l., Dia, V.P. and de Mejia, E.G. (2012). Analysis of lunasin in commercial and pilot plant produced soybean products and an improved method of lunasin purification. *J. Food Sci.,* 77: C539–45.

de Mejia, E.G. and Dia, V.P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF–κB pathway in the marcrophage. *Peptides*, 30: 2388–98.

de Mejia, E.G. and Dia, V.P. (2010). The role of nutraceuticals proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metast. Rev.,* 29: 511–28.

de Mejia, E.G., Vasconez, M., de Lumen, B.O. and Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. *J. Agric Food Chem.,* 52: 5882–87.

de Mejia, E.G., Wang, W. and Dia, V.P. (2010). Lunasin, with an arginine-glycine-aspartic acid motif, causes apoptosis to L1210 leukemia cells by activation of caspase-3. *Mol. Nutr. Food. Res.,* 54: 406–14.

de Lumen, B.O. (2005). Lunasin: A cancer-preventive soy peptide. *Nutr. Rev.,* 63: 16–21.

Dia, V.P. and de Mejia, E.G. (2011a). Differential gene expression of RAW 264.7 macrophages in response to the RGD peptide lunasin with and without lypopolysaccharide stimulation. *Peptides,* 32: 1979–88.

Dia, V.P. and de Mejia, E.G. (2011b). Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. *Mol. Nutr. Food Res.,* 55: 623–34.

Dia, V.P. and de Mejia, E.G. (2011c). Lunasin potentiates the effect of oxaliplatin preventing outgrowth of colon cancer metastasis, binds to $\alpha_5\beta_1$ integrin and suppresses FAK/ERK/NF–κB signaling. *Cancer Lett.,* 313: 167–80.

Dia, V.P. and de Mejia, E.G. (2010). Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. *Cancer Lett.,* 295: 44–53.

Dia, V.P. and de Mejia, E.G. (2013). Potential of lunasin-orally administered in comparison to intraperitoneal injection to inhibit colon cancer metastasis *in vivo*. *J. Cancer Ther.,* 4: 34–43.

Dia, V.P., Frankland-Searby, S., Laso del Hierro, F., Garcia, G. and de Mejia, E.G. (2013). Structural property of soybean lunasin and development of a method to quantify lunasin in plasma using an optimized immunoassay protocol. *Food Chem.,* 138: 334-41.

Dia, V.P., Torres, S., de Lumen, B.O., Erdman, J.W. and de Mejia, E.G. (2009a). Presence of lunasin in plasma of men after soy protein consumption. *J. Agric Food Chem.,* 57: 1260–66.

Dia, V.P., Wang, W., Oh, V.L., de Lumen, B.O. and de Mejia, E.G. (2009b). Isolation, purification and characterisation of lunasin from defatted soybean flour and *in vitro* evaluation of its anti-inflammatory activity. *Food Chem.,* 114: 108–15.

Dinelli, G., Bregola, V., Bosi, S., Fiori, J., Gotti R., Simonetti, E. *et al*. (2014). Lunasin in wheat: A chemical and molecular study on its presence or absence. *Food Chem.,* 151: 520–25.

Dzirkale, Z., Rumaks, J., Svirskis, S., Mazina, O., Allikalt, A., Rinken, A. *et al*. (2013). Lunasin-induced behavioural effects in mice: Focus on the dopaminergic system. *Behav. Brain Res.,*. 256: 5–9.

Erdmann, K., Cheung, B.W.Y. and Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J. Nutr. Biochem.,* 19: 643–54.

Esteller, M. and Herman, J.G. (2002). Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *J. Pathol.,* 196: 1–7.

FDA Talk Paper (1999). FDA approves new health claim for soy protein and coronary heart disease. FDA, United States Department of Health and Human Services. Washington, DC: United States Government Printing Office.

Fernández-Tomé, S., Ramos, S., Cordero-Herrera, I., Recio, I., Goya, L. and Hernández-Ledesma, B. (2014). *In vitro* chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells. *Food Res. Int.,* 62: 793–800.

Galvez, A.F. (2012). Abstract 10693: Identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease. *Circulation,* 126: A10693.

Galvez, A.F., Chen, N., Macasieb, J. and de Lumen, B.O. (2001). Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. *Cancer Res.,* 61: 7473–78.

Galvez, A.F. and de Lumen, B.O. (1999). A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. *Nat. Biotechnol.,* 17: 495–500.

Galvez, A.F., Huang, L., Magbanua, M.M.J., Dawson, K. and Rodriguez, R.L. (2011). Differential expression of thrombospondin (THBS1) in tumorigenic and nontumorigenic prostate epithelial cells in response to a chromatin-binding soy peptide. *Nutr. Cancer,* 63: 623-36.

Galvez, A.F., Matel, H., Ivey, J. and Bowles, D. (2013). Lunasin-enriched soy extract (LunaRich X™), in combination with the dietary supplement Reliv Now, reduces free fatty acid by increasing plasma leptin and adiponectin levels in LDL-receptor mutant pigs. PRWEB [Internet]. Available from: http://www.prweb.com/releases/lunasinlunarichxrelivnow/nutritionalepigenetics/prweb11137975.htm

Galvez, A.F., Revilleza, M.J.R. and de Lumen, B.O. (1997). A novel methionine-rich protein from soybean cotyledon: Cloning and characterization of cDNA (accession No.AF005030). *Plant Register*, #PGR97-103. *Plant Physiol.,* 114: 1567–69..

García-Nebot, M.J., Recio, I. and Hernández-Ledesma, B. (2014). Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. *Food Chem. Toxicol.,* 65: 155–61.

Gong, J., Wang, D., Sun, L., Zborowska, E., Willson, J. and Brattain, M.G. (1997). Role of alpha 5 beta 1 integrin in determining malignant properties of colon carcinoma cells. *Cell Growth*, 8: 83–90.

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*, 100: 57–70.

Hernández-Ledesma, B., Hsieh, C.C. and de Lumen, B.O. (2009a). Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun.,* 390: 803–08.

Hernández-Ledesma, B., Hsieh, C.C. and de Lumen, B.O. (2009b). Lunasin and bowman-birk protease inhibitor (BBI) in US commercial soy foods. *Food Chem.,* 115: 574–80.

Hernández-Ledesma, B., Hsieh, C.C. and de Lumen, B.O. (2011a). Relationship between lunasin's sequence and its inhibitory activity of histones H3 and H4 acetylation. *Mol. Nutr. Food Res.,* 55: 989–98.

Hsieh, C.C., Hernández-Ledesma, B. and de Lumen, B.O. (2011a). Cell proliferation inhibitory and apoptosis-inducing properties of anacardic acid and lunasin in human breast cancer MDA-MB-231 cells. *Food Chem.,* 125: 630–636.

Hsieh, C.C., Hernández-Ledesma, B. and de Lumen, B.O. (2010a). Lunasin, a novel seed peptide, sensitizes human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induced apoptosis. *Chem. Bio. Interact.,* 186: 127–34.

Hsieh, C.C., Hernández-Ledesma, B. and de Lumen, B.O. (2011b). Lunasin-aspirin combination against NIH/3T3 cells transformation induced by chemical carcinogens. *Plant Food Hum. Nutr.,*. 66: 107–13.

Hsieh, C.C., Hernández-Ledesma, B. and de Lumen, B.O. (2010b). Soybean peptide lunasin suppresses *in vitro* and *in vivo* 7,12-dimethylbenz[a]anthracene-induced tumorigenesis. *J. Food Sci.,* 75: H311–16.

Hsieh, C.C., Hernández-Ledesma, B., Jeong, H.J., Park, J.H. and de Lumen, B.O. (2010c). Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS ONE*, 5: e8890.

Jeong, J.B., de Lumen, B.O. and Jeong, H.J. (2010a). Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage by suppressing the generation of hydroxyl radical *via* blocking fenton reaction. *Cancer Lett.,* 293: 58–64.

Jeong, H.J., Jeong, J.B., Hsieh, C.C., Hernández-Ledesma, B. and de Lumen, B.O. (2010b). Lunasin is prevalent in barley and is bioavailable and bioactive in *in vivo* and *in vitro* studies. *Nutr. Cancer*, 62: 1113–19.

Jeong, H.J., Jeong, J.B., Kim, D.S. and de Lumen, B.O. (2007a). Inhibition of core hystone acetylation by the cancer preventive peptide lunasin. *J. Agric. Food Chem.*, 55: 632–37.

Jeong, H.J., Jeong, J.B., Kim, D.S., Park, J.H., Lee, J.B., Kweon, D.H. *et al*. (2007b). The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. *Cancer Lett.,* 255: 42–8.

Jeong, J.B., Jeong, J.J., Park, J.H., Lee, S.H., Lee, J.R., Lee, H.K. *et al*. (2007c). Cancer-preventive peptide lunasin from *Solanum nigrum* L. inhibits acetylation of core histone H3 and H4 and phosphorylation of Retinoblastoma Protein (Rb). *J. Agric. Food Chem.,* 55: 10707–13.

Jeong, H.J., Lam, Y. and de Lumen, B.O. (2002a). Barley lunasin suppresses *ras*-induced colony formation and inhibits core histone acetylation in mammalian cells. *J. Agr. Food Chem.,* 50: 5903–08.

Jeong, H.J., Lee, J.R., Jeong, J.B., Park, J.H., Cheong, Y.K. and de Lumen, B.O. (2009). The cancer preventive seed peptide lunasin from rye is bioavailable and bioactive. *Nutr. Cancer*, 61: 680–86.

Jeong, H.J., Park, J.H., Lam, Y. and de Lumen, B.O. (2003). Characterization of lunasin isolated from soybean. *J. Agr. Food Chem.,* 51: 7901–06.

Khan, N., Afaq, F. and Mukhtar, H. (2008). Cancer chemoprevention through dietary antioxidants: Progress and promise. *Antiox. Redox Signal*. 10: 475–510.

Lam, Y., Galvez, A. and de Lumen, B.O. (2003). Lunasin suppresses E1A-mediated transformation of mammalian cells but does not inhibit growth of immortalized and established cancer cell lines. *Nutr. Cancer*, 47: 88–94.

Lund, A.H. and van Lohuizen, M. (2004). Epigenetics and cancer. *Gen. Dev.,* 18: 2315–35.

Maldonado-Cervantes, E., Jeong, H.J., Leon-Galvan, F., Barrera-Pacheco, A., De Leo-Rodriguez, A., de Mejia, E.G. *et al*. (2010). Amaranth lunasin-like peptide internalizes into the cell nucleus and inhibits chemical carcinogen-induced transformation of NIH-3T3 cells. *Peptides,* 31: 1635–42.

Malumbres, M. and Barbacid, M. (2009). Cell cycle, CDKs and cancer: A changing paradigm. *Nat. Rev. Cancer*, 9: 156–66.

Martindale, J.L. and Holbrook, N.J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. *J. Cell Physiol.,* 192: 1–15.

Massague, J. (2004). G1 cell-cycle control and cancer. *Nature*, 432: 298–306.

Mitchell, R.A.C., Lovegrove, A. and Shewry, P.R. (2013). Lunasin in cereal seeds: What is the origin? *J. Cereal Sci.,* 57: 267–69.

Nakurte, I., Klavins, K., Kirhnere, I., Namniece, J., Adlere, L., Matvejevs, J. *et al*. (2012). Discovery of lunasin peptide in triticale (X *Triticosecale Wittmack*). *J. Cereal Sci.*, 56: 510–14.

Nakurte, I., Kirhnere, I., Namniece, J., Saleniece, K., Krigere, L., Mekss. P. *et al*. (2013). Detection of the lunasin peptide in oats (*Avena sativa* L). *J. Cereal Sci.,* 57: 319–24.

Owuor, E.D. and Kong, A.N.T. (2002). Antioxidants and oxidants regulated signal transduction pathways. *Biochem. Pharm.*, 64: 15–47.

Pabona, J.M.P., Dave, B., Su, Y., Montales, M.T.E., de Lumen, B.O., de Mejia, E. *et al*. (2013). The soybean peptide lunasin promotes apoptosis of mammary epithelial cells via induction of tumor suppressor PTEN: similarities and distinct actions from soy isoflavone genistein. *Genes Nutr.,* 8: 79–90.

Parise, L.V., Lee, J.W. and Juliano, R.L. (2000). New aspects of integrin signaling in cancer. *Semin. Cancer Biol.,* 10: 407–14.

Park, J.H., Jeong, H.J. and de Lumen, B.O. (2007). *In vitro* digestibility of the cancer-preventive soy peptides lunasin and BBI. *J. Agric Food Chem.,* 55: 10703–06.

Paucar-Menacho, L.M., Berhow, M.A., Mandarino, J.M.G., Chang, Y.K. and de Mejia, E.G. (2010a). Effect of time and temperature on bioactive compounds in germinated Brazilian soybean cultivar BRS 258. *Food Res. Int.,* 43: 1856–65.

Paucar-Menacho, L.M., Berhow, M.A., Mandarino, J.M.G., de Mejia, E.G. and Chang, Y.K. (2010b). Optimisation of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology. *Food Chem.,* 119: 636–42.

Qu, Z., Weiss, J.N. and MacLellan, R.W. (2003). Regulation of the mammalian cell cycle: A model of the G1-to-S transition. *Am. J. Physiol-Cell Physiol.,* 284: C349–64.

Rizzello, C.G., Nionelli, L. and Coda Gobbetti, M. (2011). Synthesis of the cancer preventive peptide lunasin by lactic acid bacteria during sourdough fermentation. *Nutr. Cancer*, 64: 111–20.

Rosen, G.M., Pou, S., Ramos, C.L., Cohen, M.S. and Britigan, B.E. (1995). Free radicals and phagocytic cells. *FASEB J.,* 9: 200–09.

Ruoslahti, E. and Pierchbacher, M.D. (1987). New perspectives in cell adhesion – RGD and integrins. *Science*, 238: 491–97.

Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: From the known and the unknown. *J. Cell Physiol.*, 182: 311–22.

Seber, L.E., Barnett, B.W., McConnell, E.J., Hume, S.D., Cai, J., Boles, K. *et al*. (2012). Scalable purification and characterization of the anticancer lunasin peptide from soybean. *PLoS ONE*, 7: e35409.

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D. and Hernandez-Escalante, V.M. (2011). Bioavailability of bioactive peptides. *Food Rev. Int.,* 27: 213–26.

Silva-Sánchez, C., Barba de la Rosa, A.P., Leon-Galvan, M.F., de Lumen, B.O., de Leon-Rodriguez, A. and de Mejia, E.G. (2008). Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. *J. Agric. Food Chem.,* 56: 1233–40.

Sison, J., Aragon, J., Maliwat, R. and Myrna, Y. A study on soy-based supplements as medical nutraceutical therapy in reversing lipid abnormalities [dissertation]. Medical Center Manila, Makati Medical Center, Medical City Philippines.

Stefanska, B., Karlic, H., Varga, F., Fabianowska-Majewska, K. and Haslberger, A.G. (2012). Epigenetic mechanisms in anti-cancer actions of bioactive food components – the implications in cancer prevention. *Brit. J. Pharmacol.,* 167: 279–97.

Stoeltzing, O., Liu, W., Reinmuth, N., Fan, F., Parry, G.C., Parikh, A.A. *et al*. (2003). Inhibition of integrin alpha 5 beta 1 function with a small peptide (ATN-161) plus

continuous 5-FU infusion reduces colorectal liver metastases and improves survival in mice. *Int. J. Cancer*, 104: 496–503.

Tan, Z.Q., Wortman, M., Dillehay, K.L., Seibel, W.L., Evelyn, C.R., Smith, S.J. *et al*. (2012). Small-molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. *Mol. Pharmacol.*, 81: 811–19.

Wang, S.C. (2014). PCNA: A silent housekeeper or a potential therapeutic target? *Trends Pharmacol. Sci.,* 35: 178–86.

Wang, W.Y., Dia, V.P., Vasconez, M., de Mejia, E.G. and Nelson, R.L. (2008). Analysis of soybean protein derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. *J. AOAC Int.,* 91: 936–46.

Westermarck, J. and Kahari, V.M. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.,* 13: 781–92.

Functionality of soybean compounds in the oxidative stress-related disorders

Chia-Chien Hsieh[1], Samuel Fernández-Tomé[2], Blanca Hernández-Ledesma[2,*]

[1] Department of Human Development and Family Studies, National Taiwan Normal University, 10610, Taipei, Taiwan

[2] Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain

* Corresponding author: Dr. Blanca Hernández-Ledesma
Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain
Phone: +34910017900
Email: b.hernandez@csic.es

Abstract

Soybean is source of many phytochemicals, including isoflavones, phenolic acids, phospholipids, oligosaccharides, protease inhibitors, lectins and bioactive peptides. Their demonstrated biological activities have made them to be considered excellent compounds to maintain a good health status preventing chronic disorders.

Oxidative stress and inflammation play key roles in the onset and progression of many chronic diseases, such as intestinal inflammation, cardiovascular and neurological disorders, diabetes, and cancer. This chapter summarizes the antioxidative and anti-inflammatory properties of soy phytochemicals, emphasizing on the recent evidence and the mechanisms of action.

Fermentation is not only determinant on the sensorial, technological and nutritional characteristics of soybean, but also might increase its functional value. Germination and roasting have been also recognized as economical and efficient technologies for the breakdown of macromolecules, increasing soybean's digestibility, and enhancing its nutritional and biological value. The effects of soybean processing on its antioxidative properties have also included in this chapter.

Keywords: soybean, oxidative stress, inflammation, disease, soy processing

1. Introduction: Soybean

Soybean (*Glycine max (L)* Merrill family *Leguminosae*) is one of the most cultivated plants in the world. It was originated in the Orient, probably in China [1], and mostly consumed in Asian countries, such as China, Japan, Korea, Taiwan and Indonesia. Populations from these countries consume daily an average of 20 to 80 g of traditional soy foods in many forms, including soybean, soy milk, toasted soy protein flours, soybean sprouts, tofu, and fermented soy products, such as tempeh, miso, natto, soybean paste and soy sauce [2, 3]. However, the soybean consumption by people from Western countries is much lower, ranging from 1 to 3 g per day, and this is mostly in processed forms, such as soy drinks, breakfast cereals, energy bars, soy "burgers" and “dogs”, tofu sliced meat, and tofu ice cream, among others [4, 5].

Soybean is rich in proteins (40-50%), lipids (20-30%) and carbohydrates (26-30%). 35% of soybean carbohydrates are contained in the seed and 40% in soybean meal, which are mainly composed of digestible sugars, starch, as well as non-digestible oligosaccharides [6]. The amount of protein in soybeans is larger than the protein content in other legumes (20-30%), and much larger than that of cereals (8-15%). Therefore, soy protein products have been presented as perfect substitutes for animal-based foods because they nearly offer a “complete” protein profile containing less fat than animal products, especially saturated fat. Only two factors limit the nutritional value of soybean and derived-products. By one hand, soybean is limiting in the sulphur-containing amino acids for humans, although this limitation can be easily overcome by including in the diet other protein sources rich in these amino acids, usually cereals. The second limitation of soybean is its low digestibility when the only treatment is heating, but further processing including soaking, sprouting, fermentation, grinding, and hot water extraction have been demonstrated to considerably increase digestibility [7]. Moreover, new soybean products such as soybean protein concentrate and isolate show higher digestibility than traditional soy flours [8].

Soybean has been ranked as one of the main sources of phytochemicals. They are compounds present in plants that have a beneficial effect on health or play an active role in the diseases prevention, although they are not required for normal body metabolism [9]. Soybean phytochemicals include isoflavones, saponins, sterols, phytates, phenolic acids, protease inhibitors, lectins and bioactive peptides. The presence of these phytochemicals have made soybean being considered as a functional food controlling and preventing a wide variety of chronic and degenerative diseases such as cardiovascular disease, obesity, diabetes, and cancer [10]. This chapter summarizes evidence on protective effects of soybean derived compounds against chronic diseases associated to oxidative stress and inflammation, with particular interest on intestinal disorders. Chemical nature, activity and mechanisms of action of bioactive compounds will be included.

2. Oxidative stress-associated diseases: concepts and mechanisms of ROS

Oxygen is an indispensable element for the life of aerobic organisms. Most of the energy needed for cellular survival is stored as adenosine triphosphate molecules and provided by the mitochondrial respiratory chain, with the reduction of oxygen to water and the breakdown of nutrients. As consequence of this metabolism, several chemical entities containing partially reduced oxygen,

termed as reactive oxygen species (ROS), are continuously generated in cells. ROS comprise a broad group of highly reactive and unstable molecules, because of the presence of unpaired electrons, including free radicals. Moreover, ROS can be produced from endogenous sources as well as triggered by exogenous factors such as radiation, chemotherapy, pollutants, smoking, and xenobiotics including drugs, food and alcohol. At physiological levels, they play an important role as signaling agents in multiple cellular pathways and efficient components of the immune system. On the other hand, they can be harmful to the body in case of being present to excess [11].

Cells are thus naturally provided with an extensive machinery of protective enzymatic and non-enzymatic antioxidants facing the potential injury from oxidizing agents. In the human cells, the antioxidant enzymes are mainly superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase; while the principal non-enzymatic antioxidant agent is the tripeptide glutamate-cysteine-glycine, called glutathione (GSH). There is a highly-regulated cellular balance between the physiological and deleterious effects of ROS [12]. However, in pathological states, ROS production might overcome the capacity to effectively neutralize reactive intermediates leading to the oxidative stress state (**Figure 1**). In this situation, ROS interact with membrane lipids, proteins, nucleic acids, enzymes, and some small molecules oxidizing their structures and resulting in cellular damage [13].

Large amount of ROS have been extensively implicated in the development of several human degenerative diseases, including inflammation, cardiovascular, neurodegenerative and gastrointestinal disorders, diabetes, aging and cancer [14]. Interestingly, exogenous antioxidants are able to reinforce the endogenous antioxidant defense system acting as radical scavengers, hydrogen and electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors and inducers, or metal-chelating agents.

2.1. Cardiovascular and neurodegenerative diseases, cancer, diabetes and aging are linked to oxidative stress and inflammation

Disruption of the steady-state cellular homeostasis by redox signaling may cause long-term damage, altered cellular responses, and mucosal injury that have been implicated as underlying causes of numerous degenerative diseases.

Cardiovascular diseases (CVD) have become the leading cause of morbidity and mortality worldwide, equally affecting both men and women. Besides the growing body of evidence connecting increased levels of ROS with the etiopathogenesis of CVD, ROS-induced oxidative stress further affects to various CVD-related conditions including hypertension, atherosclerosis, ischemic heart disease, cardiomyopathies, cardiac hypertrophy, and congestive heart failure. Uncoupling of the mitochondrial oxidative phosphorylation and the nitric oxide synthesis, the xanthine-oxidoreductase deregulation, and the activation of the nicotinamide adenine dinucleotide phosphate hydrogen (NAD(P)H) oxidase system by a variety of mediators have been proposed as the main mechanisms involved on these alterations [11]. Likewise, oxidative processes have been shown to participate in the development of neurodegenerative Alzheimer´s and Parkinson´s diseases. Although the origin of these disorders appears to be multifactorial, the underlying factors are clearly ROS-increased either

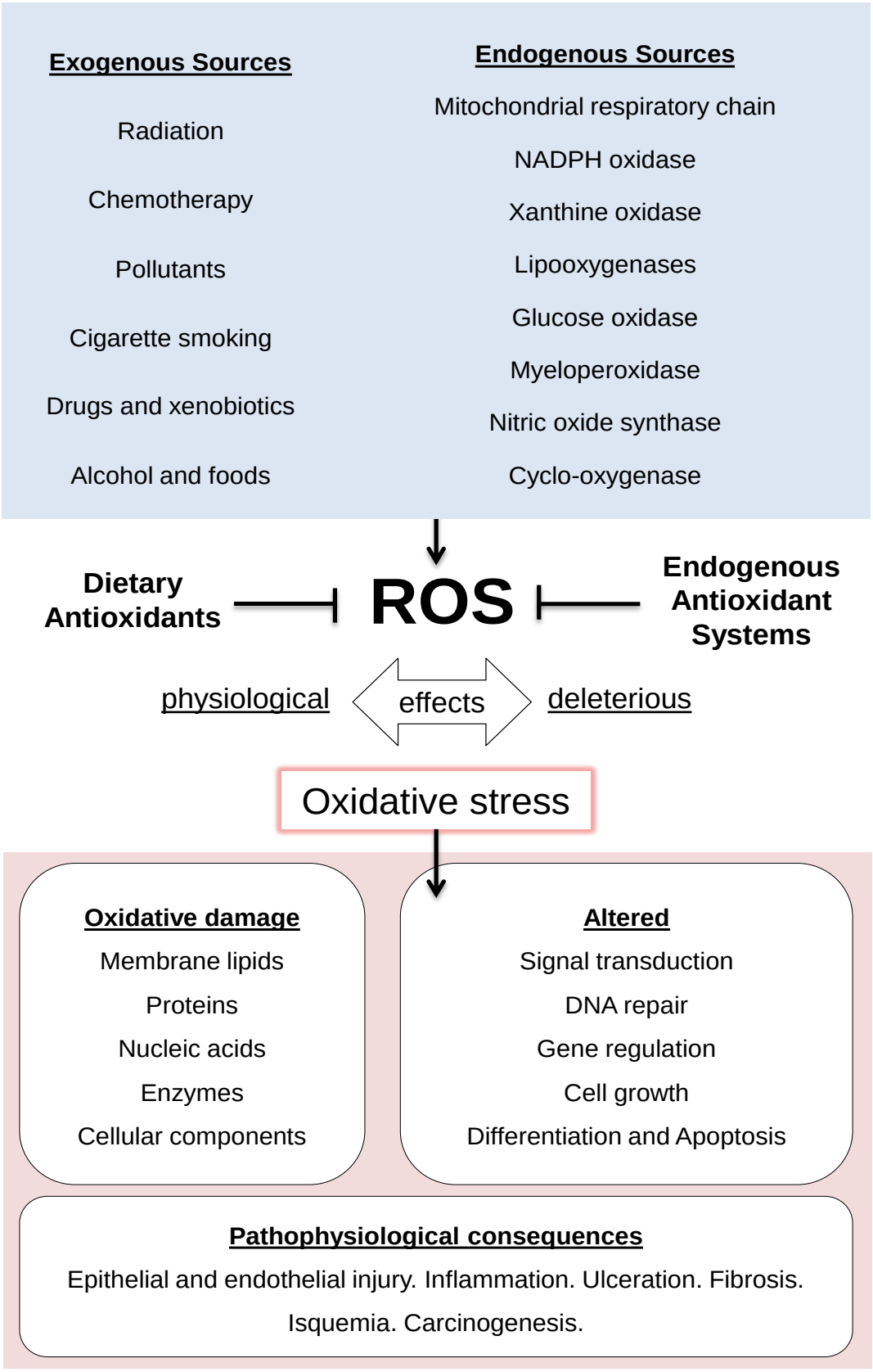


Figure 1. Schematic illustration on the factors leading to ROS generation, the induction of oxidative stress and its patho-physiological effects

by direct modification on the protein side-chains of the pathological depositions, or indirectly by the lipid peroxidation products [15]. Moreover, nervous tissues are particularly vulnerable to the oxidative damage due to the elevated rate of oxygen consumption at the nervous system, the presence of redox-active metals (Fe, Cu), and the high content of oxidisable polyunsaturated fatty acids in the brain. The cancer stages of initiation, promotion and progression have been also associated to oxidative stress as consequence of the increased DNA mutations, damage to macromolecules, genome instability, and cellular proliferation. In fact, ROS are able to interact with a variety of transcriptional factors such as nuclear factor (NF)-κB, activator protein-1 (AP-1), p53, hypoxia-inducible factor (HIF)-1α, peroxisome proliferator activated receptor (PPAR)-γ, β-catenin/Wnt, and nuclear factor-erythroid 2-related factor (Nrf2) leading to the expression of multiple genes related to the control of growth factors, inflammatory cytokines, and apoptosis and cell cycle regulatory agents [16]. Related to the metabolic disorders, diabetes mellitus is considered as one of the most common alterations and major health problems worldwide. Chronic overproduction of reactive species contributes to both the onset and evolution of diabetes and its complications, by inducing insulin resistance, β-cell dysfunction, impaired glucose tolerance, and abnormal mitochondrial activity [17]. Above-mentioned findings are all related to the process of aging and chronic diseases, absolutely linked to the harmful consequences of free radicals through lipid peroxidation, DNA damage, and protein oxidation.

2.2. Gastrointestinal disorders associated to oxidative stress and inflammation

There is a growing body of evidence into the physiology and etiopathogenesis of oxidative stress and inflammation that establish an intricate relationship between both processes. ROS are key molecules that regulate the beginning and the progression of many inflammatory disorders. On the other hand, it is well known the implication of inflammatory states in the generation of large amounts of reactive species [16, 18].

The gastrointestinal tract represents a complex interface system between the body and the external environment. Therefore, it has developed several protective mechanisms such as the mucosal gel layer, peristalsis, pH modulation, and the gut-associated lymphoid tissue. Despite this protective barrier, the gut mucosa is prone to ROS attack as continually exposed to a high concentration of reactive species with origin from endogenous compounds at the luminal surface as well as from exogenous sources and intestinal microbiota [19, 20]. ROS are able to cause inflammation through activation of epithelium, neutrophils, macrophages, and pro-inflammatory cytokines. Inflammation induces oxidative stress by stimulation of ROS-generating systems in the gastrointestinal tract, such as NAD(P)H oxidase, xanthine oxidoreductase, inducible nitric oxide synthase (iNOS), and myeloperoxidase release from inflammatory cells [18]. Moreover, disruption of redox homeostasis is related to the infiltration of inflammatory agents across the gut mucosa and the stimulation of signaling pathways, especially the redox-sensitive transcription factor NF-κB [21]. All these factors contribute to a variety of gastrointestinal alterations, and ultimately lead to the initiation and promotion of several pathophysiological conditions at this organism level. Therefore, a large

amount of evidence has highlighted the implication of redox imbalance and chronic inflammation on the development of various gastrointestinal diseases [22, 23].

The highly selective and regulated gastrointestinal epithelial barrier allows nutrients, ions, and water to be absorbed, but hampers the passage of harmful molecules, pathogens, and endotoxins. Inflammatory processes accompanied by the attack of free radicals are able to impair the epithelial barrier and disrupt the gut mucosa, leading to the alteration termed as increased intestinal permeability. As a consequence, decreased transcellular electrical resistance, increased paracellular permeability, affected epithelial tight junctions, and increased apoptosis rate have been shown at the epithelium level [24]. Integrity of gut mucosa may be compromised by both direct and indirect disorders. Excessive ROS lead to the formation of oxidative products such as lipid peroxides and protein carbonyl groups, which cause damage to the intercellular junctions [25]. On the other hand, redox imbalances are implicated in the activation of the apoptotic c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinases (MAPKs) signaling pathways [16, 26]. Regarding to increased intestinal permeability, it has been associated with Crohn´s disease and ulcerative colitis, celiac disease, gastrointestinal infections, and food allergies [24].

Inflammatory bowel disease (IBD), including both Crohn´s disease and ulcerative colitis, is a relapsing and remitting condition that involves chronic inflammation of the gastrointestinal tract. Despite the exact etiology remains to be elucidated, it is known to be mediated by several factors associated with an inflammatory-oxidative state [22, 23], leading to uncontrolled and abnormal immune reactions in the gut mucosa. Oxidative injury from free radicals as well as reduced antioxidant levels, accompanied by perpetuate inflammation with endothelial dysfunction and tissue damage, are causative findings that have been found in both IBD experimental animals and human subjects [27-29]. Evidences of IBD human patients are marked by increased levels of reactive species and oxidative biomarkers (lipid peroxidation products, and protein carbonyls) in colonic mucosa, correlated with disease severity and progression [30], and depletion of mucosal defensive agents (GSH and related-enzymatic systems, superoxide dismutase, catalase, paraoxonase-1, and metallothionein) [29], as well as at the peripheral systemic level [31].

Persistent oxidative stress and chronic inflammation contribute to tissue dysplasia. ROS are able to attack cellular molecules such as, proteins, lipids, DNA, and organelles, and trigger cytotoxic effects, altered phenotypic patterns, and the uncontrolled transformation of epithelium. Colorectal cancer in IBD patients has been found to progress from low-grade dysplasia to indefinite dysplasia, and then high-grade dysplasia with invasive adenocarcinoma as an ending step [23]. Besides, interleukin (IL)-6, IL-8, transforming growth factor (TGF)-β1, cyclooxygenase (COX)-2 mediators, and inflammation-induced protein kinase B and NF-κB activation, and JNKs and p38 inhibition may perpetuate this process [16]. In the literature, some population-based studies and meta-analysis have demonstrated an increased risk of developing gastrointestinal cancers in IBD populations [32-35].

To sum up, many gastrointestinal disorders have been associated to the inflammatory-oxidative pathophysiology, such as, gastroesophageal reflux, Barrett´s esophagus, esophageal adenocarcinoma, esophageal squamous cell cancer, gastritis, peptic ulcers, gastric adenocarcinoma, IBD, enteric infections, ischemic intestinal injury, and colorectal cancer, among others. Because of the

continuous and direct interaction between food and the digestive tract, dietary compounds may fortunately represent an interesting source of protective antioxidant and anti-inflammatory agents for gastrointestinal health [36].

3. Antioxidant and anti-inflammatory compounds from soybean

Over the past decades, accumulating evidence of experimental, epidemiological and clinical studies have linked consumption of soy foods with low incidences of several diseases, such as gastrointestinal disorders, cardiovascular diseases, metabolic disorders and cancers. These kind of diseases accompanied oxidative stress and inflammatory response in physiological changes [37-39]. The majority of soy components with antioxidant and anti-inflammatory properties include phenolic compounds, isoflavones, phospholipids, oligosaccharides, protein hydrolyzates, protease inhibitors and lunasin, which play protective roles in individuals who frequently consume soy foods [40]. This section summarizes the antioxidant and anti-inflammatory effects of soybean phytochemicals (**Figure 2**). Their properties and mechanisms of action will be also included.

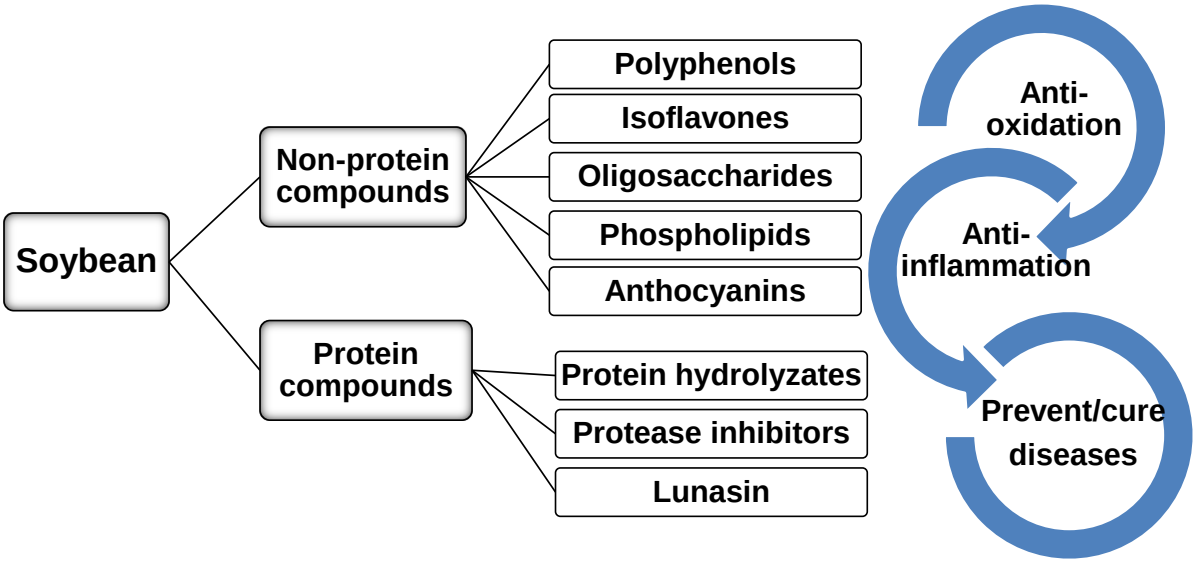


Figure 2. The schema of the health effects of phytochemicals in soybean with antioxidative and anti-inflammatory properties

3.1. Non-protein compounds: polyphenols, isoflavones, oligosaccharides, and phospholipids

The health benefits of soy consumption have received rising interest worldwide. Soybean contains several bioactive compounds, including isoflavones, phytate, saponins, phytosterol, vitamins, and minerals which exert beneficial effects on human health such as adjustment of cholesterol levels and metabolic changes. Components and amounts of phytochemicals in soy depend on environmental factors. Generally, the contents of major phytochemicals in soybean are phytic acid (1.0-2.2%), saponins (0.2-6.2%), phytosterols (0.23-0.46%), and isoflavones (0.1-0.3%) [41].

3.1.1. Polyphenols

Polyphenols are plant's compounds that show an extensive variety of chemical structures with multiple bioactivities and beneficial health effects. They are classified into two major types: flavonoids and non-flavonoids [42]. Ekor and coworkers have demonstrated that rats with acute toxic injury induced by cisplatin, and then fed the phenolic extract of soybean, improved the activities of the antioxidant enzymes such as superoxide dismutase, catalase, and GSH-S-transferase, suggesting prevention of GSH depletion and decrease of malondialdehyde levels in liver and kidney [43]. The data indicated that phenolic extract conducted antioxidant and anti-inflammatory effects, thereby, providing protective benefits against toxic injury in rats. Furthermore, antimicrobial activity of dietary phenolic compounds against gut microbes has been also reported by *in vitro* and *in vivo* studies [42].

3.1.2. Isoflavonoids

Isoflavones have been suggested as the major bioactive components in soy, receiving a growing body of considerable interest. The three major isoflavones, genistein, daidzein and glycitein have been extensively studied [44, 45]. Their structure and function are similar to 17-estradiol, the most potent mammalian estrogen, thus, they are called phytoestrogens [45]. Soy isoflavones exert both estrogenic and/or anti-estrogenic effects depending on the compounds and target tissues, interacting with estrogen receptor, blocking the binding of potent estrogens and/or regulating target genes expression [46]. This effect resulted in beneficial properties for chronic renal disease [47]. Moreover, the extracted isoflavone from soy decreased interferon (IFN)γ secretion of mitogen-stimulated T cells and autoantibodies of serum that contributed to delay inflammation in kidney [48].

Besides, non-hormonal mechanisms of isoflavones are believed to participate in their antioxidant, anti-inflammatory, and anti-proliferative properties [44, 49]. The chemical structure of soy isoflavones contributes to their antioxidative effects resulting in a decrease of lipid peroxidation and oxidative-DNA damage [44]. This antioxidative activity was demonstrated for soymilk fermented by probiotic *Lactobacillus* spp. which polyphenols inhibited oxidative reaction and increased proteolysis improving soymilk digestion by humans. In addition, isoflavone aglycones increased the antioxidative activity in soymilk during fermentation, suggesting health benefits [50]. Evidence has found that neither soyfoods nor soy isoflavones affect inflammatory mediators such as IL-6 or TNF-α expression [49]. In addition, growing literature suggests that isoflavones may have an additionally beneficial role in lipid and glucose metabolism [45]. In summary, isoflavones have beneficial effects in several chronic diseases through their anti-oxidative and anti-inflammatory activities, and more clinical trials are needed to prove these effects.

3.1.3. Phospholipids

Dietary phospholipids are contained in different food sources, such as soy, egg yolk, milk and marine sources, with demonstrated health benefits. Low phospholipid levels have been associated with various illnesses and symptoms, such as CVD, inflammation and cancer [51]. Phospholipids effectively deliver the fatty acid residues for incorporation into the membranes of cells. The altered

composition of membrane might be modulated by the activity of proteins affecting the microstructure and characteristics of membranes, or the biosynthesis of derived lipid second messengers. The essential phospholipids purified from soybeans influenced membrane-associated cellular functions and showed antioxidant, anti-inflammatory, apoptosis and regenerative, membrane repairing and protective, and cell signaling properties both in *in vitro* and *in vivo* studies [52]. Therefore, soy phospholipids might be considered as a potential therapeutic treatment and/or prevention of chronic diseases.

3.1.4. Oligosaccharides

Soybean oligosaccharides have been approved as potential prebiotic material and a safe ingredient by the Food and Drug Administration (FDA) in the US [53]. They have been shown to be a potential candidate for prevention/therapy of various diseases such as cancer, atherosclerosis and menopausal disorders [54, 55]. Their antioxidative properties have been shown in a rat model of myocardium ischemia reperfusion injury. Rats pretreated with soybean oligosaccharides showed a reduction in infarct size, and decrease in creatine kinase, aspartate transaminase and lactate dehydrogenase levels [55]. Moreover, in rats fed a high fat diet, soybean oligosaccharides were able to modify abnormal blood lipid prolife and decrease oxidative stress status [54]. In a clinical study, soybean oligosaccharides increased anti-oxidative enzyme activities, reduced malondialdehyde level in serum, and alleviated insulin resistance in women with gestational diabetes mellitus [56], suggesting that these promising compounds are beneficial to control multiple disease complications.

3.1.5. Anthocyanins

Anthocyanins are well known antioxidant and anti-inflammatory compounds. Treatment of anthocyanins in immortalized epidermal keratinocytes and human neonatal dermal fibroblasts resulted in an increase of vascular endothelial growth factor levels and wound healing, whereas thrombospondin 1 was decreased, and the translocation of NF-κB (p65) and its phosphorylation were inhibited. This data indicated that anthocyanins enhance wound healing, angiogenesis, and exert anti-inflammatory effects [57]. Recently, dietary anthocyanins supplement was found to reduce the expression of cytosolic β-catenin, COX-2 and phospholipase A2 in intestinal mucosa cells, contributing to lower the development of intestinal tumors in APC Min/+ mice [58]. The therapeutic effects of anthocyanins extracted from black soybean coats have been also shown to participate in the suppression of Th17 inflammatory signals, and to retard oxidative stress, and then to inhibit the arthritis process in an autoimmune mice model [59]. These findings showed that the anthocyanins' properties might provide support for their application as anti-inflammatory-related diseases agents.

3.2. Protein compounds: protein hydrolyzates, protease inhibitors and lunasin

In 2006, the American Cancer Society declared that soy-derived foods are excellent dietary protein sources and a good alternative to meat proteins [60]. The following section summarizes the soy proteins and peptides with antioxidative and anti-inflammatory bioactivities, including their mechanisms of action.

3.2.1. Protein hydrolyzates

Several soy-derived peptides and peptide-rich hydrolysates have been demonstrated to exert biological activities by both *in vivo* and *in vitro* studies. Soy protein hydrolysates prepared by several proteases such as alcalase, flavourzyme, trypsin, and papain, have been demonstrated to exert more potent antioxidant and anti-inflammatory activities than the intact protein [61]. As an example, glycinin and β-conglycinin of soy protein have shown radicals-scavenging activity about 3-5 times higher after enzymatic digestion by protease S from *Bacillus spp.* [62].

Soybean peptides are involved in the production of anti-inflammatory mediators by intestinal epithelial and immune cells, reduce myeloperoxidase activity, increase the suppressor T cells, and retardate the tissue damage, suggesting that these peptides might exert benefits against IBD [63, 64]. Vernaza and co-workers analyzed the oxidative and inflammatory markers of germinated Brazilian soybean protein extracts. The protein hydrolysates produced from those extracts for 18 and 72 hours showed a significant inhibition of markers nitric oxide, iNOS, prostaglandin E2 (PGE_2), COX-2, and TNF-α in RAW 264.7 macrophages stimulated by lipopolysaccharide (LPS), suggesting that germination and hydrolysis executed more potential bioactivities compared with original soybean, mainly due to the release of bioactive sequences such as RQRK and VIK [65]. Soymilks hydrolyzed by pepsin-pancreatin have been shown to inhibit of LPS-induced inflammation in RAW 264.7 cells, indicating that soy products might be considered to maintain healthy status under inflammatory stress [66].

3.2.2. Protease inhibitors (BBI and KTI)

Accumulating evidence has revealed that protease inhibitors found in plant tissues, mainly from legumes, act by targeting different enzymes or molecules, being involved in various biological processes. The most popular inhibitor, known as Bowman-Birk protease inhibitor (BBI), contains 71 amino acid residues, was isolated from soybean and characterized by its ability to inhibit trypsin and chymotrypsin [67]. Both soybean BBI and its concentrate (BBIC) are nontoxic, safe, and have been reported to exert anti-inflammatory and anti-carcinogenic activities by *in vitro* and *in vivo* assays [67]. BBIC was granted as “Investigational New Drug” status under the FDA policies, and there are several completed and ongoing human trials on its properties [68]. Arbogast and co-workers have reported that BBIC treatment retarded ROS and serine protease activity *in vitro*. In addition, animal fed supplement with BBIC protected skeletal muscle during prolonged unloading and promoted redox homeostasis in muscle fibers [69]. Moreover, oral administration of BBIC and BBI both ameliorated inflammation and demyelination in the spinal cord of the treated groups [70, 71]. It has been demonstrated the anti-inflammatory and immune regulatory effects of BBI in the experimental autoimmune encephalomyelitis model, suggesting that therapeutic effects of BBI against multiple sclerosis might be considered [71]. BBI treatment significantly inhibited IL-1β, IL-6, TNF-α and ROS productions in LPS-induced macrophages [72].

Kunitz trypsin inhibitor (KTI) is another soybean-derived protease inhibitor found to exert anti-carcinogenesis properties mainly attributed to its ability to inhibit tumor invasion and metastasis [73]. Moreover, the suppressive effects have been also shown after intraperitoneal injection or oral

administration of this inhibitor in C57BL/6 mice, where a significant reduction of the LPS-induced lethality was mediated through inhibition of MAP kinases activation and pro-inflammatory cytokines expression [74].

Accumulating evidence has showed that these protease inhibitors are safe and low cost. Moreover, they not only act as anti-proteolytic proteins, but also are good candidates for oral administration exerting physiological bioactivities without additional side effects [75, 76].

3.3.3. Lunasin

Lunasin, SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD, is a 43 amino acid peptide firstly identified in soybean in 1999 [77]. Lunasin is resistant to gastrointestinal digestion through protection by soybean protease inhibitors, such as BBI and KTI, being bioavailable to reach target tissues in an intact and active form [78]. Lunasin is considered as one of the most promising naturally agent that has been extensively studied in the last two decades on the basis of its potential against chronic diseases, such as cancer, cardiovascular and immunological disorders.

Hernández-Ledesma and co-workers firstly demonstrated that lunasin exerts antioxidant and anti-inflammation properties [79], which might contribute on its chemopreventive properties. Lunasin acts as a free radical scavenger and an inhibitor of ROS production in active RAW 264.7 cells [79]. Moreover, the ability of lunasin to protect human hepatic HepG2 and epithelial intestinal Caco2 cells from oxidative stress induced by tert-butylhydroperoxide has been also reported [80, 81]. In *Solanum nigrum L.*, purified-lunasin protected DNA oxidation by blocking fenton reaction and then, suppressing the generation of hydroxyl radical [82].

The anti-inflammatory properties of lunasin were initially revealed to be mediated through inhibition of pro-inflammatory mediators IL-6, TNF-α, and PGE_2 production in RAW 264.7 cells stimulated by LPS [79]. Subsequently, blocking signaling pathways of COX-2 and iNOS, and suppression of NF-κB pathway have also been demonstrated for this peptide [83, 84]. It has revealed that lunasin treatment inhibited the NF-κB-dependent inflammatory mediators through down-regulation of Akt phosphorylation and p65 protein expression in human LPS-challenged THP-1 macrophages by [85]. These findings indicate that the antioxidant and anti-inflammatory properties of lunasin could contribute on its protective effects against several related disorders.

4. Effects of soybean processing on antioxidant activity

4.1. Fermentation

A large number of studies have shown that the fermentative activities of different microorganisms are not only determinant on the sensorial, technological and nutritional characteristics of foods, but also may increase the functional value of natural products. Soybean fermentation has presented improved health benefits mainly due to the enhanced content in free isoflavones and peptides. Fermented soybean products have been consumed in many Asian countries for centuries. They mainly result from the microbial effect of bacteria (*Bacillus* spp., lactic acid bacteria), fungi (*Aspergillus* spp., *Mucor* spp., and *Rhizopus* spp.), or a combined action of both microbes, such as the example of the soybean paste termed doenjang [86]. **Table 1** summarizes the recent evidence on the

Table 1. Antioxidant activity of fermented soybean products

Method of preparation	Time (h)	Antioxidant activity assays	Other analysis	Antioxidant outcomes	Reference
Soy germ + *Aspergillus niger* M46	7 d	Radical (DPPH, peroxyl, hydroxyl, superoxide) scavenging	Total polyphenolic content Total flavonoid content Flavonoid analysis α-amilase and β-glucosidase activity	Higher radical scavenging activity compared with unfermented soy germ β-glucosidase and α-amylase were mainly responsible for the mobilization of phenolics during fermentation	[87]
Soybean + *Aspergillus oryzae* NL 5	7 d	Radical (DPPH) scavenging Ferric reducing power Antioxidant effects in hepatoma hepa1c1c7 cells	Isoflavone analysis	Antioxidant activity was proportional to the concentration of free isoflavones, and was significantly influenced by total isoflavone content of soybean variety and fermentation period	[88]
Soybean + *Aspergillus oryzae*	5 d	Radical (DPPH and ABTS) scavenging Ferric reducing power Antioxidant activity in stimulated- HepG2 cells Lipid peroxidation and antioxidant enzyme activities in animals Biomarkers gene expression	Protein content Isoflavone analysis Histopathological assays	Antioxidant and hepatoprotective effects on *tert*-butyl hydroperoxide-induced oxidative stress in HepG2 cells and in the rat liver	[90]
Soybean fermented paste with *Aspergillus oryzae*	72	Lipid peroxidation in mice Antioxidant enzyme activities	Hepatic glucose regulating enzyme	Diet supplementation of fermented paste inhibited oxidative stress via regulation of antioxidant enzymes	[89]
Black soybean + *Rhizopus oligosporous* NTU-5	48	Radical (DPPH) scavenging	Isoflavone quantification Polyphenol quantification Cytotoxicity Identification of bioactive compounds	High antioxidant activity that does not correspond to total polyphenol or isoflavone content An unknown compound, FBE5-A, with strong antioxidant activity was isolated	[106]
Fermented soybean paste (Doenjang) with *Aspergillus oryzae* J, *Mucor racemosus* 15,	---	Radical (DPPH) scavenging Nitrite radical scavenging	Total phenolic content Tyrosinase inhibitory	The starter culture Doenjang samples demonstrated considerable *in vitro* antioxidant, α-glucosidase inhibitory, and tyrosinase	[107]

Mucor racemosus 42, and *Bacillus subtilis* TKSP 24		Ferric reducing power	activity α-glucosidase inhibitory activity	inhibitory effects	
Soybean and soy meal + *Bacillus* natto js-1	48	Radical (DPPH, superoxide) scavenging Ferric reducing power Lipid peroxidation inhibition in rats liver Antioxidant enzyme activities and malondialdehyde in rats	Total flavonoid content Inhibition of erythrocyte haemolysis in rat blood	*In vitro* and *in vivo* antioxidant effects of fermented soy meal	[93]
Soybean + *Bacillus subtilis*	120	Radical (DPPH) scavenging Ferric reducing power Nitrite scavenging	Total phenolic content Total flavonoid content HPLC of free phenolic acid Tyrosinase inhibitory activity Conjugated diene inhibitory activity Anti-inflammatory activity	Lower antioxidant activity compared to fermented sword beans	[91]
Soybean + *Bacillus subtilis* MTCC5480 and MTCC1747	24	Radical (DPPH, superoxide) scavenging Ferric reducing power Total antioxidant activity	Total phenolic content Degree of protein hydrolysis Protein extractability Free amino acid analysis	Fermentation increased radical scavenging activity, total antioxidant activity and reducing power in comparison with unfermented soybean Higher degree of protein hydrolysis increase in free phenolics and free amino acid contents on hydrolysis	[92]
Soy whey + *Lactobacillus plantarum* B1-6	24	Radical (ABTS, hydroxyl, superoxide) scavenging Ferric reducing power Assessment of DNA damage	Total phenolic content Flavonoid analysis	Higher antioxidant activity of fermented soy whey compared with unfermented samples Higher total pehnolic and isoflavone aglycone contents	[108]
Soybean protein meal + *Lactobacillus plantarum* LP6	72	Radical (DPPH and hydroxyl) scavenging Ferric reducing power	Amino acid composition	Release of antioxidant peptides and amino acids by fermentation	[109]

Soymilk + *Lactobacillus rhamnosus* CRL981	24	Metal chelating Antioxidant enzyme activities in mice	Biochemical analysis	Fermented soymilk significantly decreased glucose levels, total cholesterol concentrations, triacylglycerols and increase antioxidant enzyme activities compared to animals that received unfermented soymilk	[94]
Soymilk fermented with *Streptococcus thermophilus* grx02	---	Radical (DPPH) scavenging Lipid peroxidation inhibition Reduced glutation content and antioxidant enzyme activities in mice	---	*In vitro* and *in vivo* antioxidant effects of fermented soymilk	[95]
Soybeans and derived-products (Soy sauce, Cheonggukjang, Meju, Doenjang, Makjang)	---	Radical (DPPH) scavenging	Total phenolic content Total flavonoid content Protein content Other biological activities Identification of bioactive compounds	Antioxidant activity increased as fermentation time increased. Total phenolic and protein contents showed strong negative correlations with antioxidant activity	[96]
Fermented soy products	---	Radical (DPPH) scavenging Ferric reducing power	Total phenolic content Total flavonoid content Flavonoid analysis Amino acid analysis	Higher antioxidant activity in fermented products compared with unfermented samples Increase of total phenolics and flavonoid during fermentation Changes in isoflavone profiles Increase of essential amino acids content	[97]

antioxidant effects of fermented soybean products, including the type of fermented preparation, the antioxidant properties demonstrated, and the analyses carried out to evaluate the chemo-preventive attributes of these food-derived compounds.

Aspergillus spp. have been widely used in the fermentation of soybean. Sheih and coworkers found that the radical 1,1-Diphenyl-2-picryl-hydrazyl, peroxyl, hydroxyl, and superoxide scavenging activity of fermented soy germ with *Aspergillus niger* M46 was higher than that demonstrated for the unfermented source [87]. Similarly, it was shown that *Aspergillus oryzae* NL 5 was able to improve the radical scavenging and ferric reducing power and the antioxidant defense systems of liver HepG2 cells, being these activities directly related to the content of free isoflavones on the soybean fermented preparation [88]. Moreover, this *Aspergillus* specie has proved functionality in *in vivo* models on biological markers at lipid peroxidation and enzymatic antioxidant levels [89, 90]. From a bacterial origin, recent studies have reported the potential of *Bacillus subtilis* to enhance the *in vitro* anti-radical activities of soybean [91], which were related to the increase in free phenolics and amino acids contents on fermentation-hydrolyzed products [92]. Similar results have been shown for different lactic acid bacteria *spp.* (**Table 1**). Moreover, biochemical analysis on the antioxidant profiles of serum and liver tissue proved the *in vivo* protective effects provided by soybean fermentation with *Bacillus* natto js-1 [93], *Lactobacillus rhamnosus* CRL981 [94], and *Streptococcus thermophilus* grx02 [95].

Interestingly, integrative studies have been recently published aiming to connect the determination of bioactive phytochemicals and their proved biological effects, related to the fermentation process. Chai and coworkers found an enhanced antioxidant radical scavenging activity as the fermentation time increased, as well as it was promoted the protein and the total phenolic contents [96]. Xu and coworkers also found a higher antioxidant activity, and total phenolic and flavonoid contents in commercially fermented soy products marketed in China, compared with the unfermented samples [97]. However, these authors hardly established a correlation between bioactivities and the observed changes in phytochemicals levels. Therefore, further studies are needed to systematically assess the bioactive compounds and antioxidant properties of commercially fermented soybean products and to clearly elucidate relationships with the presence of beneficial food components.

4.2. Germination and roasting

Germination has been recognized as an economical and efficient technology for the breakdown of macromolecules, increase of digestibility, and enhancement of nutritive values of soybean. Moreover, germinated beans are thought to contain more functional compounds, and also to eliminate or reduce anti-nutritional factors by activating the formation of enzymes, showing health benefits as compared to non-germinated beans [98]. However, many factors such as the bean cultivar, time of germination and soaking, humidity, and temperature might influence the germination process and then the contents and composition of nutrients in germinated beans [99]. Furthermore, it has been demonstrated that soybean seeds presented increased phenolic amounts correlated with antioxidant *in vitro* activities, when germinated in presence of light as compared to the dark-growth sprouts [100]. Combination of germination and fermentation was also an interesting strategy for the production of the

soybean paste doenjang, which showed greater antioxidant, and angiotensin converting enzyme- and fibrinolytic-inhibitory activities than the regular soybean doenjang [101]. In a systematic review of germinated soybean products, it was recently found that the contents of L-ascorbic acid, phenolics, isoflavones, and antioxidants were significantly improved by biochemical reactions during germination, being 4 days at 25 ºC the recommendation for optimal germination of soybean with additional functional values [102].

In food processing, roasting has been used to neutralize the anti-nutritional factors of soybean and to provide characteristic sensorial features to the final product. However, few studies have been focused on the impact of roasting conditions in the antioxidant properties of soybean. By *in vitro* assays, it has been demonstrated that the contents of isoflavone aglycones, glycosides, total phenolics and flavonoids were increased after roasting, ultimately leading to a general improved antioxidant capacity [103, 104]. Likewise, these findings were also reported by Kim and coworkers, showing further protection through suppressive effects of ROS production in rat PC12 cells stressed by H_2O_2 [105].

5. Concluding remarks

Soybean is one of the most cultivated plants in the world and ranked as one of the top sources of different functional ingredients, including some excellent proteins and peptides, phospholipids, oligosaccharides, phytosterols, along with phytoestrogens like genistein and daidzein. This chapter has summarized the evidence on the role played by the antioxidant and anti-inflammatory activity of soybean's compounds as responsible for its protective effects against intestinal and other chronic disorders related to oxidative stress and inflammation. Although soybean has been recognized as a functional food, there is still a lot to be learned about it and its beneficial properties. More researchs on the bioactivities of different soybean compounds and further elucidation of their mechanisms of action are needed. In addition, other aspects worthy of research are the bioavailability of those bioactives, the possible synergisms among them, and the influence of soybean processing on their biological effects.

References

1. Synder, H. E., and Kwon, T. W. (1987). Soybean Utilization. Van Nostrand Reinhold Co., New York.
2. Coward, L., Barnes, N., Setchell, K., and Barnes, S. (1993). Genistein, daidzein, and their betaglycoside conjugates: antitumor isoflavones in soybean food from American and Asian diets. *Journal of Agricultural and Food Chemistry* **41**, 1961-1967.
3. Wang, H., and Murphy, P. (1994). Isoflavone content in commercial soybean foods. *Journal of Agricultural and Food Chemistry* **42**, 1666-1673.
4. Omoni, A. O., and Aluko, R. E. (2005). Soybean foods and their benefits: potential mechanisms of action. *Nutrition Reviews* **63**, 272-283.
5. Lokuruka, M. N. I. (2010). Soybean nutritional properties: the good and the bad about soy foods consumption – a review. *African Journal of Food Agriculture Nutrition and Development* **10**, 1-21.
6. Karr-Lilienthal, L. K., Kadzere, C. T., Grieshop, C. M., and Fahey, G. C. (2005). Chemical and nutritional properties of soybean carbohydrates as related to nonruminants. *Livestock Production Science* **97**, 1-12.
7. Watanabe, D. J., Ebine, H. O., and Ohda, D. O. (1971). Soybean Foods. Kohrin Shoin, Tokyo.
8. Bressani, R. (1981). The role of soybeans in food systems. *Journal of American Oil Chemists' Society* **58**, 392-400.
9. Isanga, J., and Zhang, G. –N. (2008). Soybean bioactive components and their implications to health - a review. *Food Reviews International* **24**, 252-276.
10. Ahmad, A., Hayat, I., Arif, S., Masud, T., Khalid, N., and Ahmed, A. (2014). Mechanisms involved in the therapeutic effects of soybean (*Glycine max*). *International Journal of Food Properties* **17**, 1332-1354.
11. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology* **39**, 44-84.
12. Trachootham, D., Lu, W., Ogasawara, M. A., Nilsa, R. D., and Huang, P. (2008). Redox regulation of cell survival. *Antioxidant & Redox Signaling* **10**, 1343-1374.
13. Ray, P. D., Huang, B. W., and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signaling* **24**, 981-990.
14. Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Molecular Nutrition & Food Research* **52**, 507–526.
15. Jomova, K., Vondrakova, D., Lawson, M., and Valko, M. (2010). Metals, oxidative stress and neurodegenerative disorders. *Molecular and Cellular Biochemistry* **345**, 91-104.
16. Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer. How are they linked? *Free Radical Biology and Medicine* **49**, 1603-1616.
17. Rains, J. L., and Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radical Biology and Medicine* **50**, 567-575.

18. Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & Redox Signaling* **20,** 1126-1167.
19. Couto, M. R., Gonçalves, P., Catarino, T., Araújo, J. R., Correira-Branco, A., and Martel, F. (2012). The effect of oxidative stress upon the intestinal uptake of folic acid: *in vitro* studies with Caco-2 cells. *Cell Biology and Toxicology* **28**, 369-381.
20. Graham-Espey, M. (2013). Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. *Free Radical Biology and Medicine* **55**, 130-140.
21. Zhu, H., and Li, R. (2012). Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Experimental Biology and Medicine* **237**, 474-480.
22. Kim, Y. J., Kim, E. -H., and Hahm, K. B. (2012). Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. *Journal of Gastroenterology and Hepatology* **27**, 1004-1010.
23. Bhattacharyya, A., Chattopadhyay, R., Mitra, S., and Crowe, S. E. (2014). Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews* **94**, 329-354.
24. John, L. J., Fromm, M., and Schulzke, J. D. (2011). Epithelial barriers in intestinal inflammation. *Antioxidants & Redox Signaling* **15**, 1255-1270.
25. Wang, N., Wang, G., Hao, J., Ma, J., Wang, Y., Jiang, X., and Jiang, H. Q. (2012). Curcumin ameliorates hydrogen peroxide-induced epithelial barrier disruption by upregulating heme oxygenase-1 expression in human intestinal epithelial cells. *Digestive Diseases and Sciences* **57**, 1792-1801.
26. Rosillo, M. A., Sánchez-Hidalgo, M., Cardeno, A., and de la Lastra, C. A. (2011). Protective effect of ellagic acid, a natural polyphenolic compound, in a murine model of Crohn´s disease. *Biochemical Pharmacology* **82**, 737-745.
27. Damiani, C. R., Benetton, C. A., Stoffel, C., Bardini, K. C., Cardoso, V. H., Di Giunta, G., Pinho, R. A., Dal-Pizzol, F., and Streck, E. L. (2007). Oxidative stress and metabolism in animal model of colitis induced by dextran sulfate sodium. *Journal of Gastroenterology and Hepatology* **22**, 1846-1851.
28. Mizoguchi, A., and Mizoguchi, E. (2010). Animal models of IBD: linkage to human disease. *Current Opinion in Pharmacology* **10**, 578-587.
29. Catarzi, S., Favilli, F., Romagnoli, C., Marcucci, T., Picariello, L., Tonelli, F., Vincenzini, M. T., and Iantomasi, T. (2011). Oxidative stress and IL-6 production in intestinal myofibroblasts of Crohn´s disease. *Inflammatory Bowel Diseases* **17**, 1674-1684.
30. Hatsugai, M., Kurokawa, M. S., Kouro, T., Nagai, K., Arito, M., Masuko, K., Suematsu, N., Okamoto, K., Itoh, F., and Kato, T. (2010). Protein profiles of peripheral blood mononuclear cells are useful for differential diagnosis of ulcerative colitis and Crohn´s disease. *The Journal of Gastroenterology* **45**, 488-500.

31. Krzystek-Korpacka, M., Neubauer, K., Berdowska, I., Zielinski, B., Paradowski, L. and Gamian, A. (2010). Impaired erythrocyte antioxidant defense in active inflammatory bowel disease: impact of anemia and treatment. *Inflammatory Bowel Diseases* **16**, 1467-1475.
32. Bernstein, C. N., Blanchard, J. F., Kliewer, E., and Wajda, A. (2001). Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* **91**, 854-862.
33. Herrinton, L. J., Liu, L., Levin, T. R., Allison, J. E., Lewis, J. D., and Velayos, F. (2012). Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1988 to 2010. *Gastroenterology* **143**, 382-389.
34. Jess, T., Horváth-Puhó, E., Fallingborg, J., Rasmussen, H. H., and Jacobsen, B. A. (2013). Cancer risk in inflammatory bowel disease according to patient phenotype and treatment: a Danish population-based cohort study. *American Journal of Gastroenterology* **108**, 1869-1876.
35. Kappelman, M. D., Farkas, D. K., Long, M. D., Erichsen, R., Sandler, R. S., Sorensen, H. T., and Baron, J. A. (2014). Risk of cancer in patients with inflammatory bowel disease: a nationwide population-based cohort study with 30 years of follow-up evaluation. *Clinical Gastroenterology and Hepatology* **12**, 265-273.
36. Moura, F. A., Queiroz de Andrade, K., Farias dos Santos, J. C., Pimentel Araújo, O. R., and Fonseca Goulart, J. C. (2015). Antioxidant therapy for treatment of inflammatory bowel disease: Does it work? *Redox Biology* **6**, 617-639.
37. Ogura, S., and Shimosawa, T. (2014). Oxidative stress and organ damages. *Current Hypertension Reports* **16**, 452.
38. Piechota-Polanczyk, A., and Fichna, J. (2014). Review article: the role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases. *Naunyn Schmiedebergs Archives of Pharmacology* **387**, 605-620.
39. Verdile, G., Keane, K. N., Cruzat, V. F., Medic, S., Sabale, M., Rowles, J., Wijesakara, N., Martins, R. J., Fraser, P. E., and Newsholme, P. (2015). Inflammation and oxidative stress: the molecular connectivity between insulin resistance, obesity, and Alzheimer's disease. *Mediators of Inflammation* **2015,** 105828.
40. Bouchenak, M, and Lamri-Senhadji, M. (2013). Nutritional quality of legumes, and their role in cardiometabolic risk prevention: a review. *Journal of Medicinal Food* **16**,185-198.
41. Kang, J., Badger, T. M., Ronis, M. J., and Wu, X. (2010). Non-isoflavone phytochemicals in soy and their health effects. *Journal of Agricultural and Food Chemistry* **58**, 8119-8133.
42. Etxeberria, U., Fernández-Quintela, A., Milagro, F. I., Aguirre, L., Martínez, J. A., and Portillo, M. P. (2013). Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *Journal of Agricultural and Food Chemistry* **61**, 9517-9533.
43. Ekor, M., Emerole, G. O., and Farombi, E. O. (2010). Phenolic extract of soybean (Glycine max) attenuates cisplatin-induced nephrotoxicity in rats. *Food and Chemical Toxicology* **48**, 1005-1012.
44. Xiao, C. W. (2008). Health effects of soy protein and isoflavones in humans. *Journal of Nutrition* **138**, 1244S-1249S.
45. Cederroth, C. R. and Nef, S. (2009). Soy, phytoestrogens and metabolism: A review. *Molecular and Cellular Endocrinology* **304**, 30-42.

46. Morito, K., Aomori, T., Hirose, T., Kinjo, J., Hasegawa, J., Ogawa, S., Inoue, S., Muramatsu, M., and Masamune, Y. (2002). Interaction of phytoestrogens with estrogen receptors alpha and beta (II). *Biological and Pharmaceutical Bullwtin* **25**, 48-52.
47. Velasquez, M. T., and Bhathena, S. J. (2001). Dietary phytoestrogens: a possible role in renal disease protection. *American Journal of Kidney Disease* **3**, 1056-1068.
48. Hong, Y. H., Wang, T. C., Huang, C. J., Cheng, W. Y., and Lin, B. F. (2008). Soy isoflavones supplementation alleviates disease severity in autoimmune-prone MRL-lpr/lpr mice. *Lupus* **17**, 814-821.
49. Masilamani, M., Wei, J., and Sampson, H. A. (2012). Regulation of the immune response by soybean isoflavones. *Immunological Research* **54**, 95-110.
50. Subrota, H., Shilpa, V, Brij, S., Vandna, K. and Surajit, M. (2013). Antioxidative activity and polyphenol content in fermented soy milk supplemented with WPC-70 by probiotic Lactobacilli. *International Food Research Journal* **20**, 2125-2131.
51. Küllenberg, D., Taylor, L. A., Schneider, M., and Massing, U. (2012). Health effects of dietary phospholipids. *Lipids Health Disease* **11**, 3.
52. Gundermann, K. J., Kuenker, A., Kuntz, E., and Drożdzik, M. (2011). Activity of essential phospholipids (EPL) from soybean in liver diseases. *Pharmacological Reports* **63**, 643-659.
53. Zhou, X. L., Kong, X. F., Yang, X. J., and Yin, Y. L. (2012). Soybean oligosaccharides alter colon short chain fatty acid production and microbial population in vitro. *American Society of Animal Science* **90**, 37-39.
54. Chen, H., Jun, L. L., Jun, J. Z., Bo, X., and Rui, L. (2010). Effect of soybean oligosaccharides on blood lipid, glucose levels and antioxidant enzymes activity in high fat rats. *Food Chemistry* **119**, 1633-1636.
55. Zhang, M., Cai, S., and Ma, J. (2015). Evaluation of cardio-protective effect of soybean oligosaccharides. *Gene* **555**, 329-334.
56. Fei, B. B., Ling, L., Hua, C., and Ren, S. Y. (2014). Effects of soybean oligosaccharides on antioxidant enzyme activities and insulin resistance in pregnant women with gestational diabetes mellitus. *Food Chemistry* **158**, 429-432.
57. Xu, L., Choi, T. H., Kim, S., Kim, S. H., Chang, H. W., Choe, M., Kwon, S. Y., Hur, J. A., Shin, S. C., Chung, J. I., Kang, D., and Zhang, D. (2013). Anthocyanins from black soybean seed coat enhance wound healing. *Annals of Plastic Surgery* **71**, 415-420.
58. Park, M. Y., Kim, J. M., Kim, J. S., Choung, M. G., and Sung, M. K. (2015). Chemopreventive action of anthocyanin-rich black soybean fraction in APC (Min/+) intestinal polyposis model. *Journal of Cancer Prevention* **20,** 193-201.
59. Min, H. K., Kim, S. M., Baek, S. Y., Woo, J. W., Park, J. S., Cho, M. L., Lee, J., Kwok, S. K., Kim, S. W., and Park, S. H. (2015). Anthocyanin extracted from black soybean seed coats prevents autoimmune arthritis by suppressing the development of Th17 cells and synthesis of proinflammatory cytokines by such cells, via inhibition of NF-κB. *PLoS One* **10**, e0138201.
60. Doyle, C., Kushi, L. H., Byers, T., Courneya, K. S., Mark-Wahnefried, W., Grant, B., McTiernan, A., Rock, C. L., Thompson, C., Gansler, T., and Andrews, K. S. (2006). Nutrition and physical activity

during and after cancer treatment: an American Cancer Society guide for informed choices. *CA: A Cancer Journal for Clinicians* **56**, 323-353.

61. López-Barrios L, Gutiérrez-Uribe J. A., and Serna-Saldívar S. O. (2014). Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *Journal of Food Science* **79**, R273-283.
62. Chen, H. M., Muramoto, K., and Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-conglycinin. *Journal of Agricultural and Food Chemistry* **43**, 574-578.
63. Kovacs-Nolan, J., Zhang, H., Ibuki, M., Nakamori, T., Yoshiura, K., Turner, P. V., Matsui, T., and Mine, Y. (2012). The PepT1-transportable soy tripeptide VPY reduces intestinal inflammation. *Biochimica et Biophysica Acta* **1820**, 1753-1763.
64. Young, D., Ibuki, M., Nakamori, T., Fan, M., and Mine, Y. (2012) Soy-derived di- and tripeptides alleviate colon and ileum inflammation in pigs with dextran sodium sulfate-induced colitis. *Journal of Nutrition* **142**, 363-368.
65. Vernaza, M. G., Dia, V. P., de Mejia, E. G., and Chang, Y. K. (2012). Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry* **134**, 2217–2225.
66. Dia, V. P., Bringe, N. A., and de Mejia, E. G. (2014). Peptides in pepsin-pancreatin hydrolysates from commercially available soy products that inhibit lipopolysaccharide-induced inflammation in macrophages. *Food Chemistry* **152**, 423-431.
67. Losso, J. N. (2008). The biochemical and functional food properties of the Bowman-Birk Inhibitor. *Critical Reviews in Food Science & Nutrition* **48,** 94-118.
68. Armstrong, W. B., Taylor, T. H., Kennedy, A. R., Melrose, R. J., Messadi, D. V., Gu, M., Le, A. D., Perloff, M., Civantos, F., Goodwin, W. J., Wirth, L. J., Kerr, A. R., and Meyskens, F. L. (2013). Bowman Birk inhibitor concentrate and oral leukoplakia: a randomized phase IIb trial. *Cancer Prevention Research* **6**, 410-418.
69. Arbogast, S., Smith, J., Matuszczak, Y., Hardin, B. J., Moylan, J. S., Smith, J. D., Ware, J., Kennedy, A. R., and Reid, M. B. (2007). Bowman-Birk inhibitor concentrate prevents atrophy, weakness, and oxidative stress in soleus muscle of hindlimb-unloaded mice. *Journal of Applied Physiology* **102**, 956-964.
70. Gran, B., Tabibzadeh, N., Martin, A., Ventura, E. S., Ware, J. H., Zhang, G. X., Parr, J. L., Kennedy, A. R., and Rostami, A. M. (2006). The protease inhibitor, Bowman-Birk Inhibitor, suppresses experimental autoimmune encephalomyelitis: a potential oral therapy for multiple sclerosis. *Multiple Sclerosis* **12**, 688-697.
71. Safavi, F., and Rostami, A. (2012). Role of serine proteases in inflammation: Bowman-Birk protease inhibitor (BBI) as a potential therapy for autoimmune diseases. *Experimental and Molecular Pathology* **93**, 428-433.
72. Li, J., Ye, L., Cook, D. R., Wang, X., Liu, J., Kolson, D. L., Persidsky, Y., and Ho, W. Z. (2011). Soybean-derived Bowman-Birk inhibitor inhibits neurotoxicity of LPS-activated macrophages. *Journal of Neuroinflammation* **8**, 15.

73. de Mejia, E. G., and Dia, V. P. (2010). The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Reviews* **29**, 511-528.
74. Kobayashi, H., Yoshida, R., Kanada, Y., Fukuda, Y., Yagyu, T., Inagaki, K., Kondo, T., Kurita, N., Suzuki, M., Kanayama, N., and Terao, T. (2005). Dietary supplementation of soybean kunitz trypsin inhibitor reduces lipopolysaccharide-induced lethality in mouse model. *Shock* **23**, 441-447.
75. Kobayashi, H. (2013). Prevention of cancer and inflammation by soybean protease inhibitors. *Front Bioscience (Elite Edition)* **5**, 966-973.
76. Singh, B. P., Vij, S., and Hati, S. (2014). Functional significance of bioactive peptides derived from soybean. *Peptides* **54**, 171-179.
77. Galvez, A. F., and de Lumen, B. O. (1999). A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. *Nature Biotechnology* **17**, 495-500.
78. Hsieh, C. -C., Hernández-Ledesma, B., and de Lumen, B.O. (2010). Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS One* **5**, e8890.
79. Hernández-Ledesma, B., Hsieh, C. -C., and de Lumen, B.O. (2009). Anti-inflammatory and antioxidant properties of peptide lunasin in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications* **390**, 803-808.
80. Fernández-Tomé, S., Ramos, S., Cordero-Herrera, I., Recio, I., Goya, L., and Hernández-Ledesma, B. (2014). *In vitro* chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells. *Food Research International* **62,** 793-800.
81. García-Nebot, M. J., Recio, I., and Hernández-Ledesma, B. (2014). Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. *Food and Chemical Toxicology* **65**, 155-161.
82. Jeong, J. B., de Lumen, B. O., and Jeong, H. J. (2010). Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction. *Cancer Letters* **293**, 58-64.
83. de Mejia, E. G., and Dia, V. P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-kB pathway in the macrophage. *Peptides* **30**, 2388-2398.
84. Dia, V. P., Wang, W., Oh, V. L., de Lumen, B. O., and de Mejia, E. G. (2009). Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. *Food Chemistry* **114**, 108-115.
85. Cam, A., and de Mejia, E. (2012). RGD-peptide lunasin inhibits Akt-mediated NF-kB activation in human macrophages through interaction with the αVβ3 integrin. *Molecular Nutrition & Food Research* **56**, 1569-1581.
86. Sanjukta, S., and Rai, A. K. (2016). Production of bioactive peptides during soybean fermentation and their potential health benefits. *Trends in Food Science & Technology* **50**, 1-10.
87. Sheih, I. -C., Fang, T. J., Wu, T. -K., and Chen, R. -Y. (2014). Effects of fermentation on antioxidant properties and phytochemical composition of soy germ. *Journal of the Science of Food and Agriculture* **94**, 3163-3170.

88. Nam, D. H., Kim, H. J., Lim, J. S., Kim, K. H., Park, C. -S., Kim, H. J., Lim, J., Kwon, D. Y., Kim, I. -H., and Kim J. -S. (2011). Simultaneous enhancement of free isoflavone content and antioxidant potential of soybean by fermentation with *Aspergillus oryzae*. *Journal of Food Science* **76**, H194-H200.
89. Chung, S. I., Rico, C. W., and Kang, M. Y. (2014). Comparative study on the hypoglycemic and antioxidative effects of fermented paste (doenjang) prepared from soybean and brown rice mixed with rice bran or red ginseng marc in mice fed with high fat diet. *Nutrients* **6**, 4610-4624.
90. Kim, E. Y., Hong, K. -B., Suh, H. J., and Choi, H. -S. (2015). Protective effects of germinated and fermented soybean extract against *tert*-butyl hydroperoxide-induced hepatotoxicity in HepG2 cells and in rats. *Food & Function* **6**, 3512-3521.
91. Han, S. S., Hur, S. J., and Lee, S. K. (2015). A comparison of antioxidative and anti-inflammatory activities of sword beans and soybean fermented with *Bacillus subtilis*. *Food & Function* **6**, 2736-2748.
92. Sanjukta, S., Rai, A. K., Muhammed, A., Jeyaram, K., and Talukdar, N. C. (2015). Enhancement of antioxidant properties of two soybean varieties of Sikkim Himalayan region by proteolytic *Bacillus subtilis* fermentation. *Journal of Functional Foods* **14**, 650-658.
93. Yang, X., Chen, J., Zhang, C., Chen, H., and Liu, Y. (2012). Evaluation of antioxidant activity of fermented soybean meal extract. *African Journal of Pharmacy and Pharmacology* **6**, 1774-1781.
94. Marazza, J. A., LeBlanc, J. G., Savoy de Giori, G., and Garro, M. S. (2013). Soybean fermented with *Lactobacillus rhamnosus* CRL981 ameliorates hyperglycemia, lipid profiles and increases antioxidant enzyme activities in diabetic mice. *Journal of Functional Foods* **5**, 1848-1853.
95. Xu, Y., Chen, X., Lu, M., Yang, Z., Huang, Y., Liu, D., Xiao, L., Sun, Y., Gu, W., Xu, D., and Gu, R. (2012). *In vitro* and *in vivo* studies on the antioxidant effects of soymilk fermented with *Streptococcus thermophilus* grx02. *Food Biotechnology* **26**, 339-350.
96. Chai, C., Ju, H. K., Kim, S. C., Park, J. H., Lim, J., Kwon, S. W., and Lee, J. (2012). Determination of bioactive compounds in fermented soybean products using GC/MS and further investigation of correlation of their bioactivities. *Journal of Chromatography B* **880**, 42-49.
97. Xu, L., Du, B., and Xu, B. (2015). A systematic, comparatic study on the beneficial health components and antioxidant activities of commercially fermented soy products marketed in China. *Food Chemistry* **174**, 202-213.
98. Cevallos-Casals, B., and Cisneros-Zevallos, L. (2010). Impact of germination on phenolic content and antioxidant activity of 13 edible seed species. *Food Chemistry* **119**, 1485-1490.
99. Paucar-Menacho, L. M., Berhow, M. A., Mandarino, J. M. G., Chang, Y. K., and de Mejia, E. G. (2010). Effect of time and temperature on bioactive compounds in germinated Brazilian soybean cultivar BRS 258. *Food Research International* **43**, 1856-1865.
100. Yuan, M., Jia, X., Ding, C., Zeng, H., Du, L., Yuan, S., Zhang, Z., Wu, Q., Hu, C., and Liu, J. (2015). Effect of fluorescence light on phenolic compounds and antioxidant activities os soybeans (*Glycine max* L. Merrill) during germination. *Food Science and Biotechnology* **24**, 1859-1865.

101. Kim, H. -E., and Kim, Y. -S. (2014). Biological activities of fermented soybean paste (*doenjang*) prepared using germinated soybeans and germinated black soybeans during fermentation. *Food Science and Biotechnology* **23**, 1533-1540.
102. Huang, X., Cai, W., and Xu, B. (2014). Kinetic changes of nutrients and antioxidant capacities of germinated soybean (*Glycine max* L.) and mung bean (*Vigna radiata* L.) with germination time. *Food Chemistry* **143**, 268-276.
103. Lee, J. H., Lee, B. W., Kim, B., Kim, H. T., Ko, J. M., Baek, I. -Y., Seo, W. T., Kang, Y. M., and Cho, K. M. (2013). Changes in phenolic compounds (isoflavones and phenolic acids) and antioxidant properties in high-protein soybean (*Glycine max* L., cv. Saedanbaek) for different roasting conditions. *Journal of the Korean Society for Applied Biological Chemistry* **56**, 605-612.
104. Lee, J. H., Hwang, C. E., Lee, B. W., Kim, H. T., Ko, J. M., Baek, I. -Y., Ahn, M. J., Lee, H. Y., and Cho, K. M. (2015). Effects of roasting on the phytochemical contents and antioxidant activities of Korean soybean (*Glycine max* L. Merrill) cultivars. *Food Science and Biotechnology* **24**, 1573-1582.
105. Kim, H. G., Kim, G. W., Oh, H., Yoo, S. Y., Kim, Y. O., and Oh, M. S. (2011). Influence of roasting on the antioxidant activity of small black soybean (*Glycine max* L. Merrill). *LWT-Food Science and Technology* **44**, 992-998.
106. Cheng, K. -C., Lin, J. -T., and Liu, W. -H. (2011). Extracts from fermented black soybean milk exhibit antioxidant and cytotoxic activities. *Food Technology and Biotechnology* **49**, 111-117.
107. Shukla, S., Park, J., Kim, D. -H., Hong, S. -Y., Lee, J. S., and Kim, M. (2016). Total phenolic content, antioxidant, tyrosinase and □-glucosidase inhibitory activities of water soluble extracts of noble starter culture Doenjang, a Korean fermented soybean sauce variety. *Food Control* **59**, 854-861.
108. Xiao, Y., Wang, L., Rui, X., Li, W., Chen, X., Jiang, M., and Dong, M. (2015). Enhancement of the antioxidant capacity of soy whey fermentation with *Lactobacillus plantarum* B1-6. *Journal of Functional Foods* **12**, 33-44.
109. Amadou, I., Le, G. -W., Shi, Y. -H., and Jin, S. (2011). Reducing, radical scavenging, and chelation properties of fermented soy protein meal hydrolysate by *Lactobacillus plantarum* LP-6. *International Journal of Food Properties* **14**, 654-665.