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DIGESTIÓN GASTROINTESTINAL DE PROTEÍNAS ALIMENTARIAS Y BIODISPONIBILIDAD DE PÉPTIDOS RESISTENTES A LA DIGESTIÓN

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“El mayor peligro para la mayoría de nosotros no es apuntar demasiado alto y fallar, sino apuntar demasiado bajo y acertar”

Michelangelo Antonioni

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ABSTRACT

In this PhD thesis, studies related to the gastrointestinal digestion of milk proteins have been carried out. On the one hand, experiments have been carried out focused on knowing the degradation of milk proteins and the identification of the digestion products, both at the gastric and intestinal levels, in *in vivo* models of pigs and in humans, respectively, by means of proteomics and peptidomics techniques based on tandem mass spectrometry. On the other hand, these data have served to validate an internationally agreed *in vitro* gastrointestinal digestion model. In addition, the effect on digestion products of the incorporation of a brush-border intestinal membrane protease extract into the digestion protocol has been studied. Finally, the transport capacity of certain peptides generated in these digestions has been evaluated. These peptides are of physiological interest both for milk proteins and for a bioactive peptide derived from soya.

Initially, the behaviour of casein when subjected to the gastric phase of digestion was studied, obtaining samples in duodenum from cannulated pigs that were fed a micellar casein suspension. On the other hand, gastric digestions were performed *in vitro* with the same substrate. Both digestions were analysed by means of tandem mass spectrometry, where more than 4000 peptides were identified in the case of those digested *in vivo*. Peptides generated after gastric digestion were identified and it was found that only 1% of the nitrogen content corresponded to free amino acids. Furthermore, the formation of peptides and amino acids proved to be very similar between *in vivo* and *in vitro* digestions, which leads to the conclusion that the protocol of the gastric phase of digestion is a good approximation to gastric digestion for milk caseins.

The study of the intestinal phase of digestion was carried out on aspirated jejunal samples from human volunteers who had consumed caseins and whey proteins, which were provided by the INRA UMR0914 Nutrition Physiology and Ingestive Behavior de

AgroParisTech (France). More than 200 peptides were identified in the case of serum proteins and more than 400 in the case of caseins. No intact casein was detected, but β-lactoglobulin was present in the early stages of digestion. A decrease in the number of peptides found as digestion times increased was also observed, suggesting a degradation of peptides to smaller peptides or free amino acids, while resistant regions apparently coincided with areas rich in proline and negatively charged amino acids. Protein degradation and peptide generation using the standardized digestion protocol gave similar results to those observed *in vivo*, confirming that the method is a good physiological approach to the gastrointestinal digestion of milk proteins. Subsequently, we wanted to analyse whether the incorporation of brush-border peptidases as an additional step to the standardized protocol of *in vitro* digestion could represent an improvement when simulating gastrointestinal digestion. The results showed a greater generation of peptides by the action of amino- and carboxypeptidases, many of which coincided with peptides identified in jejunal digests. Although the incorporation of this extract does not represent a substantial improvement in the consensus method of *in vitro* gastrointestinal digestion, it may be important when studying the resistance of certain peptides and their possible intestinal functionality and absorption.

Finally, the transepithelial transport capacity of a group of peptides from β-casein that had shown resistance to the studied digestions, even with the addition of the brush-border peptidases, was studied. Peptides f(59-68) VYPFPGPIPN, f(63-68) PGPIPN and f(59-67) VYPFPGPI, showed a higher transport capacity through monolayers of Caco-2 cells than peptides f(60-66) YPFPGPI, f(62-66) FPGPI and f(60-62) YPF, likely due to the hydrophobic nature of Val and Pro residues located at N-terminal position. Resistance to gastrointestinal digestion and transport capacity of certain peptides from soybean lunasin peptide were also analyzed, obtaining several regions resistant to digestion and identifying the paracellular route as the main transport route for RKQLQGVN peptide. In addition, the cytotoxic activity of several lunasin fragments in

different cell cultures was studied. It was observed that both the complete lunasin and f(1-10) SKWQHQDSC had a high cytotoxic activity, although this activity depended on the cell culture used, being AGS and HT-29 cells more susceptible than Caco-2 .

RESUMEN

En esta Tesis Doctoral se han realizado estudios relacionados con la digestión gastrointestinal de proteínas lácteas. Por un lado, se han llevado a cabo experimentos centrados en conocer la degradación de las proteínas lácteas e identificación de los productos de digestión, tanto a nivel gástrico como intestinal, en modelos *in vivo* porcinos y en humanos, mediante técnicas de proteómica y peptidómica basadas en espectrometría de masas en tándem. Por otro lado, estos datos han servido para validar un modelo de digestión gastrointestinal *in vitro* consensuado internacionalmente. Además, se ha estudiado el efecto en los productos de digestión al incorporar un extracto de proteasas de la membrana intestinal del borde en cepillo en el protocolo de digestión. Finalmente, se ha evaluado la capacidad de transporte que tienen ciertos péptidos generados en estas digestiones y que además tienen interés fisiológico tanto de proteínas lácteas, como de un péptido bioactivo derivado de soja.

Inicialmente, se estudió el comportamiento de la caseína al ser sometida a la fase gástrica de la digestión, obteniéndose muestras en duodeno de cerdos canulados que fueron alimentados con una suspensión de caseína micelar. Por otro lado, se realizaron digestiones gástricas *in vitro* con este mismo sustrato. Ambas digestiones fueron analizadas mediante espectrometría de masas en tándem, en donde se identificaron más de 4000 péptidos en el caso de los digeridos *in vivo*. Se identificaron los péptidos que se generan tras la digestión gástrica y se comprobó que sólo un 1% del contenido en nitrógeno correspondió a aminoácidos libres. Además, la formación de péptidos y aminoácidos resultó ser muy similar entre las digestiones *in vivo* e *in vitro*, lo que permite concluir de que el protocolo de la fase gástrica de la digestión supone una buena aproximación a la digestión gástrica para las caseínas lácteas.

El estudio de la fase intestinal de la digestión se realizó en muestras de aspirados a nivel de yeyuno de voluntarios humanos que habían consumido caseínas y proteínas de suero lácteo, que fueron cedidas por el grupo INRA UMR0914 Nutrition Physiology

and Ingestive Behavior de AgroParisTech (Francia). Se identificaron más de 200 péptidos en el caso de las proteínas séricas y más de 400 en el caso de las caseínas. No se detectó caseína intacta, pero sí β-lactoglobulina en los primeros tiempos de la digestión. También se observó una disminución del número de péptidos encontrados conforme los tiempos de la digestión crecían, sugiriendo una degradación de los mismos a péptidos de menor tamaño o aminoácidos libres, mientras que las regiones resistentes, aparentemente, coincidieron con zonas ricas en prolina y en aminoácidos cargados negativamente. La degradación proteica y la generación peptídica en la digestión *in vitro* mediante el protocolo consensuado ofreció unos resultados de nuevo similares a lo que se observó *in vivo*, confirmando que el método supone una buena aproximación fisiológica a la digestión gastrointestinal de las proteínas lácteas. Posteriormente, se quiso analizar si la incorporación de peptidasas del borde en cepillo como paso adicional al protocolo consensuado de la digestión *in vitro* podría suponer una mejora a la hora de simular la digestión gastrointestinal *in vivo*. Los resultados mostraron una mayor generación de péptidos por la acción de amino- y carboxi-peptidasas, de los cuales, muchos de ellos coincidían con péptidos identificados en los digeridos yeyunales. Aunque la incorporación de este extracto no supone una mejora sustancial en el método consensuado de digestión gastrointestinal *in vitro*, puede ser importante a la hora de estudiar la resistencia de determinados péptidos y su posible funcionalidad y absorción intestinal.

A continuación, se estudió la capacidad de transporte transepitelial de un grupo de péptidos procedentes de la β-caseína que habían mostrado su resistencia a las digestiones estudiadas, incluso con la adición de las peptidasas del borde en cepillo. Los péptidos f(59-68) VYPFPGPIPN, f(63-68) PGPIPN y f(59-67) VYPFPGPI, mostraron una mayor capacidad de transporte a través de monocapas de células Caco-2 que los péptidos f(60-66) YPFPGPI, f(62-66) FPGPI y f(60-62) YPF, ligada muy posiblemente a la naturaleza hidrofóbica de los residuos Val and Pro en posición N-terminal. Asimismo,

se analizó la resistencia a la digestión gastrointestinal y la capacidad de transporte de ciertos péptidos procedentes del péptido lunasina de soja obteniéndose varias regiones resistentes a la digestión e identificándose a la ruta paracelular como principal ruta de transporte para el péptido RKQLQGVN. Además, se estudió la actividad citotóxica de varios fragmentos de la lunasina en diferentes cultivos celulares, observándose que, tanto la lunasina completa como el f(1-10) SKWQHQQDSC, tenían una alta actividad citotóxica, aunque esta actividad dependía del cultivo celular empleado, siendo más susceptibles las células AGS y HT-29 que las Caco-2.

LISTA DE ABREVIATURAS

BBM: enzimas del borde en cepillo del epitelio intestinal

ECA: enzima convertidora de angiotensina

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

Nano-LC-Q-TOF: nano-cromatografía líquida acoplada a espectrometría de masas de triple cuadrupolo y de tiempo de vuelo

SerP: fosfoserina

SHIME: Simulador del Ecosistema Microbiano en el Intestino Humano

SIMGI: Simulador Gastrointestinal Dinámico

TNO: Organización Holandesa para la Investigación en Ciencias Aplicadas

OBJETIVOS Y PLAN DE TRABAJO

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La digestión gastrointestinal es un proceso de gran importancia en el organismo. A lo largo de la misma ocurren múltiples reacciones enzimáticas en las que se hidrolizan complejas estructuras de los alimentos liberando nutrientes utilizables por nuestro organismo. Además, se generan una serie de moléculas que actúan como señalizadores a nivel intestinal o que pueden absorberse e interaccionar con otras dianas en el organismo actuando como reguladores y/o moduladores metabólicos.

Por lo tanto, existe un gran interés en el estudio del comportamiento de los alimentos durante la digestión gastrointestinal y en el estudio de los productos resultantes. En concreto, las proteínas alimentarias es uno de los macronutrientes que presenta mayor complejidad durante la digestión y cuyos productos pueden desempeñar no solo funciones nutricionales, sino también otros efectos fisiológicos tanto positivos como negativos, como, por ejemplo, alergias alimentarias. La digestión gastrointestinal de las proteínas alimentarias ha sido considerada durante años una "caja negra" donde se conocía la composición del alimento ingerido y finalmente podían detectarse una serie de aminoácidos en plasma, sin poder conocer los procesos que tienen lugar a lo largo del tracto gastrointestinal. El desarrollo de las técnicas de proteómica y peptidómica basadas en espectrometría de masas permite el análisis de mezclas complejas de proteínas y péptidos y conocer, por tanto, la composición de los digeridos a distintos niveles del tracto gastrointestinal.

El reconocimiento de la importancia de la digestión gastrointestinal de los alimentos en los efectos metabólicos y la salud del consumidor ha promovido el desarrollo de protocolos y modelos de digestión *in vitro*, ante la imposibilidad de llevar a cabo, por limitaciones éticas y técnicas, estos estudios en humanos. Sin embargo, es importante que estos modelos in vitro estén validados y respaldados por datos obtenidos preferiblemente en humanos o en modelos animales relevantes, como el porcino.

La absorción de los productos de digestión de las proteínas a nivel intestinal también se trata de un tema controvertido. Si bien se acepta la absorción de

aminoácidos libres y péptidos de 2 y 3 aminoácidos, la absorción de péptidos de mayor tamaño es discutida por la comunidad científica. Sin embargo, existen antecedentes que demuestran la absorción de péptidos de más de 5 aminoácidos en cantidades relevantes, aunque de nuevo, estos estudios se ven afectados por limitaciones técnicas donde la espectrometría de masas puede ayudar al generar evidencia en este tema.

Objetivo general

El objetivo general de esta tesis es el estudio de digeridos gástricos obtenidos en un modelo porcino y digeridos gastrointestinales de proteínas lácteas obtenidos en humanos mediante técnicas de proteómica y peptidómica basadas en espectrometría de masas. Además, se han empleado estos resultados para la validación de un método consensuado a nivel internacional de la digestión gastrointestinal *in vitro.* Finalmente, se ha evaluado la biodisponibilidad de algunos péptidos identificados en los digeridos intestinales en un modelo celular del epitelio intestinal.

Para llevar a cabo este objetivo se han planteado los siguientes objetivos parciales y se ha diseñado el siguiente plan de trabajo:

Objetivo 1

Estudio de efluentes obtenidos en duodeno mediante cánula en un modelo porcino tras la administración oral de caseínas. Validación de la fase gástrica del método de digestión gastrointestinal *in vitro* propuesto por la acción COST INFOGEST.

1.1 Estudio cinético de los efluentes obtenidos en duodeno porcino tras la administración de caseína micelar mediante espectrometría de masas en tándem, electroforesis en gel combinada con espectrometría de masas y técnicas de análisis general de proteínas.

1.2 Comparación de los resultados con la fase gástrica del modelo de digestión gastrointestinal INFOGEST a nivel peptídico.

Objetivo 2

Estudio de efluentes obtenidos en yeyuno mediante aspiración en humanos tras la ingesta de caseínas y proteínas de suero. Validación de la fase intestinal del método de digestión gastrointestinal *in vitro* propuesto por la acción COST INFOGEST usando los mismos sustratos.

2.1 Estudio cinético de la composición de digeridos de proteínas lácteas, caseínas y proteínas de suero, obtenidos en yeyuno humano mediante sonda nasogástrica empleando técnicas de peptidómica basadas en espectrometría de masas en tándem.

2.2 Comparación de los resultados con la fase intestinal del modelo de digestión gastrointestinal INFOGEST a nivel peptídico.

2.3 Evaluación de la complementación de la fase intestinal del protocolo consensuado con la adición de un extracto de peptidasas de la membrana de borde en cepillo.

Objetivo 3

Estudio de biodisponibilidad *in vitro* de péptidos resistentes a la digestión gastrointestinal identificados previamente.

3.1 Evaluación del transporte a través del epitelio intestinal utilizando el modelo de monocapa de células Caco-2 de péptidos derivados de caseínas identificados en los digeridos gastrointestinales a nivel del yeyuno.Evaluación del transporte del péptido de soja lunasina y de fragmentos liberados tras su digestión in vitro, a través del epitelio intestinal utilizando el modelo de monocapa de células Caco-2. Estudio de la resistencia a peptidasas celulares, así como el mecanismo implicado en el transporte.

1. INTRODUCCIÓN

INTRODUCCIÓN

Las proteínas son el principal componente estructural y funcional de las células del organismo y, como tal, tienen un rol fundamental en numerosas actividades fisiológicas que engloban funcionalidades catalíticas (enzimas), de motilidad, transporte, protección, reguladora, etc. Constituye uno de los principales macronutrientes presentes en los alimentos, tanto de origen animal como vegetal. (Gil, 2017).

Las unidades estructurales que forman las proteínas son los aminoácidos, que se unen entre sí a través de los grupos carboxilo y amino mediante el enlace peptídico con la correspondiente pérdida de agua. La secuencia de aminoácidos que componen una proteína constituye su estructura primaria. Ésta es importante desde el punto de vista nutricional, ya que además de ser una fuente de nitrógeno fundamental, ciertos aminoácidos son esenciales para nuestro organismo, es decir, que el cuerpo humano no puede sintetizar *de novo* el esqueleto hidrocarbonado y, por tanto, deben ser aportados de manera obligatoria a través de la dieta para atender a las necesidades corporales (Reeds, 2000). Estos aminoácidos que ingerimos pueden estar presentes en forma libre en los alimentos, pero la gran mayoría de ellos se obtienen tras la digestión de las proteínas en el tracto gastrointestinal, donde además, se producen una gran cantidad de péptidos que también pueden jugar un papel fisiológicamente relevante en nuestro organismo que puede, no solo ser beneficioso (Rutherfurd-Markwick, 2012), sino que también puede ser perjudicial, como es el caso de ciertos epítopos asociados a alergias alimentarias (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015).

En los últimos años, debido a la gran relevancia que estos péptidos pueden tener en ciertas actividades fisiológicas, se han desarrollado numerosos estudios relacionados con su formación, absorción y los efectos que estos pudieran ejercer en el cuerpo humano. De esta manera, se han utilizado tanto proteínas animales como vegetales para el estudio de los péptidos generados tras su hidrólisis, obteniéndose diferentes resultados en cuanto a los péptidos obtenidos y su actividad biológica dependiendo de la fuente proteica, el tipo de enzima utilizado y las condiciones

de hidrólisis (Mulero, Zafrilla, Martínez-Cachá, Leal, & Abellán, 2011). En referencia a las proteínas de origen vegetal de las que se han obtenido péptidos bioactivos, cabe destacar la soja, el trigo, el maíz, el arroz, la cebada y el girasol (Wang & De Mejia, 2005). En lo que concierne a proteína de origen animal, se han realizado estudios de péptidos bioactivos en huevo, carne, músculo de pescado (sardina, atún y bonito) y en la jalea real (Mulero et al., 2011), entre otros, pero sin duda alguna, el alimento más estudiado en relación a este grupo de proteínas de origen animal es la leche y otros productos lácteos.

1.1 Proteínas lácteas

La leche es un fluido biológico complejo compuesto principalmente por agua, lípidos, proteínas y carbohidratos, así como de otros constituyentes minoritarios como enzimas, vitaminas y sales minerales. Las proteínas lácteas son conocidas por su alto valor nutricional, ya que contienen una alta cantidad de aminoácidos esenciales y una gran capacidad de digestibilidad. En la leche bovina, el contenido proteico representa entre un 3 y 3,5% (p/v), siendo el 80% caseínas y el resto, proteínas del suero de la leche (**¡Error! No se encuentra el origen de la referencia.**) (Walstra, Wouters, & G eurts, 2005). La fracción proteica de la leche, además de poseer un alto valor nutricional, es precursora de una gran cantidad de péptidos con funcionalidades biológicas.

Tabla 1. Concentración de las proteínas presentes en la leche (Walstra et al., 2005).

Proteína	Concentración en la leche (g/L)	Porcentaje de la proteína respecto a la concentración total (p/p)
PROTEÍNA TOTAL	33,0	100,0
CASEÍNAS	26,0	79,5

α_{s1}-caseína	10,0	30,6
α_{s2}-caseína	2,6	8,0
β-caseína	9,3	28,4
κ-caseína	3,3	10,1
γ-caseína	0,8	2,4
PROTEÍNAS DE SUERO	6,3	19,3
β-lactoglobulina	3,2	9,8
α-lactoalbúmina	1,2	3,7
Inmunoglobulina	0,7	2,1
Seroalbúmina	0,4	1,2
Otras (Proteasa-peptona)	0,8	2,4
PROTEÍNAS DE LA MEMBRANA DEL GLÓBULO GRASO	0,4	1,2

1.1.1. Caseína

Las caseínas son las proteínas mayoritarias de la leche, representando alrededor del 80% del total de proteína y se definen, clásicamente, como aquella fracción proteica que precipita a 20ºC y a un pH de 4,6 (Jenness et al., 1956). Se han identificado, bajo la denominación de caseínas, cuatro tipos diferentes de cadenas polipeptídicas: α_{s1}-caseína, α_{s2}-caseína, β-caseína y κ-caseína y, además, cada una de

estas caseínas posee diferentes variantes genéticas debido a la sustitución de ciertos aminoácidos en el interior de estas cadenas (Farrell et al., 2004). Las propiedades de las caseínas son diferentes a la mayoría de las proteínas ya que son hidrófobas, pero poseen una elevada carga proporcionada por grupos fosfato que se encuentran esterificados en residuos de serina, lo que les permite poder permanecer disueltas en la leche. Por otro lado, los diferentes tipos de caseínas poseen una elevada carga de residuos de prolina distribuidos de manera uniforme, lo que impide a las caseínas un empaquetamiento denso y ordenado. De hecho, la estructura secundaria de las caseínas está poco organizada, asemejándose a estructuras de proteínas globulares desnaturalizadas, lo que les proporciona una elevada flexibilidad y una alta resistencia a la desnaturalización por agentes como la urea o el calor, comparativamente con respecto a las proteínas globulares (Walstra & Jenness, 1984). Las caseínas se disponen en la leche formando agregados coloidales, denominados micelas (**¡Error! No se encuentra el origen de la referencia.**). La κ-caseína juega un papel fundamental en la estabilidad de estas micelas, gracias al macropéptido f(106-169), que crea una capa sobre la superficie micelar de entre 5 y 10 nm que provee una estabilización estérica a las micelas y evita que entre ellas se puedan acercar demasiado (Dalgleish & Corredig, 2012). La micela no forma una estructura muy densa, sin embargo, es capaz de evitar el paso a través de ella de proteínas (β-caseína puede salir y entrar de la micela durante el enfriamiento y recalentamiento de la micela) (Creamer, 1977); las proteínas de suero pueden penetrar en la micela y formar puentes disulfuro con la parte interna de la κ-caseína (Anema & Li, 2003); la quimosina es capaz de acercarse e hidrolizar la κ-caseína y, por otro lado, la tripsina también es capaz de hidrolizar la β-caseína (Diaz, Gouldsworthy, & Leaver, 1996).

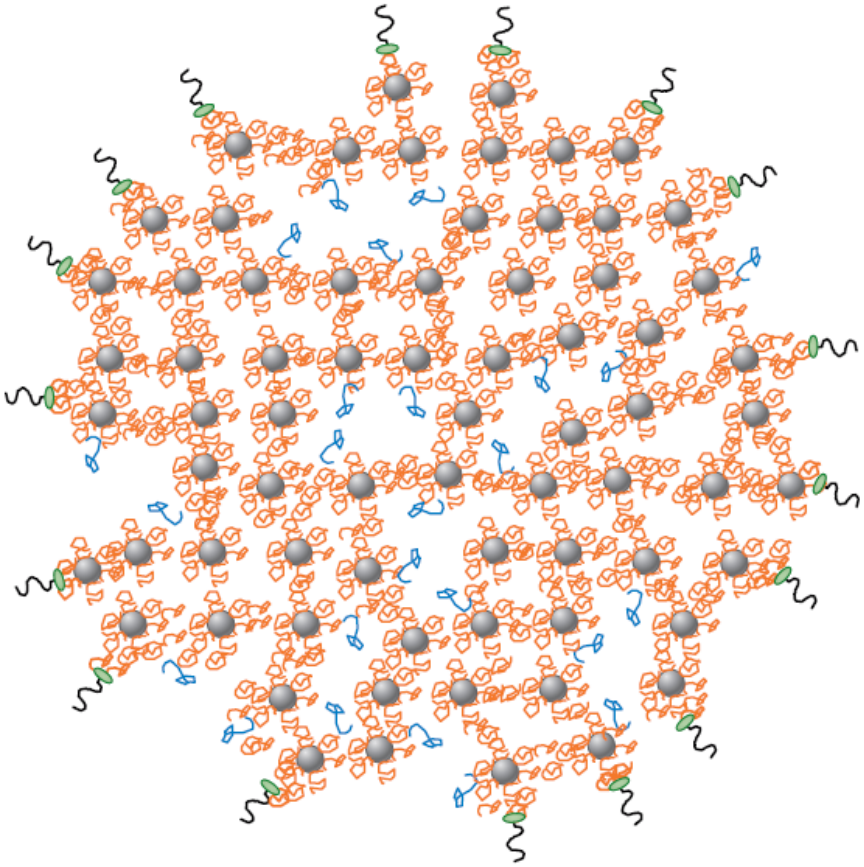

Figura 1. Esquema de la estructura micelar de las caseínas bovinas. α_s- y β-caseínas (naranja) permanecen unidas formando grupos juntos con moléculas de fosfato cálcico (Benedé et al.). Algunas β-caseínas se unen hidrofóbicamente a otras caseínas pudiendo desaparecer de la micela mediante enfriamiento. La κ-caseína (verde) y las cadenas de caseinomacropéptido (negro) permanecen en el borde exterior de la micela. Adaptada Dalgleish & Corredig, (2012).

α_{s1}-caseína

Es la proteína más abundante en la leche bovina, representando hasta el 45% del total de las caseínas. El peso molecular calculado es de 23,615 kDa, estando compuesta por 199 aminoácidos formando una cadena monomérica, de los cuales, ninguno se corresponde con cisteína. Posee 9 variantes genéticas: A, B, C, D, E, F, G, H e I. De todas ellas, se utiliza como referencia la variante B, que a su vez presenta dos subvariantes diferenciadas entre sí por poseer 8 y 9 grupos fosfatos, respectivamente. La α_{s1}-caseína bovina contiene una región altamente cargada negativamente en la región f(41-80) debido a que en esa zona se acumulan todos los grupos fosfoserina (SerP) de la proteína y tres regiones de carácter hidrofóbico f(1-44), f(90-113) y f(132-199) (Farrell et al., 2004) (Swaisgood, 2003). Estas características, junto a la elevada

cantidad de residuos de prolina distribuidos a lo largo de toda su estructura, le confiere a la α_{s1}-caseína una configuración abierta y flexible donde se pueden encontrar dominios con estructura secundaria α-hélice y lámina β-plegada (Farrell et al., 2004), lo que proporciona una gran susceptibilidad a la hidrólisis durante la digestión gastrointestinal (Astwood, Leach, & Fuchs, 1996).

α_{s2}-caseína

Constituye el 10% de la fracción total de la caseína en la leche bovina. Su peso molecular es de 25,226 kDa y está compuesta por 207 residuos repartidos en una sola cadena. Existen cuatro variantes genéticas definidas como A, B, C y D, siendo la variante A la utilizada como referencia, poseyendo 11 grupos fosfatos. Estructuralmente, la α_{s2}-caseína consta de un enlace disulfuro entre los residuos de cisteína situados en las posiciones 36 y 40, y es una proteína que se caracteriza por tener diferentes dominios con altas cargas netas negativas, f(8-16), f(56-61) y f(129-133), que le confiere una alta sensibilidad a la acción del calcio y otros cationes y se la considera como la caseína más hidrofílica de todas (Farrell et al., 2004; Schlimme, 2002).

β-caseína

Esta proteína monomérica constituye el 35% de la fracción total de la caseína. Su peso molecular es de 23,983 kDa y está compuesta por 209 aminoácidos que generan hasta 12 variantes genéticas denominadas como A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2 e I, siendo la variante de referencia la A^2 que contiene 5 grupos fosfato. Posee 35 prolinas a lo largo de toda su cadena y ninguna cisteína, lo que limita la formación de α-hélice. Es la más hidrófoba de todas las caseínas y su estructura se organiza en dos zonas diferenciadas: el extremo C-terminal es la región hidrofóbica de la proteína f (136-209) mientras que la región N-terminal es más hidrofílica gracias a la concentración de aminoácidos hidrofílicos y la presencia de los grupos fosfatos unidos a serinas. Estos

dos dominios con diferente hidrofobicidad le confieren a la β-caseína su carácter altamente anfipático (Farrell et al., 2004).

κ-caseína

Es una glicoproteína que constituye el 10% de las caseínas en la leche bovina. Tiene un peso molecular de 19,037 kDa y un total de 169 residuos de aminoácidos. Se diferencia de las otras caseínas en la unión de cinco cadenas de carbohidratos a residuos de treonina o serina. Su contenido en carbohidratos está compuesto por ácido N-acetilneuramínico, galactosa y N-acetil galactosamina (Jolles & Fiat, 1979). Existen dos dominios claramente diferenciados en la κ-caseína: el extremo C-terminal con carga negativa y carácter hidrofílico y el N-terminal hidrofóbico y con carga neta positiva (Farrell et al., 2004).

1.1.2. Proteínas de suero

Las proteínas de suero lácteo son aquellas que permanecen solubles en las condiciones de precipitación de las caseínas (pH 4,6 y 20 ºC). Las proteínas que forman este grupo son la β-lactoglobulina, la α-lactoalbúmina, la seroalbúmina bovina, las inmunoglobulinas, y otros componentes minoritarios como la lactoferrina (Wolfson & Sumner), el caseinomacropéptido que se libera de la κ-caseína y enzimas como la plasmina o la lactoperoxidasa (Farrell et al., 2004; Walstra & Jenness, 1984). Las proteínas de suero se caracterizan por tener una estructura globular y compacta donde predominan los motivos α-hélice estabilizados por puentes disulfuro, lo que las confiere de cierta resistencia a la hidrólisis, aunque son susceptibles a la desnaturalización por calentamiento (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

β-lactoglobulina

Es la proteína predominante dentro del grupo de las proteínas de suero representando hasta el 58% del total en la leche bovina. Tiene un peso molecular de 18,277 kDa y está formada por 162 aminoácidos que pueden generar, dependiendo de los aminoácidos que están presentes en ciertas posiciones de la proteína, tres variedades genéticas diferentes A, B y C (Meza-Nieto et al., 2010), siendo la A más común y difiriendo tan solo en 2 aminoácidos de la B (D^{64} y V^{118} por G^{64} y A^{118}). Estructuralmente, posee una gran capacidad para unirse a diferentes moléculas de carácter hidrófobo como el retinol, lípidos o ácidos grasos y, gracias a su estabilidad a bajo pH, la β-lactoglobulina es capaz de proteger a estas moléculas de la hidrólisis en el estómago y que permanezcan intactas hasta alcanzar el intestino, donde pueden ser absorbidas (Madureira et al., 2007).

α-lactoalbúmina

La α-lactoalbúmina es una proteína globular que representa hasta el 25% de las proteínas de suero en la leche bovina. Está formada por 123 aminoácidos, su peso molecular es de 14,175 kDa y se pueden distinguir hasta tres variedades genéticas (A, B y C), siendo la B la más común (Madureira et al., 2007). Estructuralmente se pueden observar dos dominios diferenciados, uno formado por una α-hélice y otro más pequeño con estructura de lámina β, que se unen entre sí mediante dos puentes disulfuro entre las cisteínas en posición 73 - 91 y 61 - 77. La α-lactoalbúmina posee una alta afinidad por cierto iones metálicos como magnesio, manganeso (Wolfson & Sumner), potasio, sodio, hierro y especialmente calcio (Chatterton, Smithers, Roupas, & Brodkorb, 2006; Kamau, Cheison, Chen, Liu, & Lu, 2010). En comparación con la β-lactoglobulina presenta una mayor resistencia al calor, principalmente porque la desnaturalización de la α-lactoalbúmina es reversible a una temperatura inferior a los 75º C (Dalgleish, van Mourik, & Corredig, 1997).

Seroalbúmina

Es una proteína de alto peso molecular (66,267 kDa) formada por 582 aminoácidos que representa hasta el 6% de la proteína total del suero. A lo largo de la cadena se observan 17 puentes disulfuro intramoleculares y un grupo tiol libre en la posición 34. Se organiza como monómero o como dímero, pero no se suelen observar en polimerizaciones mayores (Janatova, Fuller, & Hunter, 1968). Debido a su elevado tamaño tiene una gran capacidad para unirse a ácidos grasos libres y otros lípidos, lo que le proporciona una protección parcial frente a la hidrólisis enzimática y a la desnaturalización térmica, de manera similar a la β-lactoglobulina (Riera, Álvarez, Arguello, & Cabero, 1996).

Inmunoglobulinas

Las inmunoglobulinas constituyen un grupo complejo de compuestos producidos por los linfocitos B. Representan un 11% del total de las proteínas de suero y se agrupan en cinco clases: IgG, IgM, IgA, IgD e IgE, siendo la IgG la que predomina, representando hasta un 80% del total de las inmunoglobulinas presentes en la leche. Estructuralmente, estas proteínas se agrupan formando un complejo de cuatro subunidades consistentes en dos cadenas ligeras de aproximadamente 25 kDa y dos cadenas pesadas de entre 50 y 70 kDa. Juegan un papel fundamental a nivel inmunológico, estando muy presentes en el calostro (Hurley & Theil, 2011).

Lactoferrina

La lactoferrina es una glicoproteína monomérica formada por 689 residuos de aminoácidos y con un peso molecular de 80 kDa. Posee 17 puentes disulfuro y dos grupos carbohidratados. Se sintetiza en las células del epitelio glandular de diversos órganos, pudiéndose encontrar en leche, saliva, lágrimas, secreciones nasales e intestinales, entre otros (Madureira et al., 2007). Cumple, tanto una función de

protección en el organismo mediante la formación de un complejo de la lactoferrina con tiocianato y peróxido de hidrógeno (Wolfson & Sumner, 1993), como un importante papel en la absorción del hierro, ya que tiene la capacidad de quelar a este mineral (Sánchez et al., 1992).

1.2 Digestión gastrointestinal de los alimentos

La digestión es un proceso complejo esencial para la salud del ser humano, ya que se encarga de la transformación de los alimentos ingeridos en moléculas más simples que pueden ser absorbidas. Esta absorción de nutrientes se puede llevar a cabo tanto en el duodeno como en el yeyuno superior (Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011), para ser utilizados, posteriormente, en el mantenimiento, crecimiento celular y/o como fuente de energía por el organismo. Durante el proceso de la digestión ocurren, principalmente, dos procesos: el primero consiste en la rotura mecánica de los alimentos ingeridos, generando partículas más pequeñas que facilitan su posterior hidrólisis enzimática a través del segundo proceso, que consiste en la degradación de las macromoléculas de alimento hasta generar moléculas absorbibles por el organismo (Guerra et al., 2012).

La ingesta y deglución de los alimentos comienza en la cavidad oral, donde se produce la masticación del alimento y la hidrólisis enzimática del almidón en azúcares simples por la acción catalítica de la amilasa. Además, este acto produce la activación de la salivación, el peristaltismo esofágico y la relajación receptiva gástrica, preparando a parte del tubo digestivo para la digestión de alimentos (Edelstein, 2014). El bolo alimenticio es transportado por el esófago hasta el estómago, entrando en esta cavidad en contacto con proteasas y lipasas, encargadas de la hidrólisis de proteínas y lípidos, respectivamente. En este punto, la secreción de ácido clorhídrico al interior del estómago disminuye el pH gástrico, aumentando la actividad de la pepsina (pH óptimo 1,6-3,2) para hidrolizar las proteínas. Existe numerosa bibliografía sobre la implicación

del pH en las reacciones catalizadas por pepsina (Cornish-Bowden & Knowles, 1969). La actividad de la pepsina puede dividirse en cuatro intervalos de actividad en función de los siguientes rangos de pH: (I) Cuando el pH se encuentra entre 1 y 1,5 la actividad es máxima. (II) Cuando el pH se encuentra en valores de 2,5 a 5 su actividad máxima representa el 70%, (III) la actividad persiste de pH 3 a 5 y, (IV) cuando el pH se encuentra por encima de un valor de 6,5 no es posible ninguna actividad enzimática ya que la pepsina se encontraría inactivada irreversiblemente (Piper & Fenton, 1965). El pH actúa como activador de los precursores enzimáticos (pepsinógeno) mediante la liberación de ácido gástrico, y como desactivador enzimático de la pepsina cuando se libera jugo pancreático con pH alcalino. La acción de la pepsina finaliza cuando el contenido gástrico se mezcla con el jugo pancreático alcalino en el duodeno y yeyuno. El pH del contenido intestinal en la primera región duodenal es de 2,0 a 4,0 pero en el resto del duodeno es cerca de 6,5 donde sobre todo actúan la tripsina, quimotripsina y la elastasa, aunque también actúan aminopeptidasas, carboxipeptidasas y endopeptidasas. La digestión final de los péptidos se produce en tres regiones: lumen intestinal, las vellosidades intestinales y el citoplasma de las células de la mucosa intestinal (Ganong, Barrett, Barman, Boitano, & Brooks, 2012).

Por otro lado, también se secreta en el estómago el factor intrínseco, fundamental para la posterior absorción de la vitamina B12. El tiempo de permanencia del quimo, mezcla semilíquida del alimento, en el estómago fluctúa entre 2 y 4 horas, dependiendo de múltiples factores como, por ejemplo, el tipo de alimento. Los alimentos ricos en grasas permanecerán más tiempo que aquellos ricos en hidratos de carbono. El quimo es gradualmente enviado hacia el intestino delgado, donde el pH ácido es neutralizado y se incorporan a la mezcla la bilis y los jugos digestivos pancreáticos formados por enzimas digestivas como la tripsina, quimotripsina, lipasa pancreática, carboxipeptidasas A y B y elastasa (Boticario, 2012; Goodman, 2010). Estas enzimas catalizan la hidrólisis de las proteínas, los lípidos y el almidón, mientras que los ácidos

biliares se encargan de emulsionar los productos generados tras la digestión de los lípidos en micelas (Wickham, Faulks, & Mills, 2009). Por último, en la superficie de los enterocitos del epitelio intestinal se produce una nueva proteolisis por la acción de las enzimas presentes en las células intestinales del borde en cepillo, entre las que se encuentran la maltasa, isomaltasa, lactasa y diferentes aminopeptidasas, carboxipeptidasas y dipeptidasas (Edelstein, 2014; Shimizu, 2004). Aunque la mayor parte de los nutrientes son absorbidos a nivel del yeyuno (Langerholc et al., 2011) existen compuestos que no han sido digeridos aún en este punto de la digestión, incluyendo polisacáridos complejos, que alcanzan intactos el intestino grueso, donde pueden ser fermentadas por las bacterias presentes en él (Flint, 2012).

1.1.1. Digestión gastrointestinal de proteínas lácteas

La digestibilidad de las diferentes proteínas alimentarias en el tracto gastrointestinal humano varía en gran medida dependiendo de ciertas características fisicoquímicas como pueden ser su tamaño, carga, secuencia de aminoácidos, estructura terciaria y modificaciones post-traduccionales, especialmente la glicosilación y fosforilación. De manera general, las proteínas globulares y compactas como la β-lactoglobulina y las proteínas con un empaquetamiento compacto como la faseolina, el gluten del trigo o la glicina de la soja son menos susceptibles a la digestión en el estómago que aquellas proteínas con un menor grado de empaquetamiento estructural como la β-caseína. Por su parte, la glicosilación y la fosforilación impiden estéricamente la actividad proteasa de las enzimas gastrointestinales, dificultando la digestibilidad proteica (Dallas et al., 2017).

Las características fisicoquímicas que poseen las proteínas lácteas hacen que tengan una elevada digestibilidad, en torno al 95% (Damodaran, 1997). Sin embargo, se ha descrito la resistencia a la digestión de la β-lactoglobulina por parte de la pepsina, que se mantiene intacta hasta llegar al intestino delgado debido a sus características

estructurales que la protegen de la hidrólisis en este punto de la digestión (Guo, Fox, Flynn, & Kindstedt, 1995; Reddy, Kella, & Kinsella, 1988); mientras que la caseína alcanza el yeyuno principalmente en forma de péptidos previamente degradados (Mahé et al., 1996). En cuanto a la digestibilidad de estas proteínas durante el transcurso de la fase intestinal, numerosos estudios se han realizado testando diversos parámetros como la digestión de la β-caseína con pancreatina sin pasar por una etapa gástrica previa (Mandalari et al., 2009) y también se llevó a cabo utilizando fluido duodenal humano (Jakobsson, Lindberg, & Benediktsson, 1982). Además, también se ha estudiado la digestión gástrica de la β-caseína seguida de una fase duodenal (Dupont, Mandalari, Molle, et al., 2010; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Mandalari et al., 2009). Pese a las diferentes condiciones de pH, concentraciones de enzimas y la utilización de diferentes matrices, no se ha detectado nunca β-caseína intacta al finalizar el proceso de digestión gastrointestinal en ninguno de los ensayos descritos previamente. Por otra parte, también se ha estudiado la digestibilidad de α_{s1}-caseína y α_{s2}-caseína, observándose su susceptibilidad a la acción de las enzimas duodenales (Fu, Abbott, & Hatzos, 2002). Además, de acuerdo con la velocidad con la que las proteínas son digeridas y los aminoácidos generados tras la digestión son absorbidos, se pueden distinguir entre proteínas rápidas y lentas y esto puede tener importantes implicaciones fisiológicas. En el caso de las proteínas de la leche, las proteínas de suero se han denominado proteínas rápidas, mientras que las caseínas son proteínas lentas. Este hecho es debido a la diferente velocidad de vaciado gástrico durante la digestión, ya que las caseínas coagulan en el estómago, mientras que las proteínas de suero no lo hacen, permitiendo un vaciado rápido del mismo. (Boirie et al., 1997; Frühbeck, 1998; Miranda & Pelissier, 1983; Walrand et al., 2016).

Aunque la gran digestibilidad de las proteínas lácteas ha sido descrita tanto en ensayos *in vitro* como en estudios *in vivo*, existen ciertas regiones que presentan una gran resistencia a ser hidrolizadas por la acción de las proteínas gastrointestinales. Un

buen ejemplo lo constituyen las regiones fosforiladas de las caseínas denominadas caseinofosfopéptidos. Estos provienen de α_{s1}-caseína, α_{s2}-caseína y β-caseína y la mayoría de estas secuencias contienen una región SerP-SerP-SerP-Glu-Glu, que funciona como principal sitio de unión de minerales como calcio, hierro y zinc, entre otros (Zidane et al., 2012), aunque los residuos que hay alrededor o la longitud de las secuencias pueden tener también influencia (Cross, Huq, Palamara, Perich, & Reynolds, 2005). Se ha propuesto que los caseinofosfopéptidos juegan un papel fundamental en la biodisponibilidad de los estos minerales aumentando la solubilidad de los mismos y favoreciendo el contacto y la absorción en el lumen intestinal (Miquel et al., 2005). Existen estudios realizados *in vivo* donde se ha demostrado que en el intestino delgado se produce la liberación y la absorción de estos minerales debido a estas regiones (Chabance et al., 1998; H. Meisel & H. Frister, 1989). Por otro lado, también ha sido propuesta la resistencia a la hidrólisis de péptidos ricos en prolina debido a la posible ausencia de prolil-endopeptidasas tanto en el jugo gástrico como en el pancreático (Shan et al., 2002).

1.1.2. Modelos de digestión gastrointestinal in vitro

A pesar de que los ensayos realizados en modelos animales o en humanos generan resultados con mucha mayor relevancia fisiológica, tienen ciertas limitaciones debido implicaciones éticas y son más costosos en tiempo y dinero. Por esta razón, se han desarrollado desde hace años una gran cantidad de diferentes modelos de digestión *in vitro* que han sido utilizados para evaluar química y estructuralmente los cambios que se producen en los alimentos durante la digestión (Guerra et al., 2012; Hur, Lim, Decker, & McClements, 2011; Muttakin, Moxon, & Gouseti, 2019; Wickham et al., 2009). Existen una gran cantidad de variables fisiológicas que un modelo *in vitro* puede simular: temperatura, pH y gradiente de pH, tipo de enzima y concentración, composición y cantidad de secreciones enzimáticas, aunque los valores seleccionados de cada variable pueden ser diferentes dependiendo del protocolo experimental utilizado y de la

aplicación específica buscada en cada tipo de ensayo (Donaldson, Rush, Young, & Winger, 2014; Dupont & Mackie, 2016; Marze, 2017).

Los modelos de digestión *in vitro* pueden ser mono-compartimentales, donde todo el proceso de digestión ocurre en un mismo reactor, o multi-compartimentales, donde se utilizan diferentes reactores para simular las diferentes condiciones de distintas regiones del tracto gastrointestinal. Dependiendo de cómo el modelo simula aspectos mecánicos y fisicoquímicos de la digestión, tales como el flujo del digerido, mezclado, contracciones de estómago y peristaltismo o cambios dinámicos de pH, se pueden distinguir modelos de digestión *in vitro* dinámicos y estáticos.

Los modelos de digestión *in vitro* estáticos ofrecen la posibilidad de realizar ensayos simples, rápidos y baratos. Se llevan a cabo en uno o varios recipientes que simulan las diferentes etapas de la digestión. A menudo, se componen de 3 reactores donde se simulan las fases oral, gástrica e intestinal, con la posibilidad de agregar un cuarto que replicaría las condiciones del intestino grueso (Marze, 2017). Los experimentos suelen realizarse a 37º C bajo condiciones de agitación que generan una mezcla homogénea utilizando agitadores magnéticos, incubadores con agitación, rotadores, etc. (Englyst, Veenstra, & Hudson, 1996). Los fluidos digestivos dependen de cada protocolo experimental, pero normalmente consisten en agua con electrolitos, enzimas y, en algunos casos, otros compuestos como mucinas, sales biliares, etc. El pH se ajusta al principio de cada fase teniendo en cuenta el pH fisiológico de la misma (Marze, 2017). En cuanto a los volúmenes de muestra que posteriormente son analizados, pueden variar desde µL de material a decenas de mL (Muttakin et al., 2019).

Existen muchos métodos diferentes de digestión *in vitro* y resulta complicado comparar los diferentes resultados que proporcionan debido a la gran variabilidad de condiciones fisiológicas utilizadas como el pH o las concentraciones enzimáticas (Hur et al., 2011; Marze, 2017). En el año 2013, una revisión bibliográfica de ensayos de digestión *in vitro* para el estudio de la alergenicidad de proteínas evidenció diferencias

en las concentraciones de proteasa gástrica de hasta cuatro órdenes de magnitud (Mills et al., 2013). De manera similar, en una revisión sobre digestiones *in vitro* de almidón, se identificaron hasta 36 protocolos diferentes (Woolnough, Bird, Monro, & Brennan, 2010). Por esta razón, con el fin de armonizar los métodos estáticos de digestión *in vitro*, una red internacional financiada por la Acción COST INFOGEST ha propuesto un protocolo de digestión estandarizado basado en condiciones fisiológicas, el cual ha mostrado una gran reproducibilidad interlaboratorio (Egger et al., 2016; Minekus et al., 2014). Este protocolo ha sido extensamente empleado desde su introducción lo que ha dado lugar a su publicación como protocolo consensuado (Brodkorb et al., 2019).

En cuanto a los modelos de digestión dinámicos, ofrecen la capacidad de simular acciones digestivas complejas y, por ello, son más utilizados para estudiar los efectos de la dinámica de fluidos sobre la digestión. Sin embargo, esto implica que sean métodos mucho más laboriosos y costosos. Al igual que los métodos estáticos, los modelos de digestión *in vitro* dinámicos pueden reproducir una o más etapas del proceso digestivo (Thuenemann, 2015).

La digestión *in vitro* en fase oral ha sido simulada utilizando picadores de carne comerciales (Bornhorst, 2013), licuadoras comerciales de laboratorio (Bordoloi, Singh, & Kaur, 2012; Dhital, Dabit, Zhang, Flanagan, & Shrestha, 2015; Tamura, Okazaki, Kumagai, & Ogawa, 2017) y sofisticados modelos de bocas artificiales (Benjamin et al., 2012; Panouillé, Saint-Eve, Déléris, Le Bleis, & Souchon, 2014; Salles et al., 2007). Los modelos orales de digestión dinámica *in vitro* incorporan generalmente un elemento mecánico de digestión y medida de la rotura del alimento y/o de la liberación de compuestos volátiles. La boca es el órgano donde las características organolépticas de los alimentos son percibidas, por ello, algunos modelos han desarrollado modelos capaces de estudiar la percepción de la textura y el sabor, por ejemplo, hay modelos que cuantifican de manera dinámica la liberación de compuestos saborizantes como la sal (de Loubens et al., 2011; Mills, Spyropoulos, Norton, & Bakalis, 2011), mientras que

otros modelos se han desarrollado con el objetivo de estudiar la percepción de la textura del alimento en boca (van Aken, Vingerhoeds, & de Hoog, 2007; Vardhanabhuti, Cox, Norton, & Foegeding, 2011).

Los modelos dinámicos de digestión gástrica *in vitro* incorporan acciones mecánicas que simulan la motilidad del estómago (mezclado y fuerzas mecánicas) mediante el empleo de diferentes técnicas como la presión del contenido con las paredes del reactor o el movimiento relativo entre superficies (Chessa et al., 2014; Lo Curto et al., 2011). Algunos de estos métodos cuentan con la capacidad de controlar, además, la velocidad de flujo del digerido y de las secreciones digestivas. Estos modelos normalmente estudian la rotura del bolo alimenticio de manera mecánica y/o enzimática y también la posterior utilización del quimo como objeto de estudio mediante diferentes técnicas, entre las que se encuentran un sofisticado sistema de hardware capaz de medir presión, movimiento de fluidos y concentraciones enzimáticas (Bellmann, Lelieveld, Gorissen, Minekus, & Havenaar, 2016) o un estómago artificial capaz de reproducir y controlar temperaturas y secreción y vaciado de, mismo (Chen et al., 2016)

El quimo, a su vez, es también objeto de estudio simulando su paso por el intestino delgado. Se han desarrollado modelos de digestión *in vitro* que pretenden imitar la degradación del quimo y la bioaccesibilidad y absorción de los nutrientes generalmente cuantificados mediante la utilización de una membrana semipermeable que simula las paredes intestinales (Gouseti et al., 2014; Tharakan, Norton, Fryer, & Bakalis, 2010; Wright, Kong, Williams, & Fortner, 2016).

Por último, los modelos de digestión *in vitro* dinámicos que simulan las interacciones entre el alimento y el intestino grueso tienen en consideración, generalmente, el resto de etapas previas de la digestión (oral, gástrica e intestinal) como, por ejemplo, el Simulador Gastrointestinal Dinámico (SIMGI) ubicado en el Instituto de Investigación en Ciencias de la Alimentación (CIAL) (Barroso, Cueva, Peláez, Martínez-Cuesta, & Requena, 2015) o el Simulador del ecosistema microbiano

en el intestino humano (SHIME) localizado en *Olimp Laboratories*, Polonia (Van de Wiele, Van den Abbeele, Ossieur, Possemiers, & Marzorati, 2015) utilizado para el estudio de los procesos de fermentación en el colon. Otros modelos multicompartimentalizados como, por ejemplo, TIM-1, simulador de la digestión gástrica e intestinal y TIM-2, que se encarga de la simulación de la digestión en el intestino grueso, ambos han sido desarrollados en el *Netherlands Organisation for applied scientific research* (TNO) en los Países Bajos y ya se han comercializado (Muttakin et al., 2019).

1.1.3. Modelos de digestión gastrointestinal in vivo

En cuanto a los estudios de digestión *in vivo*, se llevan utilizando desde hace varios años. En el caso de la leche, se evaluó por primera vez cómo se digerían sus proteínas en ratas en 1983 (Miranda & Pelissier, 1983) y determinó que las proteínas α-lactoalbúmina y β-lactoglobulina no eran susceptibles a la digestión gástrica, incluso tras 240 min. En otros estudios *in vivo*, como el realizado por Yvon, Pélissier, Guilloteau, and Toullec (1985) en terneros, también se observó la resistencia de las proteínas séricas durante la fase gástrica de la digestión. Por otro lado, la cantidad de nitrógeno que permanecía en el estómago de cerdos recién nacidos, sugería que las proteínas de suero permanecían menos tiempo en el estómago que las caseínas (Newport & Henschel, 1985). Posteriormente, Moughan, Cranwell, and Smith (1991), estudiaron el ratio de vaciamiento gástrico y el cambio en el pH postprandial en terneros alimentados con fórmulas infantiles que contenían o proteínas bovinas intactas o un hidrolizado de las mismas. Los resultados obtenidos mostraron que el tipo de dieta no afectaba al pH después de la ingesta, mientras que el vaciado gástrico ocurría de manera más rápida en el caso del hidrolizado que en el de la proteína intacta, con unos valores remanentes observados en el estómago del 12% y del 22%, respectivamente, tras 3 horas de digestión. Otro estudio realizado en cerdos enanos alimentados con leche y yogur con leche marcada con Nitrógeno-15 determinó que el vaciado gástrico es el principal factor

responsable de la absorción de las proteínas. En este trabajo se observó que las proteínas de la leche fueron rápidamente absorbidas una vez estas alcanzan el intestino (Gaudichon et al., 1994). En general, el modelo porcino para estudios de digestibilidad, muestra una buena correlación interespecie con los humanos, siendo, además, un animal monogástrico y omnívoro (Deglaire, Bos, Tomé, & Moughan, 2009).

En relación a la caracterización de digeridos *in vivo* de proteínas lácteas, en 1986, el fragmento de β-caseína f(60-70), conocida como pro-casomorfina (YPFPGPIPNSL), se encontró en el contenido intestinal de cerdos enanos tras la ingesta de caseína, finalmente adoptó el nombre de β-casomorfina-11 debido a su actividad opioide (Meisel, 1986). Esta misma secuencia fue posteriormente identificada en fluidos de yeyuno distal de cerdos enanos después de haber consumido caseína. Además, también se identificó el caseín-fosfopéptido, correspondiente al fragmento f(66-74) de la α_{s1}-caseína, que posee tres fosfoserinas (Meisel & Frister, 1988, 1989).Se detectaron varios péptidos originados a partir de la digestión de caseína en el contenido estomacal de terneros tras la ingesta de una dieta basada en leche o caseína (M. Yvon & Pelissier, 1987). Entre los péptidos encontrados, se identificaron varias secuencias con actividad fisiológica: el péptido antihipertensivo de la α_{s1}-caseína AYFYPEL, f(143-149) (Contreras, Carrón, Montero, Ramos, & Recio, 2009); el péptido antibacteriano, también proveniente de la α_{s1}-caseína, conocido como isracidina, RPKHPIKHQGLPQEVLNENLLRF, f(1-23) (Lahov & Regelson, 1996) y el péptido derivado de la β-caseína con actividad inmunomoduladora, YQEPVLQPVRGPFPIIV, f(193-209) (Coste et al., 1992). Por otro lado, en 1991, se observó la presencia de varios caseín-fosfopéptidos en duodeno, yeyuno e íleon en digeridos de ratas alimentadas con proteínas de suero, caseína y caseín-fosfopéptidos (Brommage, Juillerat, & Jost, 1991). De entre los péptidos identificados, el caseín-fosfopéptido, proveniente de la β-caseína, f(59-79) mostró una gran capacidad de unión al calcio favorecida por la presencia del clúster de tres residuos de serina fosforilada seguidos de dos de ácido glutámico (SerP-

SerP-SerP-Glu-Glu) en su secuencia (Cross et al., 2005). Sin embargo, la actividad fosfatasa presente en el epitelio intestinal hace posible una fácil defosforilación en condiciones de pH neutro o ligeramente alcalino (Bäder et al., 1984; Moss, 1992). Scanff, Yvon, Thirouin, and Pelissffir (1992) identificaron el caseinmacropéptido, κ-caseína f(106-169), en estómagos de terneros. Además pudieron identificar un precursor de la β-casomorfina f(58-93) y un caseinofosfopéptido derivado de la α_{s1}-caseína [f(110-142)1P]. En 1992, Hirayama, Toyota, Hidaka, and Naito (1992) encontraron dos caseinofosfopéptidos provenientes de la α_{s1}-caseína, f(61-74) y de la β-caseína, f(7-24) tras la digestión de caseinofosfopéptidos en el intestino de ratas. Por otro lado, el caseinmacropéptido también se encontró en el estómago y duodeno de ratas alimentadas con una dieta que contenía un 48% de Phe-caseinmacropéptido-1P.

Uno de los modelos animales más utilizados para estudiar la digestibilidad proteica de alimentos en humanos son los cerdos, ya que reproduce, con un alto grado de similitud, los resultados obtenidos (Deglaire et al., 2009). Se han podido identificar una gran cantidad de péptidos liberados durante la digestión de las proteínas lácteas en el íleon y yeyuno de lechones alimentados con una fórmula láctea infantil a diferentes tiempos postprandiales utilizando un nano-LC-Q-TOF (Bouzerzour et al., 2012). En este estudio se identificó un fragmento derivado de la β-caseína f(74-91) como uno de los más resistentes a la digestión. Además identificaron un péptido, también proveniente de la β-caseína, que había mostrado actividad inhibidora de la enzima convertidora de angiotensina (ECA), TPVVVPPFLQP f(80-90) (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998), y también, la secuencia opioide β-casomorfina-7 en el yeyuno de lechones. Con esta misma técnica de identificación, se consiguieron detectar entre 3000 y 4000 secuencias provenientes de efluentes duodenales de cerdos enanos alimentados con leche cruda y calentada (Barbé et al., 2014). En este estudio, la estructura de la matriz alimentaria tuvo una pequeña influencia en el sitio de ruptura de los péptidos, pero principalmente afectó al número de secuencias identificadas. Aproximadamente 29 de

las secuencias encontradas en este estudio se habían identificado previamente como péptidos bioactivos. Se encontraron varias regiones resistentes a la digestión tanto en el caso de la α_{s1}-caseína, como en el de la β-caseína (Figura 2). Entre estas regiones resistentes cabe destacar la región f(30-60) y la región C-terminal f(170-209) para la β-caseína. En el caso de la α_{s1}-caseína, la región N-terminal 1-20, especialmente a tiempos cortos de digestión, la región f(110-140) y la región C-terminal de la proteína son las más resistentes a la digestión. Durante la realización de esta Tesis Doctoral, se han utilizado cerdos alimentados con leche en polvo y leche en polvo desnatada con el objetivo de comparar los resultados con el protocolo de digestión *in vitro* estático publicado por (Minekus et al., 2014). Esto demuestra el interés a nivel internacional por este tipo de estudios de validación con datos in vivo (Egger et al., 2017).

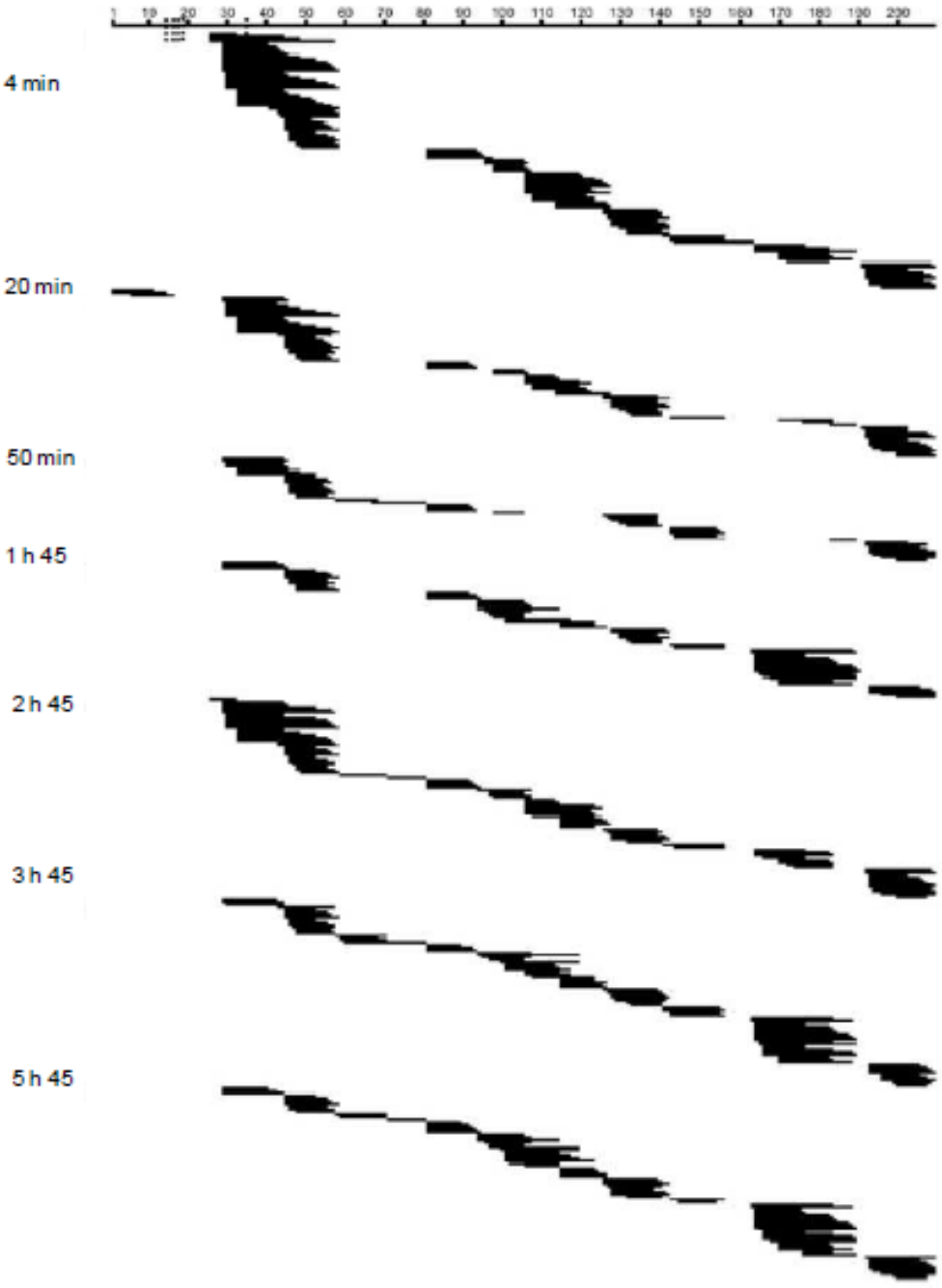

Figura 2 . Péptidos de β-caseína provenientes efluentes duodenales de la digestión de leche cruda en cerdos enanos (Barbé et al., 2014)

1.1.4. Validación de modelos in vitro con datos in vivo

Como se ha expuesto en apartados anteriores, los modelos de digestión *in vitro* se utilizan desde hace años y se han desarrollado para resolver cuestiones científicas tales como la bioaccesibilidad y la digestibilidad de productos farmacéuticos y alimentarios. Estos modelos se han constituido como la principal alternativa a los modelos *in vivo*, ya que tienen la ventaja de ser más rápidos, menos costosos y no tienen restricciones éticas. Esto posibilita que se analicen, simultáneamente, un número relativamente grande de muestras, que a su vez sean reproducibles y permite la elección de condiciones controladas y facilidad de muestreo (Minekus et al., 2014).

No obstante, reproducir fielmente las condiciones fisiológicas que se dan en el organismo es complicado ya que es un proceso complejo en el que varias variables afectan al desarrollo del mismo, entre los que se encuentran: Por una parte, la especificidad de una enzima determina los enlaces que se hidrolizan, aunque la hidrólisis de un enlace en particular depende del acceso que tenga la enzima al sustrato y los péptidos que se originan por dicha rotura. Por otro lado, la regulación del pH, ya que en el organismo este proceso es dinámico a lo largo del tracto gastrointestinal y afecta a la activación y desactivación de las enzimas. También cabe destacar la gran importancia de que las enzimas utilizadas en estos modelos de digestión sean aquellas que se encuentren en el tracto digestivo, junto con poder realizar una simulación de fluidos que reproduzcan las condiciones fisiológicas del tracto digestivo para que las enzimas puedan actuar correctamente (Boisen & Eggum, 1991). Por último, la concentración enzimática es crucial a la hora de diseñar modelos de digestión *in vitro*. La concentración a la que se añaden las enzimas afecta significativamente al proceso de digestión, ya que, cuando las concentraciones son altas, el proceso de digestión o degradación de los componentes de los alimentos se acelera y viceversa, por lo que es importante utilizar niveles fisiológicamente relevantes. Estas concentraciones varían dependiendo de la persona involucrada, edad y estado de salud, la hora del día a la cual

se consume el alimento, y el tipo y la cantidad de alimentos que se consumen (Hur et al., 2011; Minekus et al., 2014). Sin embargo, las digestiones gastrointestinales *in vitro* no solo presentan dificultades en cuanto a la simulación de las condiciones fisiológicas, sino también en la equiparación y la reproducibilidad de los resultados obtenidos en diferentes laboratorios y por diferentes investigadores. Es por esta razón que se elaboró el método publicado por Minekus et al. (2014), cuyo objetivo general ha sido definir las condiciones de digestión, desarrollar un protocolo que sea fácil de aplicar y que proporcione resultados fácilmente comparables entre la comunidad científica. Para ello, el método desarrollado comprende tres fases: fase oral, fase gástrica y fase intestinal. Para cada una de ellas se definieron unas condiciones establecidas de digestión, entre las que se incluyen: la medida de la actividad enzimática, la preparación de fluidos simulados oral, gástrico e intestinal y las condiciones de pH y temperatura requeridas para la realización de los ensayos.

Los protocolos de digestión *in vitro* se han utilizado durante años con el fin de obtener resultados significativos en el área de la investigación alimentaria. Sin embargo, se han utilizado tantos diferentes que hacer una comparación entre los distintos estudios resulta muy complicado (Hur et al., 2011; Kopf-Bolanz et al., 2012; McClements & Li, 2010; Picariello et al., 2010; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Wickham et al., 2009). A partir de la publicación del método consensuado de digestión *in vitro (Minekus et al., 2014)*, se estudió si la comparabilidad entre distintos laboratorios mejoraba mediante la digestión de un mismo lote de leche desnatada en polvo cuyo resultado fue positivo, ya que confirmaron que el método armonizado mejora la comparabilidad de los datos de las digestiones gastrointestinales interlaboratorio, mostrando a este protocolo como una herramienta sencilla y robusta para el estudio digestivo de los alimentos tras la ingesta oral del mismo (Egger et al., 2016). Para comprobar con datos *in vivo* la fiabilidad de este protocolo, se alimentó a ocho cerdos con la misma leche desnatada en polvo tres veces: 6, 3 y 1,5 horas antes del sacrificio

de los animales. Las muestras se recogieron en cuatro zonas diferentes del tracto digestivo: una gástrica en el estómago, una duodenal y dos intestinales en la parte inferior y superior. Para el análisis de las muestras se utilizó la misma metodología que en el ensayo anterior. Los resultados mostraron valores comparables entre sí entre las muestras *in vivo e in vitro* en cuanto a degradación proteica, de proteínas a péptidos y aminoácidos. No obstante, se necesitan más estudios y comparaciones con ensayos *in vivo* y especialmente con datos obtenidos en humanos (Egger et al., 2017).

1.3 Péptidos liberados durante la digestión gastrointestinal. Funcionalidad biológica

El papel que juega la digestión de las proteínas en el organismo es innegable, no sólo desde el punto de vista nutricional sino debido a la acción que pueden ejercer los péptidos liberados en la salud, ya que pueden desencadenar una respuesta negativa, ocasionando algunos tipos de reacciones alérgicas, o bien pueden actuar como un factor beneficioso para el organismo. En este contexto se observa que, en general, determinadas regiones de las proteínas de la leche resistentes a la hidrólisis gastrointestinal son, o bien precursores de péptidos bioactivos, o bien péptidos que ejercen alguna bioactividad (**¡Error! No se encuentra el origen de la referencia.**). Por t anto, existen ciertas regiones de las proteínas lácteas que poseen gran importancia fisiológica, y que han llegado a ser consideradas como "hormonas alimentarias" (Teschemacher, Koch, & Brantl, 1997). Es también importante considerar que la forma activa del péptido a veces se libera sólo después de la digestión. Un ejemplo es el fragmento f(169-175) de la β-caseína KVLPVPQ, para el que se observó una baja actividad inhibidora de la ECA *in vitro*, pero produjo un importante efecto antihipertensivo tras su administración oral a ratas espontáneamente hipertensas. Se demostró que esta secuencia perdía el aminoácido Gln en el extremo C-terminal durante una simulación de

digestión gastrointestinal, dando lugar a la KVLPVP, que es, presumiblemente, la forma activa *in vivo* (Maeno, Yamamoto, & Takano, 1996).

Tabla 2 Péptidos Bioactivos derivados de proteínas lácteas

Proteína	Péptido bioactivo	Bioactividad	Referencia
α_{S1}-caseína	TTMPLW	Antihipertensivo, antioxidante, inhibidor DPP-IV, inmunomodulante, opioide	(Skrzypczak, Gustaw, Szwajgier, Fornal, & Waśko, 2017)
α_{S1}- caseína	YLGYLEQLLR	Antihipertensivo, opioide	Skrzypczak et al. (2017)
α_{S1}- caseína	RPKHPIKHQGLPQEVLNENLLRF	Antibacteriano, inmunomodulante	Chabance et al. (1995); Barbé et al. (2014)
α_{S1}- caseína	FFVAPFPEVFGK, FVAPFPEV, FVAPFPEVF, FVAPFPEVFG, VAPFPEVF, KKYKVPQL, YKVPQL, AYFYPEL, DAYPSGAW	Antihipertensivo	Chabance et al. (1995, 1998); Boutrou et al. (2013); Barbé et al. (2014)
α_{S1}- caseína	HIQKEDVPSER, YFYPEL	Antioxidante	Chabance et al. (1998); Boutrou et al. (2013)
α_{S1}- caseína	RYLGYL	Opioide	(Chabance et al., 1995)
α_{S1}- caseína	YLGYLEQ, YLGYLEQL, YLGYLEQLLR	Antiestrés	Boutrou et al. (2013)

α_{S1}- caseína	LRLKKYKVPQL, SDIPNPIGSENSEK	Antimicrobiano	Barbé et al. (2014)
α_{S2}- caseína	NAVPITPTLNR	Antihipertensivo, inhibidor DPP-IV	(Skrzypczak et al., 2017)
α_{S2}- caseína	LTEEEK	Inhibidor DPP-IV	Skrzypczak et al. (2017)
α_{S2}- caseína	AMKPWIQPK	Antihipertensivo, inhibidor DPP-IV	Skrzypczak et al. (2017)
α_{S2}- caseína	AMKPWIQPKTK	Antihipertensivo, inhibidor DPP-IV	Skrzypczak et al. (2017)
α_{S2}- caseína	TKVIPYVRYL	Antihipertensivo, inhibidor DPP-IV, antibacteriano, antioxidante	Skrzypczak et al. (2017)
α_{S2}- caseína	VIPYVRYL	Antihipertensivo, inhibidor DPP-IV	Skrzypczak et al. (2017)
α_{S2}- caseína	f(150–188), f(164–179), f(183–207), f(164–207), f(175–207), f (181–207)	Antimicrobiano	(López Expósito & Recio, 2006); Barbé et al. (2014)
α_{S2}- caseína	VYQHQKAMKPWIQPKTKVIPYVRY	Inhibidor fosfodiesterasa, inhibidor peptidasas	Barbé et al. (2014)
α_{S2}- caseína	AMKPWIQPK, MKPWIQPK	Antihipertensivo	Barbé et al. (2014)
α_{S2}- caseína	AMKPWIQPKTKVIPYVRYL	Inhibidor fosfodiesterasa	Barbé et al. (2014)

β- caseína, κ-caseína	IPP, VPP	Antihipertensivo	Boutrou et al. (2013)
β- caseína	KVLPVP	Antihipertensivo	Sun et al. (2009)
β- caseína	GPFPIIV	Antihipertensivo, inhibidor DPP-IV	Skrzypczak et al. (2017)
β- caseína	f(184–210)	Antimicrobiano	López Expósito & Recio (2006)
β- caseína	LNVPGEIV, NVPGEIVE, DKIHPF, SLVYPFPGPI	Antihipertensivo	Boutrou et al. (2013)
β- caseína	LVYPFPGPIPNSLPQ	Antihipertensivo, inhibidor peptidasas	Barbé et al. (2014)
β- caseína	VYPFPGPI, VYPFPGPIPN, YPFPGPIPN	Antihipertensivo, opioide	Boutrou et al. (2013); Barbé et al. (2014)
β- caseína	VYPFPGPIP	Inhibidor peptidasas	Boutrou et al. (2013); Barbé et al. (2014)
β- caseína	YPFP, YPFPGP, YPFPGPI, YPFPGPIP, YPFPGPIPNSL, YPVEPF, YPFPGPIPNSLPQNIPPLTQT	Opioide	Boutrou et al. (2013); Barbé et al. (2014)
β- caseína	PGPIPN,	Inmunomodulante	Boutrou et al. (2013)

	LYQEPVLQPVRGPFPIIV, YQEPVLQPVR, YQEPVLQPVRGPFPIIV		
β- caseína	NIPPLTQTPV, PPLTQTPV, TPVVVPPFLQP, EMPFPK, LHLPLP, HLPLPLL, SQSKVLPVPQ, SKVLPVPQK, VENLHLPLPLL, NLHLPLPLL, AVPYPQR, LLYQQPVLGPVRGPFPIIV, QEPVLQPVRGPFPIIV, EPVLQPVRGPFP	Antihipertensivo	Boutrou et al. (2013)
β- caseína	VKEAMAPK, KVLPVPQK, VLPVPQK	Antioxidante	Boutrou et al. (2013); Barbé et al. (2014)
β- caseína	YQEPVLGPVRGPFPI	Antimicrobiano	Barbé et al. (2014)
κ- caseína	f(96–106)	Antioxidante	Barbé et al. (2014)

κ- caseína	f(106–169), f(18–24), f(30–32), f (139–146)	Antimicrobiano	López Expósito & Recio (2006)
κ- caseína	f(106–111), f(106–116), f(112–116)	Antitrombótico	Chabance et al. (1998); Boutrou et al. (2013)
κ- caseína	f(4–11), f(6–7), f(17–25), f(19–26), f(22–23), f(25–32), f(26–32), f(26–34), f(35–40), f(35–41), f(35–44), f(37–38), f(37–39), f(40–47), f(41–48), f(43–51), f(44–49), f(46–53), f(56–58), f(57–58) f(59–62), f(59–69), f(66–72), f(67–73), f(74–80), f(74–83), f(76–86), f(77–82), f(76–81), f(80–84), f(80–85), f(81–89), f(82–85), f(82–88), f(89–92), f(98–105), f(100–106), f (102–110), f(102–112), f(103–	Antihipertensivo	Barbé et al. (2014); Skrzypczak et al. (2017)

	111), f(105–113), f(110–119), f (114–118), f(115–122), f(116–121), f(118–127), f(119–127), f (121–132), f(124–126), f(124–127), f(130–135), f(136–142), f (136–147), f(137–147), f(138–144), f(139–147), f(139–148), f (141–144), f(141–151), f(146–149), f(154–164), f(155–161), f (155–162), f(155–164), f(157–167), f(158–167), f(159–167), f (164–169), f(168–178), f(171–173), f(171–180), f(173–181), f (173–182), f(174–185), f(176–181), f(176–184), f(176–186), f (182–190), f(183–189), f(183–		

	190), f(185–190), f(187–190)		
α-lactoalbúmina	f(1–5), f(17–31)S-S(109–114), f (61–68)S-S(75–80)	Antimicrobiano	López Expósito & Recio (2006)
β-lactoglobulina	f(15–20), f(25–40), f(78–83), f(92–100)	Antimicrobiano	López Expósito & Recio (2006)
Lactoferrina	f(17–41), f(17–42)	Antimicrobiano	López Expósito & Recio (2006)

Las β-casomorfinas bovinas son fragmentos con actividad opioide derivados de la β-caseína bovina procedentes de la región f(60-66; β-casomorfina-7) y de pequeños fragmentos de la región C-terminal que fueron por primera vez aislados a partir de un digerido enzimático de caseína (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). Estos péptidos poseen la secuencia de aminoácidos N-terminal YPFP y se comportan, preferentemente, como agonistas de los receptores opioides del tipo μ (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981). En voluntarios adultos después de la ingesta de leche, se identificaron en la sección duodenal del intestino delgado, β-casomorfina-7 y cantidades reducidas β-casomorfina-4 y β-casomorfina-6, junto con un fragmento más grande que fue identificado como el precursor de la β-casomorfina-7 (Svedberg, de Haas, Leimenstoll, Paul, & Teschemacher, 1985). De hecho, un fragmento relacionado con este precursor se aisló más tarde en el quimo duodenal de cerdos enanos tras alimentarlos con caseína y se denominó β-casomorfina-11, presentando cuatro aminoácidos más que la β-casomorfina-7 en el extremo C-terminal (Meisel, 1986). La baja cantidad encontrada de este péptido en el duodeno se atribuyó a la rápida degradación generada por las enzimas del borde en cepillo y/o a la alta tasa de absorción de estos fragmentos. Más recientemente, en el duodeno de cerdos enanos alimentados con leche desnatada gelificada, se han identificado dos precursores adicionales de la β-casomorfina-7, β-caseína f(58-72) y β-caseína f(59-67) (Barbé et al., 2014). También se han identificado β-casomorfina-7, numerosos precursores de este péptido y β-casomorfina-5 en yeyuno humano tras la ingesta de caseína (Boutrou et al., 2013).

Jinsmaa y Yoshikawa examinaron las condiciones necesarias para la liberación de β-casomorfinas durante la digestión gastrointestinal *in vitro* (Jinsmaa & Yoshikawa, 1999). La pepsina rompe el péptido en el enlace Leu^{58}-Val^{59} y la leucina aminopeptidasa elimina la Val^{59}, dejando a la Tyr en el extremo N-terminal. En cuanto al segmento C-terminal, se ha identificado a la elastasa pancreática como la enzima responsable de la

hidrólisis del enlace peptídico Ile^{66}-His^{67} dando lugar a la β-casomorfina-7 (YPFPGPI). Esto sugiere que la adición de las enzimas pancreáticas que no sean tripsina y quimotripsina en el protocolo *in vitro* determinará la generación de las roturas peptídicas necesarias para la liberación de la β-casomorfina-7 a partir de sus precursores. La β-casomorfina-7 también ha sido identificada en la digestión de fórmulas infantiles con pepsina y Corolase PP® en condiciones que simulan la digestión infantil, con un pH gástrico de 3,5 (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). Noni, hidrolizando β-caseína en condiciones similares, también ha reportado la aparición del péptido opiáceo y observó una mayor cantidad del mismo cuando la pepsina actuaba a un pH de 2.0 (Noni, 2008). Tras la digestión de queso o leche con pepsina y pancreatina se encontraron algunos péptidos de la región de la β-caseína 57-68 (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Sánchez-Rivera et al., 2014) o también al digerirlos con secreciones gastrointestinales humanas (Qureshi, Vegarud, Abrahamsen, & Skeie, 2013). Curiosamente, el realizar un paso de hidrólisis adicional con peptidasas del borde en cepillo también libera β-casomorfina-7 (Picariello et al., 2015).

La neocasomorfina-6, f(114-119) de la β-caseína (YPVEPF) se identificó por primera vez en un digerido gastrointestinal *in vitro (*Jinsmaa *&* Yoshikawa*,* 1999*)* y, más recientemente, se ha identificado en yeyuno humano tras la ingesta de leche (Boutrou et al., 2013). Asimismo, esta secuencia apareció en los efluentes duodenales de cerdos enanos tras la ingesta de leche líquida sometida a un tratamiento térmico (Barbé et al., 2014). También se ha identificado la neocasomorfina-6 después de la digestión *in vitro* de leche y queso (Sánchez-Rivera et al., 2014), y en condiciones fisiológicamente relevantes de digestión tanto para adulto como para niños (Dupont, Mandalari, Molle, et al., 2010; Hernández-Ledesma et al., 2007). En cambio, otros autores han reportado la liberación de un precursor de este péptido, en concreto, el fragmento de la β-caseína f(114-124) (Picariello et al., 2010).

Los péptidos antihipertensivos son probablemente los compuestos bioactivos que han recibido una mayor atención en los últimos años, siendo la leche la principal fuente de péptidos con esta actividad. Sin embargo, no existe una correlación completa entre la actividad inhibitoria de la ECA *in vitro* y el efecto *in vivo* (reducción de la presión sanguínea), y estas discrepancias se han atribuido a la degradación de los péptidos durante la digestión gastrointestinal o a una pobre biodisponibilidad de los mismos. Por lo tanto, la presencia de secuencias activas en la digestión marcan el primer paso para conocer su bioaccesibilidad y su posible absorción (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012).

Las secuencias KVLPIPQ y VVPYPQR procedentes de la β-caseína de la leche humana, correspondientes a los péptidos antihipertensivos procedentes de leche bovina β-caseína f(169-175), KVLPVPQ y f(177-183), AVPYPQR con el reemplazo de los aminoácidos Ile- y Val- por Val- y Ala-, respectivamente, se han identificado en el estómago de los recién nacidos, pero también en leche no digerida, lo que sugiere que la proteólisis comienza durante la lactancia o justo antes de ella (Dallas et al., 2014). Sin embargo, se han encontrado muchos péptidos en digeridos gástricos que no estaban presentes en la leche materna, sugiriendo que también existe un extenso proceso de proteólisis en el estómago infantil. La secuencia de β-caseína f(133-138), LHLPLP, fue identificada en el yeyuno de voluntarios que ingirieron caseína comercial (Boutrou et al., 2013). Los precursores de esta secuencia, f(132-140) y f(130-140), se encontraron en duodeno porcino tras la administración oral de leche calentada y geles ácidos preparados a partir de leche (Barbé et al., 2014). Esta secuencia pertenece a una región muy conservada de la β-caseína en diferentes mamíferos y es resistente a la digestión debido a la abundancia de residuos de Pro. Se han encontrado numerosos péptidos relacionados con esta secuencia tras la digestión gastrointestinal simulada de leche humana (Hernández-Ledesma et al., 2007) y bovina (Benedé et al., 2014; Qureshi et al., 2013; Sánchez-Rivera et al., 2014). La inclusión de las hidrolasas del borde en cepillo

en el modelo de digestión favoreció la liberación de diferentes formas que contenían Pro^{138} en el extremo C-terminal (Picariello et al., 2015). Por otro lado, a partir de la secuencia LHLPLP, por la acción de las peptidasas plasmáticas se formaron varios fragmentos procedentes de la misma: HLPLP, HLPL, LPLP, HLP, LPL y PLP, los cuales han demostrado poseer también actividad antihipertensiva (Quirós et al., 2007; Sánchez-Rivera et al., 2016).

En el caso de la α_{s1}-caseína, el f(143-149), correspondiente con la secuencia AYFYPEL, se encontró en el estómago y el duodeno de humanos después de ingerir leche o yogur (Chabance et al., 1998), mientras que en el duodeno la secuencia encontrada fue f(144-149), YFYPEL. Estos péptidos han sido descritos como antihipertensivos e identificados en un hidrolizado de caseína (Contreras et al., 2009; Sánchez-Rivera et al., 2014), pero recientemente, se ha demostrado su capacidad para interactuar de manera agonista con los receptores opiáceos (Fernández-Tomé et al., 2016). Tras la digestión gástrica de leche en condiciones dinámicas se han encontrado diferentes formas peptídicas procedentes de esta región de la α_{s1}-caseína (Sánchez-Rivera, Ménard, Recio, & Dupont, 2015), también tras la digestión *in vitro* de leche (Dupont, Mandalari, Mollé, et al., 2010), queso (Sánchez-Rivera et al., 2014) y fórmulas infantiles (Hernández-Ledesma et al., 2007).

Las caseínas bovinas son una importante fuente de caseinofosfopéptidos, los cuales se ha demostrado que previenen la precipitación de iones metálicos a pH alcalino en el intestino delgado (Meisel & FitzGerald, 2003). Curiosamente, las regiones fosforiladas son relativamente resistentes a la hidrólisis durante la fase gastrointestinal de la digestión. Se demostró que además de la fosforilación, la presencia de minerales unidos también aumenta la resistencia de los caseinofosfopéptidos a la digestión gastrointestinal (Boutrou, Coirre, Jardin, & Léonil, 2010). El análisis de digeridos de Fe-β-caseína f(1-25) en una preparación *ex vivo* de intestino de rata evidenció que el fragmento que contiene cuatro fosfoserinas, β-caseína f(15-25), es resistente a las

proteasas. Asimismo, se han identificado en el estómago humano, los fragmentos fosforilados de β-caseína f(6-17), f(29-40), f(29-41), f(30-41) y f(33-44) tras la ingesta de leche. En duodeno humano, se encontraron formas similares a estas secuencias (Chabance et al., 1998). En el duodeno de cerdos, se han identificado caseinofosfopéptidos procedentes de la β-caseína junto con secuencias monofosforiladas de α_{s1}-caseína y α_{s2}-caseína. Sin embargo, otras regiones fosforiladas que poseen la secuencia SerP-SerP-SerP-Glu-Glu podrían no ser identificadas debido a las condiciones de análisis, ya que poseen una baja capacidad de ionización (Barbé et al., 2014). En el yeyuno y el íleon de humanos, se han encontrado secuencias similares, lo que implica que los caseinofosfopéptidos pueden sobrevivir al paso intestinal *in vivo* (Boutrou et al., 2013; Meisel & FitzGerald, 2003).

Se ha estudiado la formación de caseinofosfopéptidos y su resistencia durante la digestión gastrointestinal simulada. Se ha demostrado que la hidrólisis con pepsina y, posteriormente, con pancreatina, libera secuencias fosforiladas previamente identificadas *in vivo*, incluso en fórmulas infantiles empleando condiciones adecuadas (Miquel et al., 2005). Además, la separación de los caseinofosfopéptidos mediante precipitación selectiva o cromatografía de TiO_2 permitió identificar varias secuencias que contenían SerP-SerP-SerP-Glu-Glu (Miquel, Alegría, Barberá, & Farré, 2006; Picariello et al., 2010). Se comparó el número de sitios de fosforilación en muestras de queso *Beaufort* digerido y no digerido, y se encontró una disminución en el número de péptidos fosforilados tras la digestión (Adt et al., 2011). Aun así, una parte importante de los péptidos identificados contenían la secuencia SerP-SerP-SerP-Glu-Glu. El hecho de que este análisis se realizara mediante una precipitación selectiva de caseinofosfopéptidos pone en relieve la importancia de este paso en la identificación minuciosa de los posibles péptidos fosforilados.

En resumen, estos resultados muestran que la digestión *in vitro* en diferentes condiciones es capaz de liberar ciertos péptidos resistentes a la digestión

gastrointestinal que también se encuentran en las digestiones humanas y, que además podrían ejercer o ejercen alguna actividad biológica en el cuerpo. Además, los ejemplos anteriores demuestran que, no sólo las enzimas, sino su concentración, así como la composición iónica y el pH de los jugos digestivos, afectan, en gran medida, a los resultados obtenidos y ponen en relieve, una vez más, la importancia de emplear parámetros similares a los encontrados *in vivo* en humanos para obtener resultados fisiológicamente relevantes.

1.4 Absorción de péptidos liberados durante la digestión

Algunos de los péptidos generados por la digestión gastrointestinal pueden ser transportados, a través de las células de la membrana del borde en cepillo, al torrente sanguíneo gracias a una de las siguientes rutas: (1) transportador PepT1, (2) ruta paracelular a través de uniones intercelulares, (3) transcitosis y (4) difusión pasiva transcelular (Figura 3) (Daniel, 2004; Horner, Drummond, & Brennan, 2016; Matsui, 2018; Xu, Fan, Yu, Hong, & Wu, 2017).

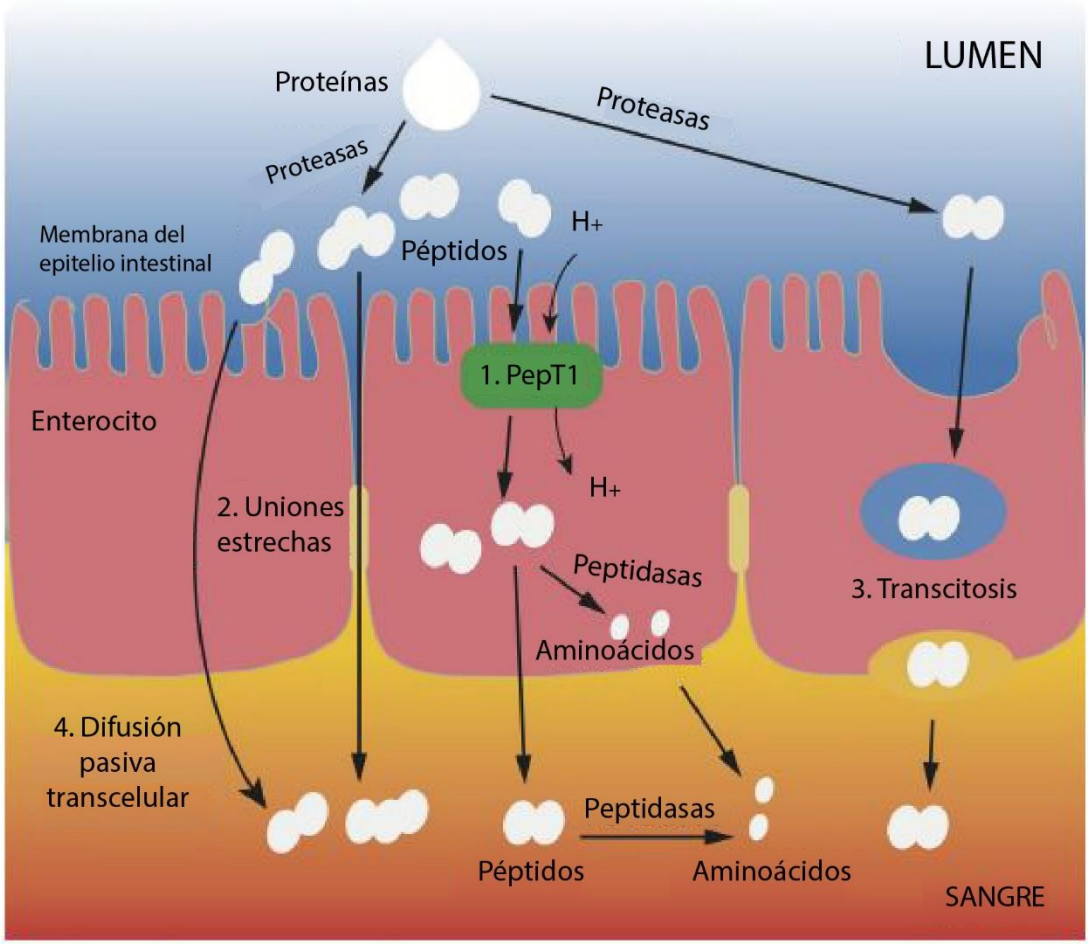

Figura 3. Posibles rutas de transporte peptídico a través de la membrana apical de las células epiteliales hasta el torrente sanguíneo.

TRANSPORTADOR PepT1

Una gran variedad de pequeños péptidos (di- y tripéptidos) se transportan a través del transportador PepT1 debido a su alta capacidad de transporte, junto con su baja afinidad por secuencias específicas de péptidos y, además, por su alta expresión en el epitelio intestinal (Daniel, 2004). PepT1 conduce a los péptidos desde el lumen intestinal hasta el interior celular gracias al gradiente electroquímico de protones que existe entre ambos espacios (Daniel, 2004; Xu et al., 2018; Xu et al., 2014). Se han descrito varios péptidos que son transportados vía PepT1, como por ejemplo IPP (Gleeson, Brayden, & Ryan, 2017; Gleeson, Frías, Ryan, & Brayden, 2018), LKP (Gleeson et al., 2017; Gleeson et al., 2018; Xu et al., 2017), β-AH (Shimizu, 2004) y YPI (Miguel et al., 2008). Sin embargo, PepT1 no puede transportar el péptido C, procedente

de la β-caseína (VLPVPQK) o la β-casomorfina-5 (YPFPG) (Vij, Reddi, Kapila, & Kapila, 2016) quedando, su capacidad de transporte, limitada a los di- y tripéptidos. El transportador PepT1 reconoce preferentemente cadenas cortas de péptidos con carga neutra y de carácter hidrofóbico (por ejemplo, AF, FY y YY), pero apenas muestra afinidad por dipéptidos más voluminosos o cargados positivamente (KK, RR, RK y PK) debido al tamaño limitado y a la carga positiva del sitio de unión de la PepT1 (Omkvist et al., 2010; Vig et al., 2006).

RUTA PARACELULAR

Algunos péptidos podrían ser transportados a través de los espacios intercelulares que existen entre las células que forman la monocapa celular, también denominados uniones estrechas. El tracto gastrointestinal humano tiene muchos poros de difusión paracelular, por los cuales una gran variedad de péptidos puede transportarse sin gasto energético asociado. Esta es la ruta propuesta para algunos péptidos alimentarios como por ejemplo HLPLP (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008), KVLPVP (Sun, Liu, Li, & Qin, 2009), LKP (Gleeson et al., 2017; Xu et al., 2017), RLSFNP (Guo et al., 2018), RWQ, WQ (Fernández-Musoles et al., 2013), SRYPSY, YPFPG, YPFPGPI (Sienkiewicz-Szłapka et al., 2009), VLPVP (Lei, Sun, Liu, Liu, & Li, 2008) y VPP (Satake et al., 2002). Sin embargo, el fragmento de la β-caseína f(193-209) no puede transportarse por difusión debido a su larga longitud y a su hidrofobicidad (Regazzo et al., 2010).

La presencia de estas uniones estrechas puede explicar por qué los pequeños péptidos tienen una mayor biodisponibilidad que los péptidos de mayor tamaño (Shen & Matsui, 2017). Los poros de las uniones estrechas en células humanas de adenocarcinoma colorrectal (Caco-2) tienen un diámetro de aproximadamente 2,1 nm, lo que explicaría que no pueden transportar péptidos de gran tamaño. Debido al limitado tamaño de los poros y a sus características electroestáticas, los péptidos hidrofílicos y

de bajo peso molecular, cargados negativamente pueden ser transportados con mayor facilidad a través de esta ruta (Linnankoski et al., 2010; Shimizu, Tsunogai, & Arai, 1997).

Este tipo de transporte permite que los péptidos no sean digeridos por peptidasas intracelulares (Shimizu, 2004). Por ejemplo, el péptido VPP es rápidamente hidrolizado por peptidasas citosólicas una vez que está en el interior de células Caco-2, por lo tanto, el VPP que alcanza el torrente sanguíneo es principalmente el transportado a través de la ruta paracelular (Satake et al., 2002). Se ha descrito que ocurre algo similar para el transporte de la bradiquinina, RPPGFSPFR (Shimizu et al., 1997; Vij et al., 2016). La Citocalasina D (Shimizu et al., 1997), las sales biliares (Celine et al., 2011) y el desoxicolato de sodio (Sun et al., 2009) pueden desestabilizar las uniones estrechas, provocando un aumento del transporte paracelular de péptidos. Además, las teaflavinas, los principales pigmentos polifenólicos en el té negro, podrían aumentar la capacidad de barrera mediante un aumento de la expresión de las uniones estrechas. Todos estos compuestos han sido utilizados para investigar el papel de las uniones estrechas en el transporte de péptidos a través de monocapas Caco-2 (Lin et al., 2017; Shen & Matsui, 2017; Vij et al., 2016; Xu et al., 2017).

RUTA DE LA TRANSCITOSIS

La transcitosis es una vía transcelular dependiente de energía que implica la captación apical endocítica de los péptidos a través de la internalización, transporte vesicular y secreción basolateral. Esta vía es la principal ruta de transporte para los péptidos de cadena larga (Regazzo et al., 2010), tales como la bradiquinina (Shimizu et al., 1997; Vij et al., 2016), los fragmentos de β-caseína: VLPVPQK, YPFPG (Vij et al., 2016), YPFPGPI (Sienkiewicz-Szłapka et al., 2009) y el fragmento f(193-209) (Regazzo et al., 2010) y el fragmento de la κ-caseína, SRYPSY (Sienkiewicz-Szłapka et al., 2009). En particular, los péptidos transportados a través de la transcitosis son altamente

hidrofóbicos. Esto se debe a que los péptidos hidrofóbicos deben interactuar con la superficie lipídica apical a través de interacciones hidrofóbicas previas a la internalización (Shimizu et al., 1997). Para estudiar el papel de la transcitosis en el transporte de péptidos se ha empleado wortmanina, que es un inhibidor de la fosfoinositol 3-quinasa, proteína responsable del proceso de la transcitosis (Fernández-Musoles et al., 2013; Quirós et al., 2008; Vij et al., 2016; Wang & Li, 2017).

DIFUSIÓN PASIVA

También se ha descrito el transporte de algunos péptidos alimentarios a través de la membrana del borde en cepillo intestinal mediante difusión pasiva, lo que implica la incorporación pasiva en las células, el transporte intracelular y la salida basolateral. El transporte de los péptidos por esta vía está influido por muchos factores, incluyendo su tamaño, carga e hidrofobicidad. Los péptidos altamente hidrofóbicos podrían ser transportados a través de esta ruta (Miguel et al., 2008). Es difícil cuantificar la cantidad de péptidos que son transportados mediante difusión pasiva debido a la falta reguladores de la misma, sin embargo, esta ruta está aceptada universalmente.

La permeabilidad aparente de la mayoría de los péptidos que atraviesan la monocapa de Caco-2 es baja (alrededor de 1×10^{-7} cm s^{-1};Tabla 3). La biodisponibilidad de los péptidos depende de sus características moleculares, tales como su tamaño, peso molecular, estabilidad, hidrofobicidad y carga (Celine et al., 2011; Horner et al., 2016; Shimizu et al., 1997). Por ejemplo, en un estudio de los siguientes péptidos, WR, IPI, IPIQY, LPYPY y LKPTPEGDL, la permeabilidad de WR a través de la monocapa de Caco-2 fue la más alta, mientras que de LKPTPEGDL fue la más baja (Lacroix, Chen, Kitts, & Li-Chan, 2017). En un estudio que investigaba la biodisponibilidad de algunas fracciones derivadas de la caseína, la biodisponibilidad de los fragmentos con alto peso molecular era menor que el de las fracciones con bajo peso molecular (Wang & Li, 2017). Estos estudios sugieren que los péptidos con bajo peso molecular tienen generalmente

una mayor biodisponibilidad. La degradación peptídica generada por las peptidasas del borde en cepillo también juega un papel fundamental en la baja permeabilidad de los péptidos (Celine et al., 2011; Lacroix et al., 2017; Shimizu et al., 1997). Por ejemplo, el péptido RLSFNP se hidroliza parcialmente durante el transporte a través de las monocapa de Caco-2 (Guo et al., 2018) , por lo que mostró una baja permeabilidad; los péptidos LHLPLP (Quirós et al., 2008) y VLPVPQK (Vij et al., 2016) también tenían menos permeabilidad, aunque en el caso de LHLPLP se ha observado su degradación formando HLPLP, el cual sí muestra ser biodisponible *in vitro* e *in vivo* (Quirós et al., 2008; Sánchez-Rivera et al., 2016). Derivados de la lactoferricina, RRWQWR, no puede traspasar la monocapa de Caco-2 de manera intacta debido a la hidrólisis que sufre; sin embargo, los fragmentos, WQ y RWQ, pueden ser transportados a través de las uniones estrechas debido a su pequeño tamaño y resistencia a las peptidasas (Fernández-Musoles et al., 2013). Sin embargo, algunas regiones específicas podrían resistir a la degradación enzimática, como las regiones hidrofóbicas de la β-caseína que son ricas en prolina (Horner et al., 2016). Además, la resistencia a las peptidasas del fragmento de la β-caseína f(193-209) está asociada con su secuencia rica en prolina (cuatro residuos de prolina) (Regazzo et al., 2010). La dipeptidil peptidasa IV es otro factor limitante en la vida media de los péptidos transportados por el tracto gastrointestinal y, por lo tanto, los inhibidores de la dipeptidil peptidasa IV pueden jugar un papel fundamental en la no degradación de ciertos péptidos (Iwan et al., 2008; Lacroix et al., 2017; Sienkiewicz-Szłapka et al., 2009). Además, la carga que poseen los péptidos es otro factor que afecta a la biodisponibilidad. Los péptidos hidrofóbicos con carga negativa derivados de la caseína mostraron una mayor biodisponibilidad en comparación con los péptidos con carga positiva (Tabla 3) (Wang & Li, 2018; Xie, Wang, Jiang, Liu, & Li, 2015).

Tabla 3 Rutas de transporte y coeficientes de permeabilidad de diferentes péptidos bioactivos provenientes de la leche a través de monocapa de células Caco-2

Péptido bioactivo	PepT1	TJs	Transcitosis	P_{app} (x 10^{-7} cm s^{-1}) o ratio	Referencia
AYFYPEL, RYLGY	ND	ND	ND	2.2, 2.6	(Contreras, Sancho, Recio, & Mills, 2012)
HLPLP	x	√	x	ND	Quirós et al. (2008)
LKP, IPP	√	√	ND	68, 89	Gleeson et al. (2017, 2018)
LKPTPEGDL, LPYPY, IPIQY, IPI, WR	ND	ND	ND	0.05–0.47%	Lacroix et al. (2017)
RLSFNP	x	√	x	ND	Guo et al. (2018)
RWQ, WQ	x	√	x	0.07, 0.39	Fernández-Musoles et al. (2013)
SRYPSY, YPFPG, YPFPGPI	x	√	√	0.17, 1.65, 1.54	Sienkiewicz-Szłapka et al. (2009)
VPP	x	√	x	24.3	Satake et al. (2002)
VLPVPQK	x	x	√	1%	Vij et al. (2016)
YPFPG	x	x	√	0.03%	Vij et al. (2016)
α_{S1}-CN-f(91–97)	ND	√	x	6.5	Cakir-Kiefer et al. (2011)
b-Ala-His	√	ND	ND	ND	Shimizu (2004)

β-casomorfina-5	x	√	x	6.3	Iwan et al. (2008)
β-casomorfina-7	x	√	x	67	Iwan et al. (2008)
β-CN-f(193–209)	x	x	√	ND	Regazzo et al. (2010)

Basándose en estos factores, se ha evaluado el empleo de varios compuestos que podrían mejorar la biodisponibilidad de los péptidos, como los inhibidores de enzimas, potenciadores de permeabilidad, nanopartículas, micropartículas, emulsiones, péptidos de penetración celular y transportadores lipídicos (Choonara et al., 2014; Nongonierma & FitzGerald, 2016; Renukuntla, Vadlapudi, Patel, Boddu, & Mitra, 2013). Entre ellos, los nano-transportadores pueden mejorar la biodisponibilidad peptídica evitando su degradación y mejorando su transporte.

Las moléculas que son capaces de aumentar la permeabilidad pueden mejorar sustancialmente la biodisponibilidad de los péptidos, sin embargo, esto podría permitir que algunas sustancias tóxicas pudieran entrar fácil y rápidamente al torrente sanguíneo, pudiendo provocar problemas para la salud. En un estudio en animales se utilizó caprato de sodio para incrementar la permeabilidad de los péptidos IPP y LKP a través de la inhibición de PepT1 (Gleeson et al., 2018). Los nano-transportadores y los péptidos de penetración celular podría ser herramientas poderosas a tener en cuenta en el futuro para mejorar el transporte de péptidos con baja permeabilidad a través de las células epiteliales del intestino.

2. RESULTADOS

2.1 Publicación I: **Digestion of micellar casein in duodenum cannulated pigs. Correlation between *in vitro* simulated gastric digestion and *in vivo* data**

FOOD CHEMISTRY, article in press.

2.2 Publicación II: **Peptidomic data in porcine duodenal effluents after oral administration of micellar casein**

DATA IN BRIEF, aceptado para publicación

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Digestion of micellar casein in duodenum cannulated pigs. Correlation between *in vitro* simulated gastric digestion and *in vivo* data

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ABSTRACT

Correlation and validation of the results of simulated gastrointestinal digestion of food compounds towards *in vivo* data is essential. The objective of this work was to monitor the digestion of milk micellar casein in the porcine upper intestinal tract and to match the outcome with the gastric *in vitro* digestion following the Infogest harmonized protocol. In pig duodenum, small amounts of intact caseins were present in all samples, while caseins were observed up to 60 min of gastric *in vitro* digestion. The peptide profile generated after *in vitro* and *in vivo* digestion showed clear similarities with specific overrepresented regions rich in proline and other hydrophobic residues. The statistical comparison of the *in vivo* and *in vitro* peptidome resulted in satisfactory correlation coefficients, up to 0.8. Therefore, the *in vitro* protocol used was a robust and simple model that provides a similar peptide profile than that found in porcine duodenum.

1. Introduction

Food, after being ingested, suffers a series of complex physical and chemical modifications during digestion that will originate the release and absorption of nutrients. Food proteins are hydrolyzed by gastric and pancreatic enzymes, and epithelial brush border membrane peptidases into a complex mixture of peptides and free amino acids that transit and are progressively absorbed along the small intestine (Goodman, 2010). In fact, several released peptides during the digestion of proteins could entail physiological implications, acting as signals in regulatory functions of the organism (Rutherfurd-Markwick, 2012; Santos-Hernández, Miralles, Amigo, & Recio, 2018) or pose a toxicological risk through the formation of epitopes capable of inducing allergic reactions (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015).

In this context, it is critical to know the changes that food proteins undergo in the stomach in relation to degradation and the identification of resistant protein regions during the digestive process. Gastric acid denatures and unfolds food proteins to make them more accessible to the action of pepsin. This endopeptidase has a higher specificity for hydrolyzing peptide bonds C-terminal to aromatic amino acids and leucine, and degrades 10 to 15% of the common dietary proteins into oligopeptides (Antonov, Rumsh, & Tikhodeeva, 1974; Luo, Boom, & Janssen, 2015). However, the behaviour of food proteins and susceptibility to the action of pepsin varies with the protein source depending mainly on amino acid sequence, tertiary structure and post-translational modifications (Wang, et al., 2017). While globular and compact proteins like β-lactoglobulin are resistant to the action of this gastric enzyme, other proteins with a looser structure like milk caseins undergo disaggregation of micellar structure with submicelle formation and clotting, and are hydrolysed by the action of pepsin into smaller polypeptides (Dallas et al., 2017). Due to clotting in the stomach, transit rate to the intestinal tract of milk casein is delayed with regard to other food proteins. This clotting/aggregation behavior of caseins is also influenced by the manufacture conditions of the product including heat treatment, drying or gelation. In a previous study with mini-pigs cannulated at duodenum and jejunum, it was found that the structure of dairy products (stirred or not stirred gels and rennet or acid gels) strongly affected gastric kinetics of protein digestion (Barbé, Le Feunteun, et al., 2014). It was proposed that milk rennet gels had longer residence time at the stomach due to the formation of a coagulum with higher stiffness than acid gels (Barbé, Ménard, et al., 2014). The coagulation behavior and kinetics of protein hydrolysis of different milk protein ingredients has been evaluated by using an *in vitro* dynamic model. The formation of different structured curds under gastric conditions conditioned the course of protein digestion with skim milk

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powder exhibiting slower hydrolysis rate than sodium caseinate (Wang, Ye, Lin, Han, & Singh, 2018). Similarly, it has been reported that the consistency of the casein coagulum from UHT milk was softer than that formed from pasteurized milk and this affects protein hydrolysis at the end of the gastric digestion (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019). Casein obtained by isoelectric precipitation or by enzymatic hydrolysis with chymosin has long been used as a popular food ingredient in many applications. A more recently adopted technology consists in milk microfiltration and diafiltration to obtain products with high protein content where 85–95% corresponds to casein. This micellar casein is successfully applied in cheese making and in high-protein nutritional and sport beverages, among others. Although the technological properties of micellar casein powders and concentrates have been evaluated (Crowley et al., 2018), the kinetics of protein hydrolysis in the stomach has not been attempted.

Pig is an animal model well suited to predict protein digestibility in humans (Deglaire, Bos, Tomé, & Moughan, 2009). This model has been previously employed for studying the resistance of selected milk protein fragments in the neonate (Bouzerzour et al., 2012) or the kinetics of protein digestion with regard to the heating or gelation process (Barbé et al., 2013). Although methods evaluating *in vivo* digestion, both in humans or animals, contribute the most to get relevant physiological results, in order to give a general prediction of any nutrient digestibility, it is necessary to develop *in vitro* models of gastrointestinal simulation (Bohn et al., 2018). *In vitro* methods employ less manpower and are expected to reduce the variation associated with inter-individual variability. The development of the harmonized model of digestion in the Infogest network based on physiologically relevant conditions provides a consensus set of conditions that has already proved to produce reproducible results in inter-laboratory trials (Egger et al., 2016). This *in vitro* digestion protocol provides also comparable results with skim milk powder digestion in pigs (Egger et al., 2017) and with casein and whey protein digests obtained in human jejunum (Sanchón et al., 2018). The suitability of the protocol to mimic the behaviour of micellar casein in the upper intestinal is hypothesized.

Therefore, the specific objectives of this work were: (a) to study the behaviour of milk micellar casein along the digestion process in the upper intestinal tract through the use a porcine model cannulated in duodenum, an experimental design that permits to collect early duodenal effluents in the course of the stomach emptying and (b) to compare the casein degradation products upon the application of the Infogest harmonized digestion protocol to those delivered *in vivo* in order to evaluate the digestion model.

2. Materials and methods

2.1. Samples and reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Commercial micellar casein was purchased from Ingredia S.A, Arras, France (Prodiet 85B, batch no. 141179). Its protein content was 77.84% measured by Kjeldahl.

2.2. Animal experiments

All procedures were in accordance with the European Community guidelines for the use of laboratory animals (L358-86/609/EEC). The study was approved by the local committee for ethics in animal experimentation. The animals were 10 pigs Large White × Landrace × Piétrain of about 40 kg live weight. Two weeks before starting the trials, they were surgically fitted with a T-shaped cannula in the duodenum (10 cm downstream from the pylorus). Following the operation, the animals were housed in individual slatted pens within a ventilated room with controlled temperature (21 °C). During convalescence following the surgical procedure and also between the sampling days, pigs were fed with 800 g/d of a pig feed concentrate (Cooperl Arc Atlantique®, Pelstan, France) containing 16% proteins, 1% fat, 4% cellulose and 5% mineral matter. Animals had free access to water.

Within the experimental period (two weeks), the days of sampling were separated by at least 2 days. Test meal (casein powder reconstituted in water 5% (w/v) in sufficient quantity to cover the protein daily requirements of 50 g/kg) was offered to the pigs for 10 min. Chromium-EDTA (110.8 ppm, w/w) was added to the meal as marker of the liquid phase, as previously described (Barbé et al., 2013). The animals had no access to water from 1 h before to 7 h after the meal delivery. Duodenum effluents were collected in plastic bottles 15 min before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after ingestion of test meal. The sampling was stopped when 40 mL were collected or after a maximum of 3 min of sampling time. A protease inhibitor (Pefabloc®, Sigma-Aldrich, St. Louis, MO, USA at 2.5 mM final concentration) was added to the collecting bottles. The effluents were weighed and freeze-dried.

2.3. In vitro simulated digestion

Casein was digested following the oral and gastric phase of the *in vitro* harmonized protocol (Brodkorb et al., 2019). The protein concentration in the simulated oral fluid was 6% (w/v) and human amylase was not used in this step due to the absence of starch in the substrate. Gastric phase was initiated by the addition of pepsin from porcine gastric mucosa (EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA) mixed in simulated gastric fluid (2000 U/mL of digesta), at a ratio of 50:50 (v/v). Samples were collected at different times of digestion (10, 20, 30, 60, 90 and 120 min) and the reaction was stopped by adjusting the pH to 7 with NaOH 1 M. Digests were snap freezed in liquid nitrogen, freeze-dried and kept at −20 °C until analysis.

2.4. Analysis of digests

2.4.1. Chromium and nitrogen analysis in duodenal effluents

The chromium content was determined using an inductively coupled plasma mass spectrometry (ICP-MS) instrument NexION 300XX (Perkin-Elmer, Waltham, MA, USA). Nitrogen content was determined by elemental analysis in a LECO CHNS-932 analyzer. The analyses were performed at the Mass spectrometry and Elemental analysis units from the Interdepartmental Investigation Service from the Autonomous University of Madrid.

2.4.2. Protein characterization by SDS-PAGE, in-gel digestion and identification.

Protein load of duodenal effluents and *in vitro* gastrointestinal digests was adjusted to 1 mg of protein/mL based on the nitrogen content of the samples determined by elemental analysis. Freeze-dried digests were treated as previously reported (Cruz-Huerta, García-Nebot, Miralles, Recio, & Amigo, 2015). A molecular weight marker (Precision Plus Protein™ Unstained standard, Bio-Rad Laboratories, Hercules, CA, USA) and undigested casein (0.8 mg of protein/mL) were included on each gel. Thereafter, the 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 Laboratories, Hercules, CA, USA) and processed with Quantity One®1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Coomassie Blue-stained bands were manually excised and decolored with 25 mM ammonium bicarbonate and acetonitrile (1:1; v:v). Then, the bands were reduced with 10 mM 1,4-dithiothreitol (DTT, Sigma-Aldrich) in 25 mM ammonium bicarbonate for 1 h at 56 °C prior to 30 min incubation with 55 mM iodoacetamide at room temperature. Bands were washed (×2) with 25 mM ammonium bicarbonate: acetonitrile (1:1; v:v) and then dehydrated in 100% acetonitrile. The in-gel digestion was carried out at 37 °C, overnight, with trypsin (V5117, Promega Biotech Ibérica S. L., Spain) at a final concentration of

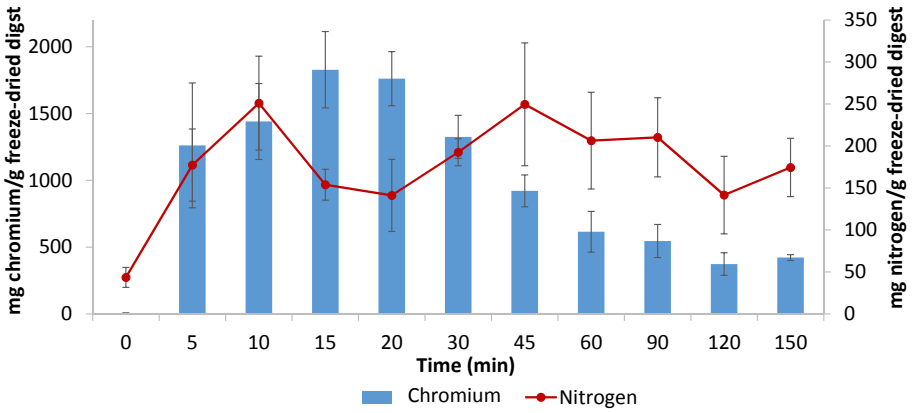

Fig. 1. Chromium-EDTA concentration (blue bars) and nitrogen content (red trace) of pig duodenal effluents from 5 to 150 min of digestion after oral administration of caseins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

12.5 ng/μL in 25 mM ammonium bicarbonate. Soluble peptides were recovered by centrifugation at 7000 × *g* for 1 min and bands were washed twice with acetonitrile: 10% formic acid (1:1; v:v) and centrifuged; the supernatants were merged and dried using a Speed Vac prior to reconstitution in acetonitrile 33% containing trifluoroacetic acid 0.1%. An Autoflex SpeedTM (Bruker Daltonics, Bremen, Germany) was used. Samples were spotted into a MALDI target plate with α-cyano-4-hidroxycinnamic acid matrix. Ions were detected in positive reflection mode and were collected from the sum of 100–600 lasers shots. Protein calibration Standard I, (Bruker Daltonics) was employed for external calibration of spectra. Monoisotopic peaks generated using FlexAnalysis software were fragmented using the lift method for MS/MS analysis. For peptide identification the MASCOT v2.4 software (Matrix Science) Server 2.1 and Biotools version 2.1 (Bruker Daltonics) were used.

2.4.3. Peptidomic analysis

Freeze-dried digests were reconstituted in solvent A (water:formic acid, 100:0.1, v/v) and centrifuged at 13000 × *g*, 10 min. 50 μL of supernatant was injected for each sample. Additionally, samples were submitted to a selective precipitation by adding calcium chloride (1% w/v) and ethanol (50% v/v) to prepare an enriched phosphopeptide fraction (Sánchez-Rivera et al., 2014).

HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranea Sea C_{18} column (150 × 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. A linear gradient from 0 to 45% of solvent B (acetonitrile:formic acid, 100:0.1, v/v) in 120 min was performed for peptide elution and the flow was set at 0.2 mL/min. Spectra were recorded over the mass/charge (*m/z*) range 100–1200 and the target mass was set at 900 *m/z*.

Data processing was done by using Data Analysis (version 4.0, Bruker Daltonics GmbH, Bremen, Germany). Peptide identifications were obtained using the MASCOT v2.4 protein search engine (Matrix Science) against a homemade database of major genetics variants of bovine caseins. A homemade database of porcine digestive enzymes was also used (Tables S1 and S2 in Supplementary material, respectively). No enzyme was selected. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. No post-translational modifications were considered. The matched MS/MS spectra were interpreted by using Biotools version 3.2 (Bruker Daltonics). The relative abundance of peptides was that provided by the software output. A manual revision was done for each identification peptide spectrum, regardless of its *P* value. The analysis of enzymatic cleavages was performed in the peptide lists by EnzymePredictor tool (Vijayakumar et al., 2012).

2.4.4. Analysis of free amino acids

Protein from freeze-dried digests was precipitated with 4-sulphosalicylic acid (12.5 mg/mg of protein) by allowing to stand 1 h on ice. Centrifugation for 15 min at 15000 × *g*, 4 °C, was followed by filtration of the supernatant by 0.45 μm membranes and adjusting to pH 2.2 with 0.3 M NaOH. Amino acid analysis was performed on a Biochrom 30 amino acid analyser (Biochrom Ltd, Cambridge, UK). Results were expressed as mmol of amino acid per kg of digest. Under our analysis conditions, tryptophan, glutamine and asparagine are not determined.

2.5. Statistical analysis

Spearman correlation matrices were built on the basis of frequency of appearance of amino acids belonging to peptides, ranging from 1 to 30. Statistica software (StatSoft Incl., Tulsa, OK, USA) was used for the calculations.

3. Results and discussion

3.1. Digestion kinetics and protein degradation in vivo

Chromium was added to the casein meal as marker of the liquid phase. Its concentration in the digests underwent an increase up to 20 min and decreased to a steady level from this time (Fig. 1). A return to the basal values could not be observed in the analysed time interval. This is consistent with previous reported data in pig after consumption of a normal diet, where the liquid marker remained in the stomach 300 min post-ingestion with a half time of 48.8 min (Chiang, Croom, Chuang, Chiou, & Yu, 2008). The increased concentration in chromium-EDTA between 5 and 30 min corresponded to the rapid emptying of soluble compounds. However, the protein content of the effluents reached a maximum at shorter time points (around 10 min), decreased up to 20 min, and a second maximum was reached at 45 min. This bimodal curve for the protein content of the effluents is compatible with the gastric emptying of soluble whey proteins present in small amounts in the casein product at short time points and the draining of casein degradation products at longer times.

The protein in the duodenal effluents was characterized by SDS-PAGE. Fig. 2A shows a representative electrophoretic profile of successive duodenal samples taken from a single animal. The oral administered micellar casein also contained β-lactoglobulin (18 kDa), α-lactalbumin (14 kDa) and several bands corresponding to casein fragments with MWs between 10 and 15 kDa, as identified by in-gel digestion and MALDI TOF/TOF analysis. Table S3 in Supplementary data summarizes the identified proteins and protein fragments. Most intense

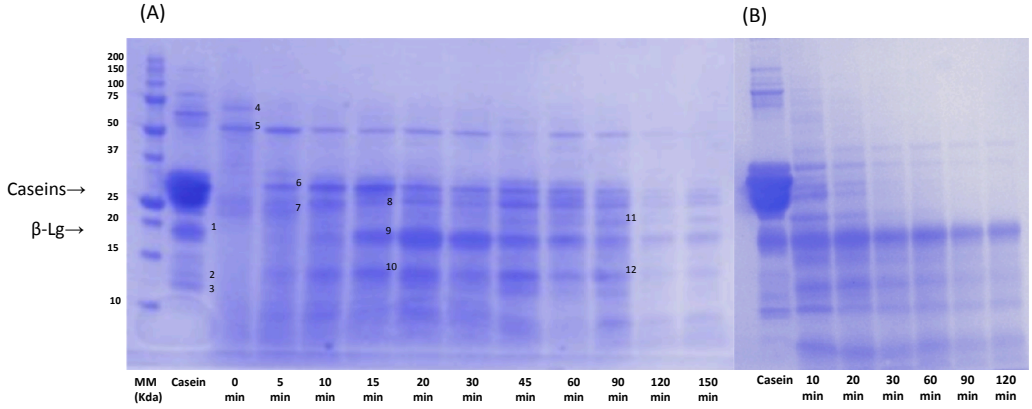

Fig. 2. SDS-PAGE profile of (A) *in vivo* and (B) *in vitro* casein digests. Each lane in A corresponds to an effluent sampling time. Each lane in B corresponds to a gastric digestion time. MM, molecular weight marker. Identified bands:1: β-lactoglobulin, 2: β-casein fragment, 3: β-casein fragment, 4: Porcine serum albumin, 5: Porcine pancreatic α-amylase, 6: α_{S1}-casein + trypsin, 7: α_{S1}-casein fragment + chymotrypsin, 8: κ-casein, 9: β-lactoglobulin, 10: κ-casein + α_{S1}-casein fragment, 11: α_{S1}-casein fragment, 12: κ-casein fragment.

electrophoretic bands observed in the effluents taken 15 min before oral feeding (lane 0), corresponded to porcine serum albumin and pancreatic α-amylase (Fig. 2A). Low amounts of non-degraded caseins could be identified in the 15 to 120 min range, with time points 45, 60 and 90 min showing particular abundance of caseins. Small traces of undigested caseins were also found in duodenum of cannulated pigs at short times after the oral administration of milk by Barbé, Le Feunteun et al. (2014). A study in pigs did not detect intact casein in duodenum after administration of reconstituted skim milk powder, although in that case the total duodenum content was analysed instead duodenal effluents (Egger et al., 2017). In addition to the different sampling method, cannula *vs* intestinal contents, the nature of the product could influence clotting and solubility of the sample. A fraction of the colloidal calcium phosphate may be dissolved during the manufacturing process of the micellar casein used in our study resulting in looser and smaller micellar structure (Wang et al., 2018). In addition, clotted casein is exposed to the action of gastric pepsin and empties slowly from the stomach in the form of degraded products, as reflected in the protein content of the effluents (Fig. 1) and in the bands between 10 and 20 kDa identified as casein fragments SDS-PAGE by in-gel digestion and MALDI-TOF/TOF analysis (Supplementary data).

Moreover, all digests contained bands with MW < 10 kDa corresponding to small size peptides which were identified by HPLC-MS/MS (ion trap, see Section 3.2). The electrophoretic band corresponding to β-lactoglobulin was also present at short time points (up to 30 min) being emptied with soluble phase and showing a similar behaviour than chromium. This indicates that the small amount of β-lactoglobulin present in the casein substrate resists the first digestion stage in the stomach, as it was found in the early duodenal effluents.

3.2. Peptide and amino acid composition in duodenal effluents

Peptide sequencing was conducted by HPLC-MS/MS by matching with the main casein genetic variants. All identified peptide sequences in the effluents collected at different time points for each individual are detailed in Miralles et al., Data in brief, co-submitted with this manuscript. On the whole, the 652 distinct casein-derived peptides identified over digestion time are listed in the Supplementary Excel file. Their distribution for β-, α_{s1}-, α_{s2}-, and κ-casein was 32, 29, 18 and 20%, respectively. Peptide size ranged between 5 and 34 amino acid residues and peptides masses between 520 and 3502 Da. The identified peptides were mapped and aligned on their parent protein sequences with the Peptigram tool (Manguy et al., 2017). Phosphorylated regions are not included in this mapping since they were analysed separately in an enriched fraction, given their lower ionization capacity. Fig. 3A and 3B show the β- and α_{s1}-casein derived peptides found in the duodenal digests over the sampling time. Peptides from α_{s2}- and κ-casein are shown in Fig. 4. Each vertical bar corresponds to an amino acid, the peptide abundance is represented by the height of the bars, while the intensity of the green colour is proportional to the intensities of overlapping peptides. The protein coverage ranged from 76 to 94% in the case of β-casein, and from 70 to 91% in the case of α_{s1}-casein. For all proteins, a rather similar pattern of peptides with regard to time could be observed, which suggests certain homogeneity of the outflowed material from the stomach. In fact, peptides showed a similar size at the different collected time points. Around 70% of peptides comprised < 10 amino acids, which reflects the susceptibility of caseins to pepsin. For β- and α_{s1}-casein, an important number of peptides with high intensity were found at the C-terminal region of the protein, which may correlate with peptide abundance and/or the ionization capacity of the peptides belonging to these domains. In addition, peptides from regions 24–40 and 53–64 from α_{s1}-casein were abundant in duodenal effluents at all-time points. Lower protein coverage was observed for α_{s2}- and κ-casein, ranged 51–78% and 60–83%, respectively. Those regions with a higher number of overlapping peptides are between residues 89 and 124 from α_{s2}-casein, residues 31 to 89 from κ-casein, and the C-terminal region of κ-casein. The lower coverage observed, especially in the *N*-terminal part of these two proteins can be due to the absence of charged residues in this part of both proteins chains, as compared with α_{s1}- and β-casein.

The analysis of phosphorylated peptides was performed separately in an enriched Ca/ethanol fraction prepared from the duodenal effluents. Several phosphorylated fragments from α_{s1}- and α_{s2}-, β-, and κ-casein could be identified (Fig. 5). In the case of β-casein, the region covered by most phosphopeptides corresponded to ^{30}IEKFQSpEEQQQ^{40}, comprising phosphorylated serine 35. In α_{s1}-casein, the determined sequences covered the three regions where phosphorylations occur, including the phosphorylation sites 46, 48, 64, 66–68, 75, and 115. Other phosphorylated fragments were identified from α_{s2}- and κ-casein. There are only scarce reports on the appearance of phosphorylated peptides in digests from *in vivo* assays. Nevertheless, fragments ^{7}NVPGEIVESpLSpSpSpE^{20} and ^{7}NVPGEIVESpLSpSp^{18} from β-casein had been previously found in human duodenum after milk ingestion after 20 and 40 min, respectively (Chabance et al., 1998). Various sequences comprised in these regions have also been identified in human jejunum effluents (Sanchón et al., 2018) or have proven to be bioavailable after consumption of cheese (Caira et al., 2016). These peptides are expected to form soluble complexes with different minerals such as calcium, zinc, and iron with a positive role in mineral absorption (Cross et al., 2007). Although the role of casein phosphopeptides needs to be elucidated, the present study showed the release of, at least, 70 phosphorylated fragments from casein at early times of intestinal digestion in pig after casein intake.

Most peptides found in cannulated duodenum were compatible with the action of pepsin with a high number of cleavages explained by the action of this enzyme, as calculated by using the EnzymePredictor tool (results not shown). Some of the identified sequences had been previously reported as biologically active peptides (Table S4 in

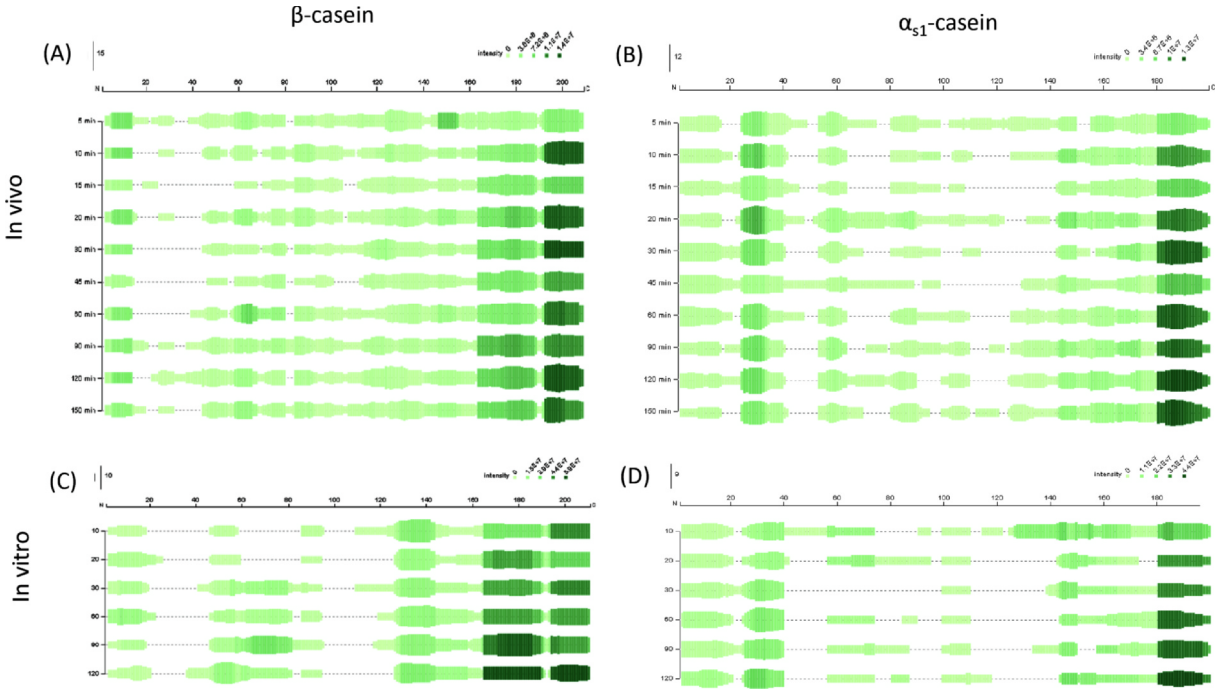

Fig. 3. Peptides derived from β- and α_{s1}-casein in the duodenal (*in vivo*, A, B) and *in vitro* (C, D) digests over sampling time (5 to 150 min) identified by HPLC-MS/MS. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence. Peptide overlapping is represented by the height of the bars while the intensity of the green colour is proportional to the sum of intensities of overlapping peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Supplementary Material). For instance, various peptides found in the digests at all times had been previously described as opioid peptides, such as fragments β-casein ^{114}YPVEPF^{119} (Jinsmaa & Yoshikawa, 1999), α_{s1}-casein ^{144}YFYPEL^{149} (Fernández-Tomé et al 2016), and κ-casein ^{33}SRYPSY^{38} (Yoshikawa et al., 1994). Thirteen sequences reported as antibacterial or antimicrobial were identified in nearly all samples, while antioxidant, antihypertensive or immunomodulatory peptides were observed at certain collection times. Other activities reported for the identified peptides include ACE, DPP-IV and Prolyl-endopeptidase inhibition, increased mucin genes expression, bradykinin potentiation and antithrombin. Some of these peptides might exert their activity by their interaction at the intestinal level (i.e.: opioid, antioxidant), while others are expected to be absorbed to yield their known biological effect (i.e.: antihypertensive, immunomodulatory).

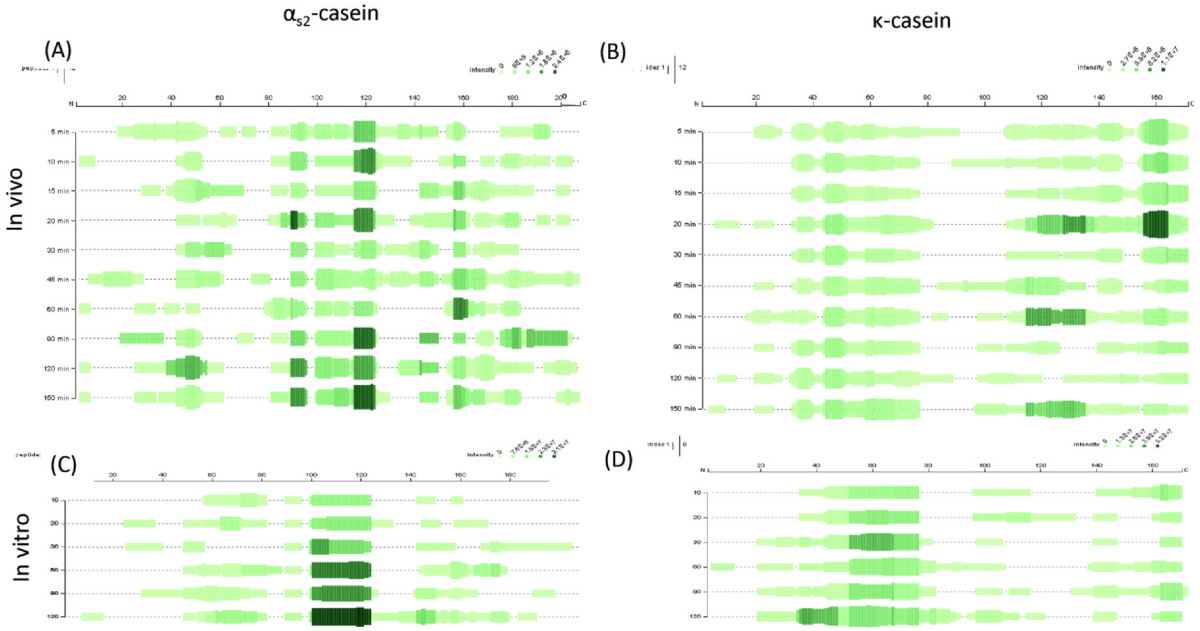

Fig. 4. Peptides derived from α_{s1}- and κ-casein in the duodenal (*in vivo*, A, B) and *in vitro* (C, D) digests over sampling time (5 to 150 min) identified by HPLC-MS/MS. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence. Peptide overlapping is represented by the height of the bars while the intensity of the green colour is proportional to the sum of intensities of overlapping peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Caseinophosphopeptides from β-, α-, and κ-caseins identified in the pig duodenum digests. X, denotes a phosphorylated serine residue.

Irrespective of this aspect, the rate of appearance of peptides raises the question on the role of the concentration of selected protein fragments with biological activity during digestion. A continuous flow might permit to reach a peak concentration able to overcome the limit threshold and trigger a physiological effect.

In order to portray the global composition of the material coming out from the stomach the profile of free amino acids was also studied over time (Fig. 6). Since this determination was conducted after normalization of the digests by nitrogen content, the average concentration trend is representative of the proportion of amino acids and small peptides in the total protein fraction in the digest. Time point zero in the graph corresponds to free amino acids determined in the effluent taken 15 min prior casein feeding. The global concentration in the samples taken after casein supplementation ranged between 50 and 140 mmol/kg and represented from 0.3 to 1% of the total nitrogen content; a small proportion if compared with the reported amount at the end of the small intestine, 30% (Goodman, 2010). Results in the same order of magnitude have been previously shown for milk proteins in pig stomach and duodenum (Egger et al., 2017). Regarding the amino acid distribution, the duodenal content before casein ingestion showed a marked abundance of glycine followed by alanine and leucine. In contrast, after casein intake, the pattern changed to high occurrence of phenylalanine followed by leucine, lysine, and tyrosine, with average relative shares of 30, 13, 12, and 10%, respectively (Fig. 6). The rest of amino acids were present at much lower amounts while proline concentration was below 0.2 mmol/kg in the duodenal effluents after casein ingestion. In the analysis of the amino acid composition of the identified peptides, it was observed that proline was the most abundant amino acid (Miralles et al., Data in brief). This suggests that this amino acid remains preferentially in the form of peptides. Only minor changes in the amino acid abundance distribution could be detected over time, which is consistent with the mentioned homogeneous composition of the material outflowed from the stomach.

3.3. *In vitro* simulated digestion. Comparison with *in vivo* data

The *in vitro* oral and gastric digestion of the same substrate was performed according to the standardized Infogest protocol (Brodkorb et al., 2019). *In vitro* gastric digestion of the micellar casein product was tracked by stopping the reaction at 10, 20, 30, 60, 90 and 120 min. Considering that the duodenal effluents are representative of the gastric contents, these were compared with the end point of the *in vitro* gastric

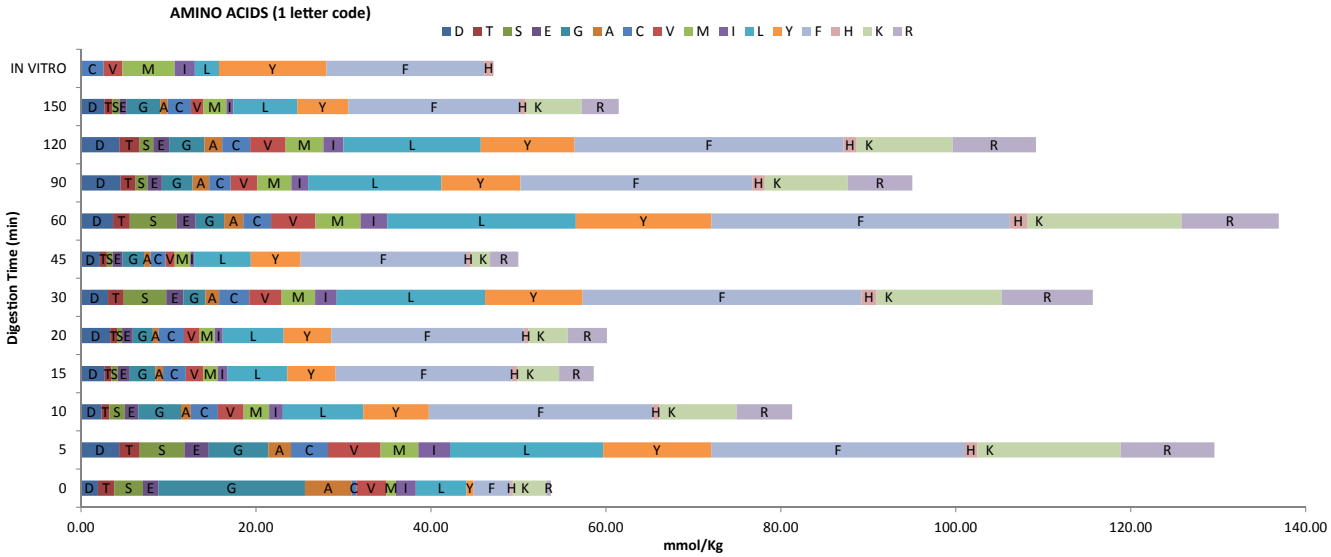

Fig. 6. Distribution of free amino acids in the duodenal (5–150 min) and *in vitro* digests of caseins at 120 min. Tryptophan, glutamine and asparagine were not determined. * Proline showed a value under 0.2 mmol/kg and is not represented.

digestion of the same substrate. The electrophoretic bands corresponding to caseins could be clearly detected up to 20 min of the *in vitro* gastric digestion (Fig. 2B). After that time, although faint bands of caseins were still distinguishable at 30 and 60 min, a profile dominated by bands of whey proteins and small molecular weight compounds, together with digestive enzymes was found. The *in vitro* digestion electrophoretic pattern reflects the rapid degradation of caseins and the resistance of β-lactoglobulin to the action of pepsin. The absence of intact casein and the presence of the β-lactoglobulin band in the *in vitro* gastric electrophoretic pattern fits the duodenal effluents outflowed during the first 30 min (Fig. 2A).

The *in vitro* gastric digests were analysed by HPLC-MS/MS under the same conditions that the duodenal contents and the set of identified protein fragments were mapped to their parent proteins with the Peptigram tool over sampling time. As observed in the *in vivo* effluents, the more abundant and intense peptides belonged to the N- and, especially, the C-terminal domains of β- and α_{s1}-casein. In the *in vitro* digests, peptides from regions 41–59 and 122–144 from β-casein and 20–40 from α_{s1}-casein were especially abundant which matched with the peptides found in duodenal contents (Fig. 3 C,D). In the case of α_{s2}-casein, the region 99–124 was remarkably intense while in κ-casein the 52–76 and the C-terminal regions comprised a higher number of peptides, in good correspondence with the *in vivo* pattern, despite a lower number of distinct identified sequences (285) (Fig. 4).

The frequency of appearance of each amino acid as part of β-casein and α_{s1}-casein-derived peptides were used to contrast *in vitro* with *in vivo* results. The comparison of different time points of digestion provided correlation coefficients between 0.66 and 0.79 at the final point of gastric digestion, while the correlation coefficient average between animals ranged from 0.51 to 0.75. The application of this statistical analysis for the comparison of peptidomic results was previously used to evaluate an interlaboratory trial with the Infogest digestion protocol (Egger et al., 2016) or to compare peptides found in human jejunum with those released by this *in vitro* digestion protocol (Sanchón et al., 2018). As expected, the amino acid composition of the *in vivo* and *in vitro* identified peptides was also similar (Miralles et al., Data in brief, submitted). The most abundant amino acid in peptides identified both *in vivo* and *in vitro* is proline reaching a 20%, followed by valine, leucine, glutamic acid, and glutamine, which ranged from 11 to 6%.

The distribution of free amino acids at 120 min of *in vitro* digestion was compared with those determined in the *in vivo* digests (Fig. 6). The total concentration of free amino acids in the *in vitro* gastric digests reached on average 50 mmol/kg, an amount at the lower limit of values observed in the duodenum after casein consumption. This can be attributed to the absence of endogenous amino acids that are present in the *in vivo* samples or to the lack of enzymatic activities giving rise to single amino acids. With regard to amino acid composition, the most abundant free amino acids, phenylalanine and tyrosine, matched with the *in vivo* effluents, whereas lysine, leucine, and arginine were found at lower concentration in the *in vitro* digests. It has been reported that arginine, lysine, tyrosine and phenylalanine are the most readily liberated free amino acids, while cysteine and proline were the least digested in four protein sources comprising casein, cod protein, soy and gluten. The different occurrence of these amino acids had been observed after the sequential hydrolysis with pepsin and pancreatin (Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005). In the present study the pattern is displayed from the early duodenal content in pigs and largely matches with the application of the simulated gastric phase.

4. Conclusion

The analysis of the duodenum effluents shows the behavior of caseins in the stomach, with a nearly emptying of low protein-liquid phase which corresponds to the initial clotting of caseins. With regard to peptide release, many resistant sequences that include physiologically active fragments have been identified and monitored during gastric emptying. On the other hand, the determined free amino acids in the digests ranged from 60 to 140 mmol/kg and showed a distinctive pattern with regard to the basal one, although the fraction of free amino acid represented < 1% of the total nitrogen content. From the point of view of protein degradation, it is only possible to establish a correspondence between the end point of the gastric phase and the *in vivo* profile at certain points. In this case, kinetic studies where emptying of the stomach is considered, should better mimic the evolution of the protein content. In view of the similarities, this *in vitro* gastric protocol could be accepted to simulate protein degradation at duodenal level.

CRediT authorship contribution statement

B. Miralles: Investigation, Formal analysis, Writing - original draft. **J. Sanchón:** Investigation, Formal analysis. **L. Sánchez-Rivera:** Investigation, Formal analysis. **D. Martínez-Maqueda:** Investigation, Formal analysis. **Y. Le Gouar:** Investigation, Formal analysis. **D. Dupont:** Conceptualization, Supervision. **L. Amigo:** Writing - original

draft. **I. Recio:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128424.

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Peptidomic data in porcine duodenal effluents after oral administration of micellar casein

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ABSTRACT

The data in this article are related to the Research publication “Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data” (Miralles et al., co-submission to Food Chemistry). Pig duodenum effluents were collected with a T-shaped cannula 15 min before and during digestion over 150 min after casein intake. The casein degradation profile of individual pigs during digestion is presented. All identified peptide sequences at different digestion times for six subjects are provided. The peptide profile of digests in the form of heat maps is shown for α_{s1}-, α_{s2}-, β- and κ-casein. The sum of amino acids belonging to peptides released from β- and α_{s1}-casein has been used to determine correlation coefficients and range the inter-individual variability. Finally, the global amino acid composition, isoelectric point and sequence length of all released peptides has been determined.

Keywords: Digestion, pig duodenum, micellar casein, peptidomic analysis

Specifications Table

Subject	Food Science
More specific subject area	Proteomics and biochemistry
Type of data	Table, Figures
How data was acquired	PAGE-SDS + imaging (VersaDoc™ MP 5000 system, Bio-Rad Laboratories) RP-HPLC-MS/MS (Ion-trap Esquire 3000 (Bruker Daltonics)
Data format	Raw and analyzed

Parameters for data collection	Six Large White × Landrace × Piétrain adults pigs of about 40 kg live weight were fitted with a T-shaped cannula surgically placed 10 cm downstream from the pylorus to collect duodenum effluents 15 min before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after ingestion of micellar casein.
Description of data collection	LC-MS/MS files were processed with Data analysis and Biotools (Bruker Daltonics). Sequencing was performed with Mascot. The frequency of appearance of each amino acid identified as part of a peptide sequence was used to build heat maps. Spearman correlation coefficient calculated with the frequency of appearance of each amino acid was used to show the inter-individual variability. In the identified peptides the amino acids composition was calculated by using ProtParam tool (Expasy) and isoelectric point and number of amino acids were ranged using Peptide Analyzer (Thermofisher Scientific).
Data source location	Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM) Madrid, Spain
Data accessibility	With the article
Related research article	B. Miralles, J. Sanchón, L. Sánchez-Rivera D. Martínez-Maqueda Y. Le Gouar, D. Dupont, L. Amigo, I. Recio. Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data. Food Chem. DOI, submitted.

Value of the data

- The duodenal contents in the pig can be representative of micellar casein digestion in humans
- Peptide sequences released in the upper intestinal tract which may have physiological implications, are available for future assays
- Illustration of the inter-individual variability of the duodenal contents after casein digestion.
- These *in vivo* data are useful to validate static and dynamic gastrointestinal simulations in terms of protein degradation and peptide formation

Data description

Figure 1 shows the SDS-PAGE protein profiles at duodenum during pig digestion of casein in three different individuals. Intact caseins, whey proteins, degradation products thereof and digestive enzymes were identified by in-gel digestion and MALDI-MS/MS in the electrophoretic profile from one of the subjects [1].

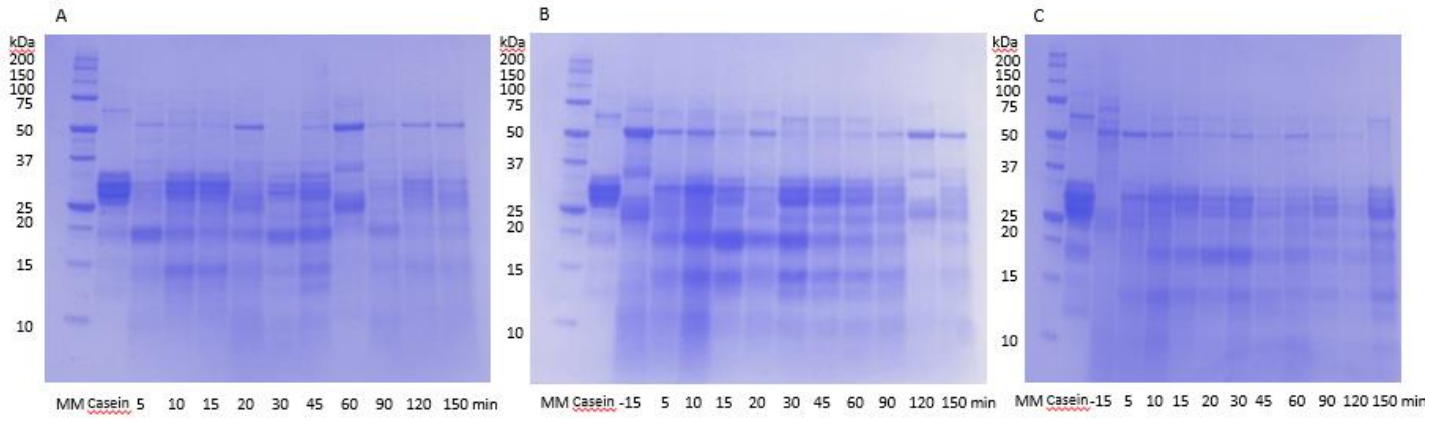

Fig.1. SDS-PAGE of duodenal micellar casein digests from three individual animals. Each lane corresponds to micellar casein or a collected duodenal sample (A) from 5 to 150 min, (B, C) from 15 min before casein ingestion to 150 min.

Peptide sequencing was conducted by HPLC-MS/MS by matching with the main genetic variants from the casein fraction of milk. All peptide sequences identified in each individual and time of sample collection are listed in Dataset 1. In total, 3895 peptides

were sequenced, 1221 from α_{s1}-casein, 346 from α_{s2}-casein, 1489 from β-casein and 784 from κ-casein.

Heat maps built with the frequency of appearance of each amino acid identified as part of a peptide sequence from α_{s1}-, α_{s2}-, β- and κ-casein, after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration are shown in Fig. 2, 3, 4, and 5, respectively. The green color represents low frequency and red high frequency. For α_{s1}-casein and β-casein, an important number of peptides were found at the C-terminal region of the protein. In addition, peptides from regions 24-40 from α_{s1}-casein were also abundant in duodenal effluents at all-time points. For α_{s2}- and κ-casein, those regions with the largest number of identified peptides are around residues 85-90 and 110-120 from α_{s2}-casein, and residues 40 to 50 from κ-casein. Inter-individual variability (Spearman correlation coefficient) calculated with the frequency of appearance of each amino acid identified as part of an identified peptide sequence after micellar casein administration is shown in Fig.6.

The amino acid composition in the identified peptides in duodenal effluents from 6 individuals after 5 to 150 min of micellar casein administration is shown in Fig 7. A high occurrence of proline and glutamic acid (13 and 10%, respectively) is followed by valine and leucine, with average relative shares of 8% each (Fig. 7).

Identified sequences distributed by isoelectric point range are shown in Fig 8. The highest percentage of peptides, 28%, showed isoelectric points in the range from 3 to 4. The rest are widely distributed with relative shares from 3 to 14% at all isoelectric point ranges except range 8 to 9 where less than 1% of peptides are comprised.

Figure 2. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from α_{s1}-casein, after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration.

The green color represents low frequency and red high frequency.

Figure 3. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from α_{s2}-casein, after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration. The green color represents low frequency and red high frequency.

Figure 4. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from β-casein, after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration. The green color represents low frequency and red high frequency.

Figure 5. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from κ-casein, after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration. The green color represents low frequency and red high frequency.

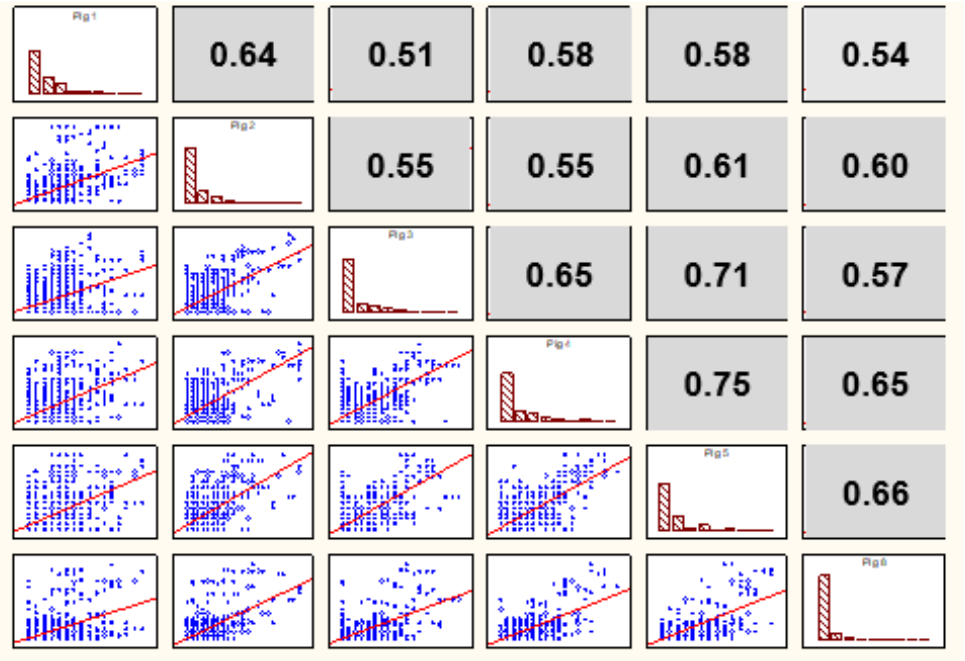

Figure 6. Interindividual variability (Spearman correlation coefficient) calculated with the frequency of appearance of each amino acid identified as part of an identified peptide sequence after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration.

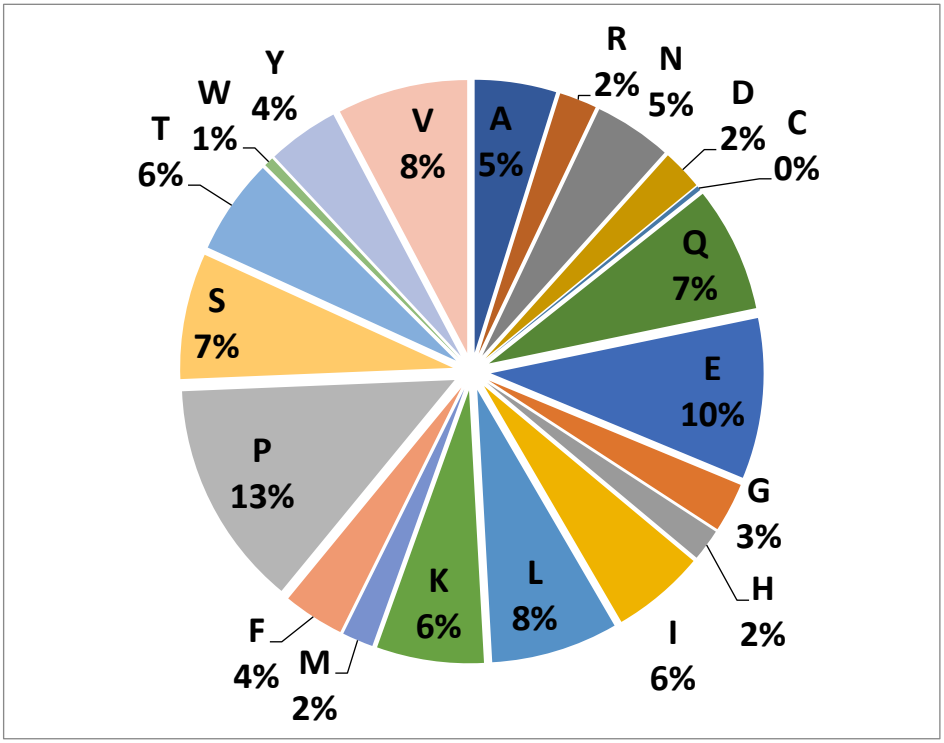

Figure 7. Amino acid composition of the identified peptides in duodenal effluents from 6 individuals after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration.

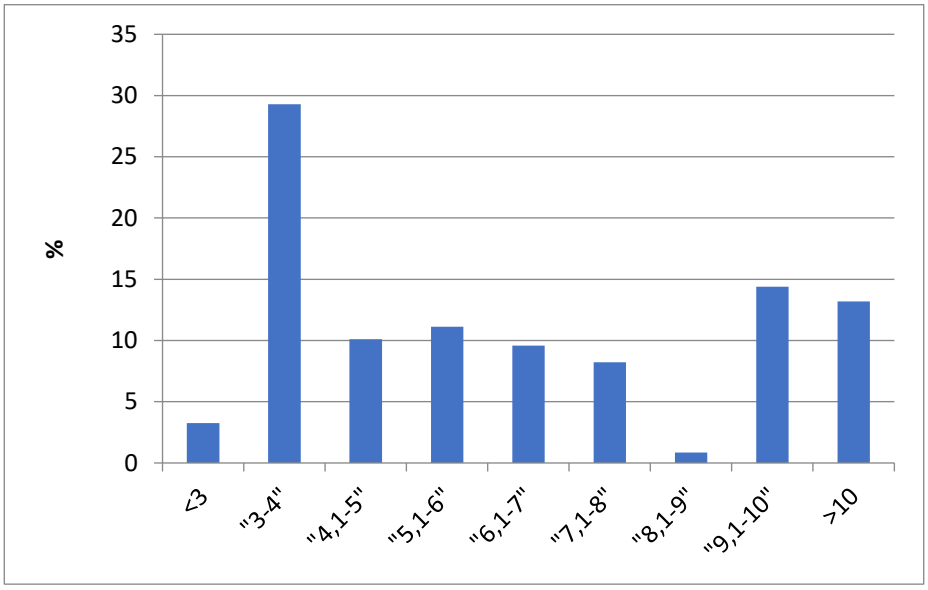

Figure 8. Percentage of identified peptides within each isoelectric point range. Identified peptides in duodenal effluents from 6 individuals after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration.

Fig 9 shows the stratified bar graph with percentage of identified peptides by number of amino acid residues. The group of peptides with a number of amino acid residues between 6 and 10 is the most important, with relative shares between 12 and 16%, while percentages lower than 3.5% were observed for the rest.

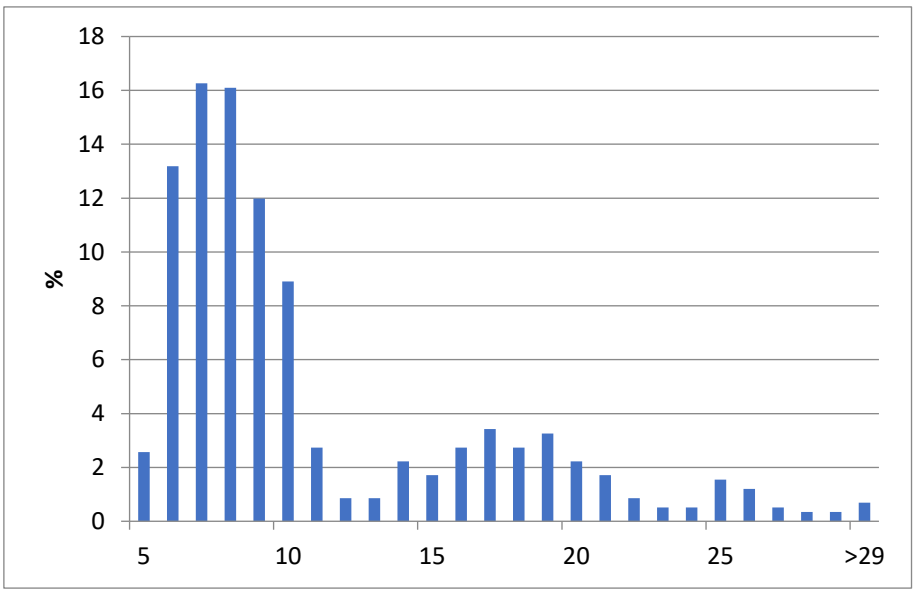

Figure 9. Percentage of identified peptides by number of amino acid residues. Identified peptides in duodenal effluents from 6 individuals after 5, 10, 15, 20, 30 45, 60, 90, 120, and 150 min of micellar casein administration.

Experimental design, materials, and methods

2.1 Animal experiments

The animals were male pigs Large White × Landrace × Piétrain of ca. 40 kg live weight. Two weeks before starting the trials, 10 animals were surgically fitted with a T-shaped cannula in the duodenum (10 cm downstream from the pylorus). After surgery, the animals were housed within a ventilated room with controlled temperature (21 ºC) in individual slatted pens. During convalescence and over the period of the trial, excluding sampling days, pigs received a diet appropriate for their age and physiological state, which consists of 800 g/d of a pig feed concentrate (Cooperl Arc Atlantique®, Pelstan, France) containing 16% proteins, 1% fat, 4% cellulose and 5% mineral matter. Furthermore, they had free access to water.

The trial period lasted two weeks, whereby the sampling phases were separated by at least 2 days. Test meal (casein powder reconstituted in water 5% (w/v) adequate in quantity to cover the protein daily requirements of 50 g/kg) was provided to the pigs for 10min. Chromium-EDTA (110.8 ppm, w/w) had been previously added to the casein preparation as marker of the liquid phase.The animals had no access to water neither 1h before nor 7h after the meal delivery. Duodenum effluents were collected 15min before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after ingestion of test meal in plastic bottles. The sampling was ended when 40ml were pooled or after a maximum of 3 min of sampling time. A protease inhibitor (Pefabloc®, Sigma-Aldrich, St. Louis, MO, USA) at 2.5 mM final concentration) was added to the collecting containers. The digestive contents were subsequently weighed and freeze-dried.

All procedures were in accordance with the European Community guidelines for the use of laboratory animals (L358-86/609/EEC). The study was approved by the local committee for ethics in animal experimentation.

2.2 Samples and reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Commercial micellar casein was purchased from Ingredia S.A, Arras, France (Prodiet 85B, batch no. 141179). Its protein content was 77.84% measured by Kjeldahl.

2.3 Analysis of porcine duodenal effluents

2.3.1 Protein characterization by SDS-PAGE

SDS-PAGE was performed as previously described [2] but protein load of digestive contents was adjusted to 1 mg of protein/mL. Undigested casein was dissolved at 0.8 mg of protein/mL. A molecular weight marker (Precision Plus Protein™ Unstained standard, Bio-Rad Laboratories, Hercules, CA, USA) was included on each gel. Subsequently, the 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 Laboratories, Hercules, CA, USA) and processed with *Quantity One*®1-D analysis *software (Bio-Rad* Laboratories, Hercules, CA, USA).

2.3.2 Peptide identification

Freeze-dried digests were reconstituted in solvent A (water:formic acid, 100:0.1, v/v) and centrifuged at 13000 × *g*, 10 min. 50 µL of supernatant was injected for each sample. Besides, an enriched phosphopeptide fraction from the samples was prepared by selective precipitation with addition of calcium chloride (1% w/v) and ethanol (50% v/v) [2].

HPLC-MS/MS was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), with a Mediterranea Sea C_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain). HPLC system was connected to an Esquire 3000 linear

ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) fitted with an electrospray ionization source. For peptide elution, the flow was set at 0.2 mL/min and a linear gradient over 120 min from 0 to 45% of solvent B (acetonitrile:formic acid, 100:0.1, v/v) was employed. The mass/charge (*m/z*) ranged from 100 to 1200 and the target mass was set at 900 m/z. Data were processed by using Data Analysis (version 4.0, Bruker DaltonicsGmbH, Bremen, Germany). Peptide sequencing was performed using the MASCOT v2.4 protein search engine (Matrix Science) against a homemade database of major genetics variants of bovine caseins and porcine digestive enzymes [3]. No cleaving enzyme was selected. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. The matched MS/MS spectra were interpreted by using Biotools version 3.2. Regardless of its *P* value, a manual revision was done for each identification peptide spectrum.

2.3.3. Peptidomic analysis

The frequency of appearance of each amino acid identified as part of a peptide sequence was used to build heat maps with alignment in each parental protein (β-, α_{s1}-, α_{s2}, and κ-casein). Spearman correlation coefficient calculated with the frequency of appearance of each amino acid was used to determine the inter-individual variability. The amino acids composition of the identified peptides was calculated by using ProtParam tool (Expasy) and isoelectric point and number of amino acids were ranged using Peptide Analyzer (Thermofisher Scientific).

2.4 Statistical analysis

Spearman correlation matrices were built on the basis of frequency of appearance of amino acids included in the identified peptides. Statistica software (StatSoft Incl., Tulsa, OK, USA) was used for the calculations.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have influenced the work reported in this article.

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2.3 Publicación III: **Protein degradation and peptide release from milk proteins in human jejunum. Comparison with *in vitro* gastrointestinal simulation.**

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Protein degradation and peptide release from milk proteins in human jejunum. Comparison with *in vitro* gastrointestinal simulation

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ABSTRACT

Human jejunal digests after oral ingestion of casein and whey protein were collected by a nasogastric tube and protein degradation and peptide release was compared with that found in the digests of the same substrates using a standardised protocol. No intact casein was detected in the jejunal nor in the *in vitro* samples taken during the intestinal phase, while β-lactoglobulin was found in one hour-jejunal samples in agreement with the *in vitro* digestion. *In vivo* and *in vitro* digests showed comparable peptide profiles and high number of common sequences. A selective precipitation step was used to strengthen the identification of phosphorylated peptides. Most of the sequences found in jejunum, some of them not previously described, were also identified in the simulated digests. Common resistant regions to digestion were identified, revealing that the *in vitro* protocol constitutes a good approximation to the physiological gastrointestinal digestion of milk proteins.

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 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, Benedé, Molina, & López-Expósito, 2015).

Because of the potential health implications of dietary peptides, several studies aimed at the identification of the peptides released in the gastrointestinal tract during protein digestion have previously been performed (Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013; Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014). Among food proteins, milk proteins are a main source of exogenous biologically active peptides (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Nongonierma & FitzGerald, 2015) and represent a stable complex food matrix commonly used as a suitable model in digestion experiments. Some *in vivo* studies have been performed on the presence of milk-derived peptides in the gastrointestinal lumen of humans (Boutrou et al., 2013; Chabance et al., 1998; Svedberg, de Haas, Leimenstoll, Paul, & Teschemacher, 1985) and animals (Barbé et al., 2014; Bouzerzour et al., 2012) after ingestion of dairy products, but the reported data although essential is still scarce and a notable diversity has been recently stated (Boutrou, Henry, & Sánchez-Rivera, 2015).

Knowledge on the factors that influence protein degradation and lead to peptide release and stability, is also provided by several *in vitro* methods, simulating the digestion of food and pharmaceuticals (Dupont et al., 2010; Kaukonen, Boyd, Charman, & Porter, 2004; Kopf-Bolanz et al., 2012; Martos, Contreras, Molina, & López-Fandiño, 2010; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In this context, a harmonized static *in vitro* digestion method was recently developed by the COST Action INFOGEST FA1005 network (Minekus et al., 2014), but it remains difficult to overcome the wide variation between the individual parameters used on these methodologies, and to fully mimic the kinetic behaviour of the complex *in vivo* digestion process by a static model, prompting further experiments to directly compare the *in vitro* results towards *in vivo* data (Egger et al., 2016).

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The aim of the present study was to directly compare the *in vitro* results with *in vivo* data to provide additional information on the interaction between milk proteins and the digestive tract, and to shed light on their physiological-derived consequences. Accordingly, this work compared 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 were compared with *in vivo* jejunal samples obtained 1, 2, 3 and 4 h after protein ingestion. Protein degradation was determined by SDS-PAGE, and the characterisation at a peptide level of the digestion products was evaluated by HPLC-tandem mass spectrometry (HPLC-MS/MS). Moreover, enrichment by selective precipitation was performed on casein samples to study the caseinphosphopeptide (CPP) fraction.

2. Materials and methods

2.1. Human jejunal effluents

Human jejunal effluents were obtained at the Human Nutrition Research Centre of Bobigny, as previously described (Boutrou et al., 2013; Mahé et al., 1996; Marsset-Baglieri et al., 2014). All procedures were approved by the Ethics Committee for Saint-Germain-en-Laye Hospital. Written informed consent was obtained from all participants. The study was registered under www.ClinicalTrials.gov (NCT00862329). Briefly, individuals ingested in fasting conditions 30 g of 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 h after oral administration of the protein solution. Jejunal effluents were collected on ice, 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, jejunal samples collected from a total of 9 volunteers were analysed: 5 who had ingested casein and 4 who had ingested whey protein.

2.2. In vitro simulated gastrointestinal digestion

Casein and whey 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 digest, 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 porcine bile extract (10 mM in the final mixture, Sigma-Aldrich). All simulated fluids were tempered at 37 °C before use. Digestions of each protein powder 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, 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 and Western blotting

Samples were dissolved at 1 mg of protein/mL in sample buffer that contained Tris-HCl (0.05 M, pH 6.8, Sigma-Aldrich), SDS (1.6%, w:v, Merck, Darmstadt, Germany), glycerol (8%, v:v, Panreac Química 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™ Unstained 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).

Western blotting detection of intact casein was performed by SDS-PAGE analysis of the human jejunal digests after casein ingestion. Gels were run in duplicate and after separation one gel was stained using Coomasie Blue as described above and the other was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 30 min. Proteins were electroblotted onto nitrocellulose membranes using a trans-Blot semi-dry transfer cell (BioRad) at 18 V for 30 min. Membranes were blocked by incubation in pH 7.6 Tris buffered saline containing 0.05% Tween 20 (TBST) and 1% bovine serum albumin (BSA) for 3 h at room temperature. After three washing steps with TBST, the membrane was incubated with casein polyclonal antibody developed in rabbit (Biorbyt Ltd., Cambridge, UK) diluted 1/1000 (v:v) in TBST containing 0.1% BSA overnight at 4 °C. The incubation was followed by five washing steps with TBST of 5 min each. Finally, the membrane was incubated overnight at 4 °C with monoclonal peroxidase-conjugated swine anti-rabbit IgG antibody (Dako, Glostrup, Denmark) 1/5000 diluted in TBST containing 0.1% BSA followed by six washing steps with TBST of 5 min each. The membrane was developed by chemiluminescence with Amersham TM ECL Prime (GE Healthcare, Chalfont St Giles, UK). Image acquisition was performed using the VersaDoc Imaging System. The positive control contained 20 μg of casein for the SDS-PAGE and western blot analyses.

2.4. Analysis by HPLC–tandem mass spectrometry (HPLC–MS/MS)

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 casein samples, an enrichment selective precipitation of CPPs was performed by adding calcium chloride (1%, w:v) and ethanol (50%, v:v) as described by Sánchez-Rivera et al. (2014). 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.

Samples were analysed by HPLC-MS/MS in duplicate using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranea Sea_{18} column (150 × 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. The spectra were recorded

over the mass/charge (m/z) range 100–700, 100–1700 and 100–2000, selecting 500, 1200, and 750 as target mass, respectively. For CPPs, same solvents and gradient, although in 60 min, were used, and spectra were 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 most abundant bovine casein and whey protein, including major genetic variants, was used for the peptide sequencing in MASCOT v2.4 software (Matrix Science). 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. Moreover, Biotools version 3.2 was used for the interpretation of the matched MS/MS spectra. Each identification peptide spectrum was revised manually, regardless of its P value.

2.5. Statistical analysis

Spearman correlation matrices were built on the basis of frequency of appearance of amino acids data. This frequency of appearance of each amino acid in the protein sequences was also used to perform Principal Component Analysis (PCA). Statistica software for Windows version 7.0 (StatSoft Inc., Tulsa, OK, USA) was used for the calculations.

3. Results and discussion

3.1. Protein degradation during human and in vitro 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 Fig. 1 (A and C, respectively). In all the jejunal digests from casein (Fig. 1A), there was a clear band with molecular weight (MW) 50 kDa, which likely corresponds to human pancreatic lipase, as previously described by other authors (Kopf-Bolanz et al., 2012). In addition, there were several light bands with mobility 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 (Supporting Information Fig 1), 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

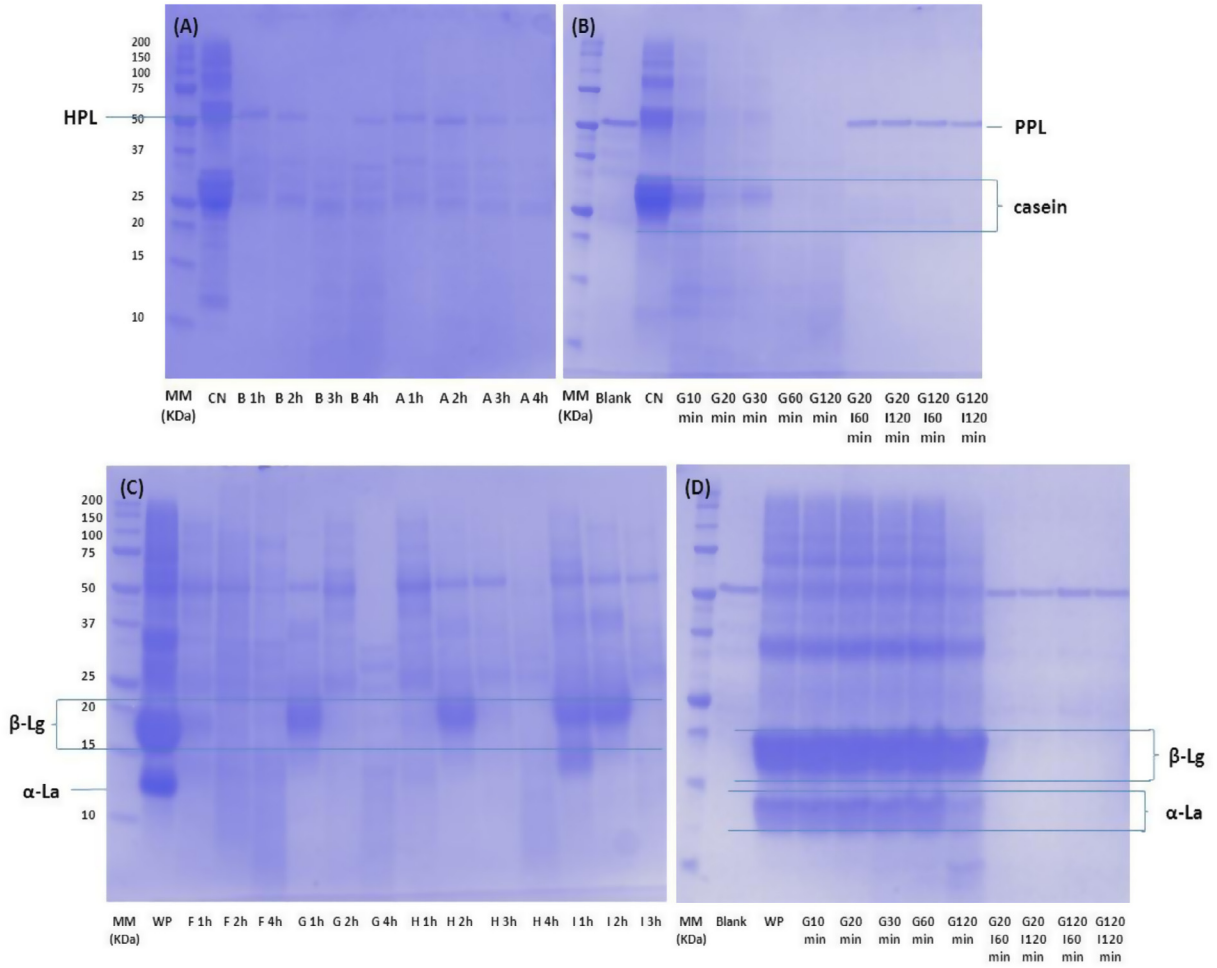

Fig. 1. SDS-PAGE comparison of jejunal casein digests with *in vitro* simulated gastrointestinal casein digestion (A and B) and jejunal whey protein digests with *in vitro* simulated gastrointestinal whey protein digestion (C and D). Each lane in A and C corresponds to different subjects (referred to 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.

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 (Barbé et al., 2014).

When applying the standardised *in vitro* digestion conditions on the same casein substrate (Fig. 1B), 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 (Kopf-Bolanz et al., 2012). The rapid degradation of the casein fraction of milk during *in vitro* gastric digestion agrees with previous reports using this standardised method in 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 (Kopf-Bolanz et al., 2012; Ménard et al., 2014; Picariello et al., 2010).

Fig. 1C shows the SDS-PAGE analyses of the jejunal contents of different volunteers at different times after whey protein intake. The electrophoretic band corresponding to β-lactoglobulin was visible in the samples taken at 1 h in all volunteers with variations in intensity. In some volunteers, this protein was also detected in the sample taken at 2 h after oral administration of whey protein (volunteers H and I). Again, this agrees with previous results on nitrogen content in jejunum where β-lactoglobulin could be recovered, mostly in the form of intact protein (Mahé et al., 1996). Fig. 1C shows that in volunteer I, an important amount of α-lactalbumin could be found 1 h after administration. As expected, during *in vitro* digestion of whey protein (Fig. 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* (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., 2014; Sánchez-Rivera, Ménard, Recio, & Dupont, 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 phases were analysed under equal conditions by HPLC-MS/MS. The peptides identified in the jejunal digests from β-casein, α_{s1}-casein, β-lactoglobulin, and α-lactalbumin are shown in Supplementary Tables S1–S4. To compare the peptide profile obtained from the analysis of the jejunal digests and *in vitro* digests, heat maps were built (Fig. 2). These graphs represent the appearance frequency of each amino acid identified as part of a peptide sequence for a given protein. This representation gives qualitative information about the protein coverage and about those protein regions where peptides were identified. Each line in the heat map corresponds to a human volunteer (Fig. 2A and C) or a different duplicate in the *in vitro* assays (Fig. 2B and D). 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

Fig. 2. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from β- and α_{s1}-casein. A and C correspond to profiles obtained from human jejunal digests obtained at 1, 2, 3 and 4 h after oral administration of casein and B and D correspond 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inferred. In addition, the peptide profiles of jejunal digests are comparable to the intestinal phase of the *in vitro* simulated gastrointestinal digestion (Fig. 2A and B). The β-casein *N*-terminal region and the region 57–99 are highly represented in both cases, but the peptides in the central and final region of the protein (residue 115–175) were more abundant in the jejunal digests. For α_{s1}-casein (Fig. 2C and D), the peptide profile was much more complex *in vivo* than *in vitro*. While in the simulated *in vitro* digestions peptides corresponded to the N- and C-terminal regions, in the jejunal digests, peptides were released from 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 and α_{s1}-casein were used to compare the *in vitro* and the *in vivo* digestion. Interindividual and *in vivo*/*in vitro* Spearman correlations were calculated. The correlation coefficient for the *in vivo*/*in vitro* intestinal digests comparison reached 0.71 ± 0.08 which is within the range found when comparing different individuals 0.58–0.73. To compare these results, a PCA was applied to the frequency of appearance of each amino acid for β-casein and α_{s1}-casein. Fig. 3 shows the clustering of the *in vivo* and *in vitro* digestion samples in the PC1-PC2 score plot. As expected, the samples from the *in vitro* gastric phase were plotted separately from jejunal digests and from *in vitro* simulated gastrointestinal digestion, and these two latter types of digests could be grouped in the sample cluster. There were two jejunal samples separated from the rest (C 2 h and B 4 h) that reflects the high *in vivo* variability. This analysis confirmed the similarity between the jejunal and the *in vitro* intestinal digests at peptide level for the digestion of the casein fraction.

Fig. 4 shows the peptide pattern of the two major proteins, β-lactoglobulin (Fig. 4A and B) and α-lactalbumin (Fig. 4C and D), obtained when whey protein was digested. In this case, although some β-lactoglobulin-derived peptides were found in the 30 min-gastric phase, most of peptides were identified in the intestinal phase during *in vitro* gastrointestinal simulation. This agrees with the low protein degradation pattern obtained by SDS-PAGE and the previously mentioned resistance of β-lactoglobulin to the action of pepsin. Similarly, other *in vitro* digestion studies have reported the release of certain peptide sequences from β-lactoglobulin during gastric digestion (Benedé et al., 2014; Egger et al., 2016). Discrepancies between different studies are explained because the susceptibility of this protein to peptic hydrolysis may vary with the denaturation degree of β-lactoglobulin by heating or other treatments (Singh, Oiseth, Lundin, & Day, 2014; Sánchez-Rivera et al., 2015). Likewise, only few α-lactalbumin peptides were identified at the end of the gastric phase, consistent with the late degradation of this protein observed by SDS-PAGE. During the *in vitro* intestinal phase, several β-lactoglobulin sequences were released mainly from regions comprised between the 42–68 and 107–135, with few peptides from the N- and C-terminal regions of the protein (Fig. 4B). The peptidic profile obtained from jejunal digests was more complex than the *in vitro* one, with a higher number of 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) (Fig. 4B). Previous studies have also shown a higher abundancy of β-lactoglobulin peptides from region 40 to 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, and 80–90 (Fig. 4C and D). Interestingly, peptides were found in jejunal 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 maintained their presence along the digestion times evaluated (Fig. 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 human jejunum.

A correlation matrix for the *in vitro*/*in vivo* comparison of β-lactoglobulin and α-lactalbumin-derived peptides was also built. In this case, the first two samples from the *in vitro* gastric phase were not included neither jejunal samples at 4 h due to the low

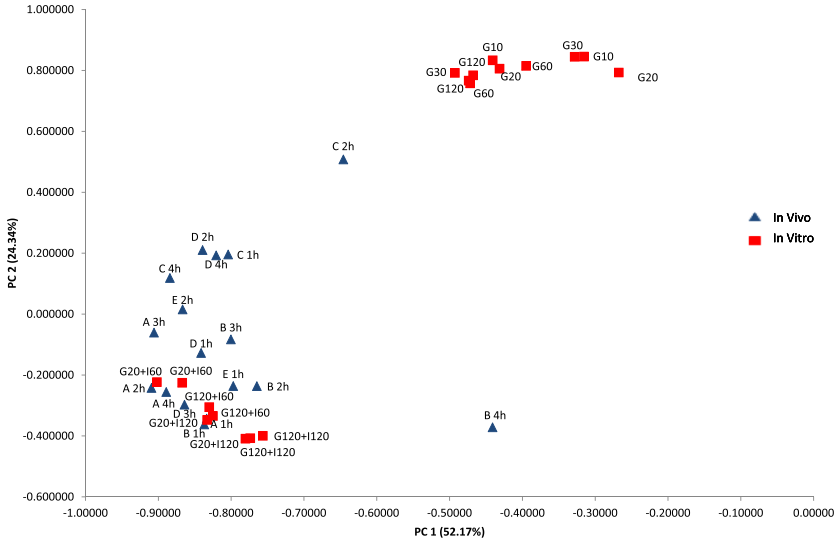

Fig. 3. Principal component analysis score plot calculated with the frequency of appearance of each amino acid identified as part of a peptide from β-casein and α_{s1}-casein. Different human subjects (blue triangles) are referred to with capital letters from A to E followed by the time of jejunal sampling (1, 2, 3 and 4 h). *In vitro* gastrointestinal digests are represented with red squares. G, gastric; I, intestinal, followed by the time expressed in minutes of *in vitro* digestion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

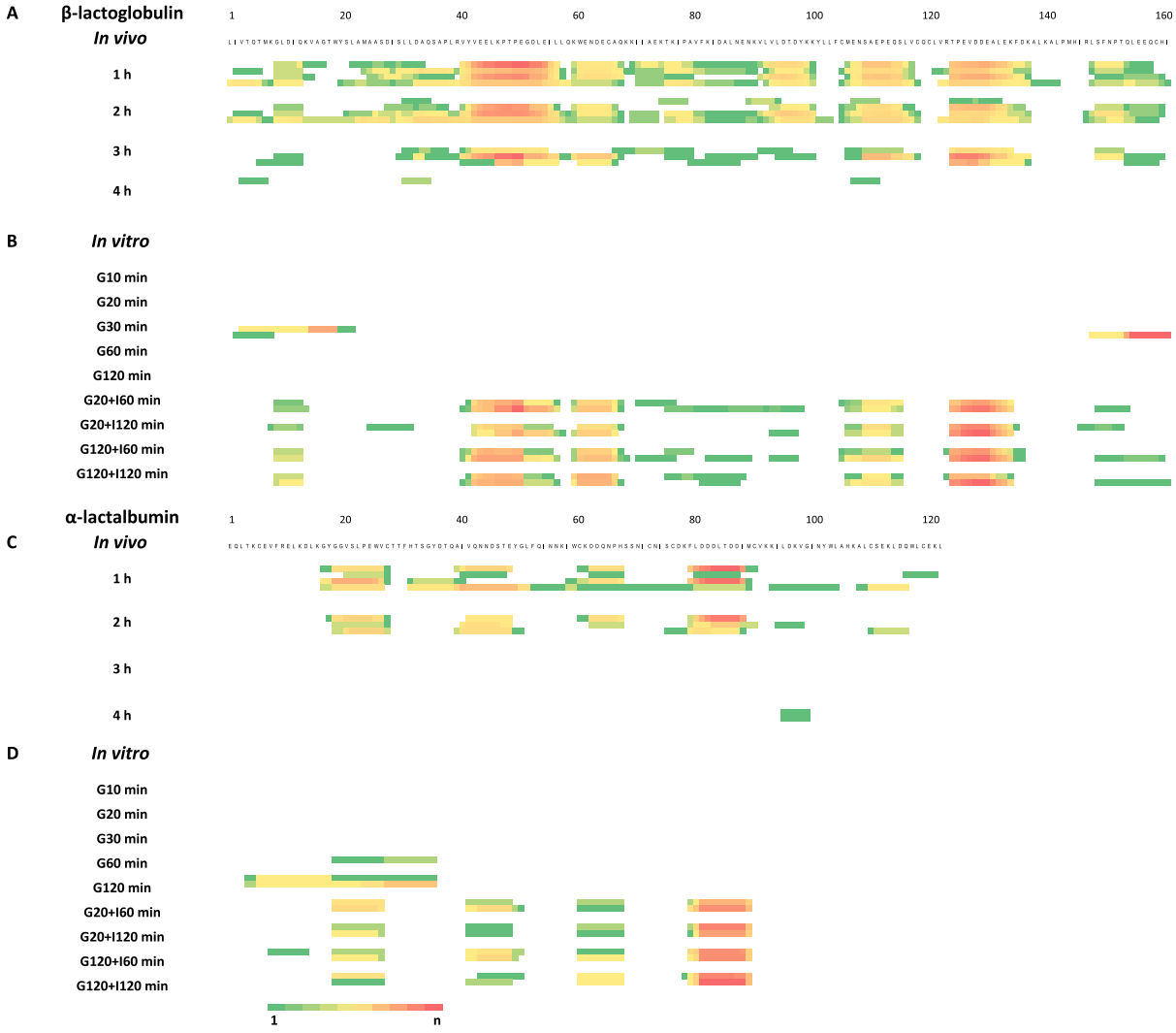

Fig. 4. Heat map built with the frequency of appearance of each amino acid identified as part of a peptide sequence from β-lactoglobulin and α-lactalbumin protein chain. A and C correspond to profiles obtained from human jejunal digests obtained at 1, 2, 3 and 4 h after oral administration of whey proteins and B and D correspond 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number of identified peptides. The average of Spearman correlation coefficient within jejunum digests from different individuals ranged from 0.43 to 0.76, similar to the average Spearman correlation coefficient found for the *in vivo/in vitro* comparison, 0.74 ± 0.16. Moreover, these results were examined by PCA and two groups were distinguished. One group was composed by the intestinal digests (*in vitro* and *in vivo*) while the other cluster comprised the *in vitro* gastric digests (data not shown). These results were similar to those found with casein as substrate.

3.3. Identification of phosphorylated peptides

All casein digests were subjected to a selective precipitation procedure with $CaCl_2$ to identify phosphorylated regions in the *in vivo* and *in vitro* digests. A total of 19 different phosphorylated sequences from β-casein were found in the jejunum digests (Fig. 5). They contained from one to four phosphorylated Ser but only β-casein f15–24 included the cluster sequence SerP-SerP-SerP-Glu-Glu, typically recognised as the binding region of minerals (Zidane et al., 2012). In addition, other CPPs with residues of Glu close to phosphorylated Ser residues were identified. Various phosphorylated fragments belonging to the *N*-terminal region, β-casein f6-16, f7-16, f8-16 and f9-16, were identified in all jejunal digests, but especially, in the samples taken at 1 and 2 h where these phosphorylated peptides were found in all volunteers (Fig. 5). One of the sequences, β-casein f7-16, had been previously identified by Chabance et al. (1998) in the human duodenum after milk ingestion. In addition, these authors had identified the sequence 155–165, that comprises β-casein f157-165, one of the fragments found in the jejunum. Most of the phosphorylated peptides from β-casein found in jejunum were also identified in the simulated intestinal digests (Supplementary Fig. 2), although in some cases, longer sequences were shown. This was the case of the peptide f7-35 that comprised some of the previously mentioned jejunal sequences. Regarding α_{s1}-casein, 15 different phosphopeptides were identified in the jejunum digests (Fig. 6). The most consistent region was α_{s1}-casein f42-52 that appeared at all jejunal time points and was also persistently found *in vitro* (Supplementary Fig. 3). Peptides belonging to the α_{s1}-casein domain 43–52 have also been identified in human plasma after cheese consumption (Caira et al., 2016). The region α_{s1}-casein f66-70 merits being mentioned because it includes the sequence SerP-SerP-SerP-Glu-Glu and, although it had been found in digests from rats (Hirayama, Toyota, Hidaka, & Naito, 1992) and mini pigs (Barbé et al., 2014;

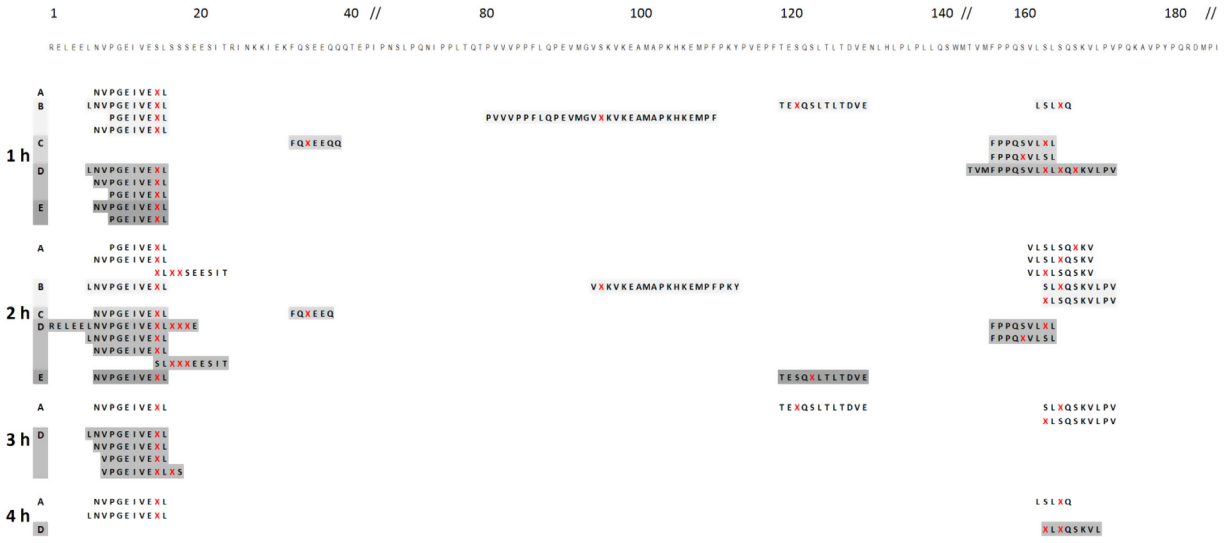

Fig. 5. Phosphorylated peptides derived from β-casein identified in jejunal digests at different times from ingestion. Each grey colour denotes a different subject. In peptides with multiple phosphorylation sites the position of the phosphorylated residues may vary. Phosphorylated serine residues are represented by a red coloured X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

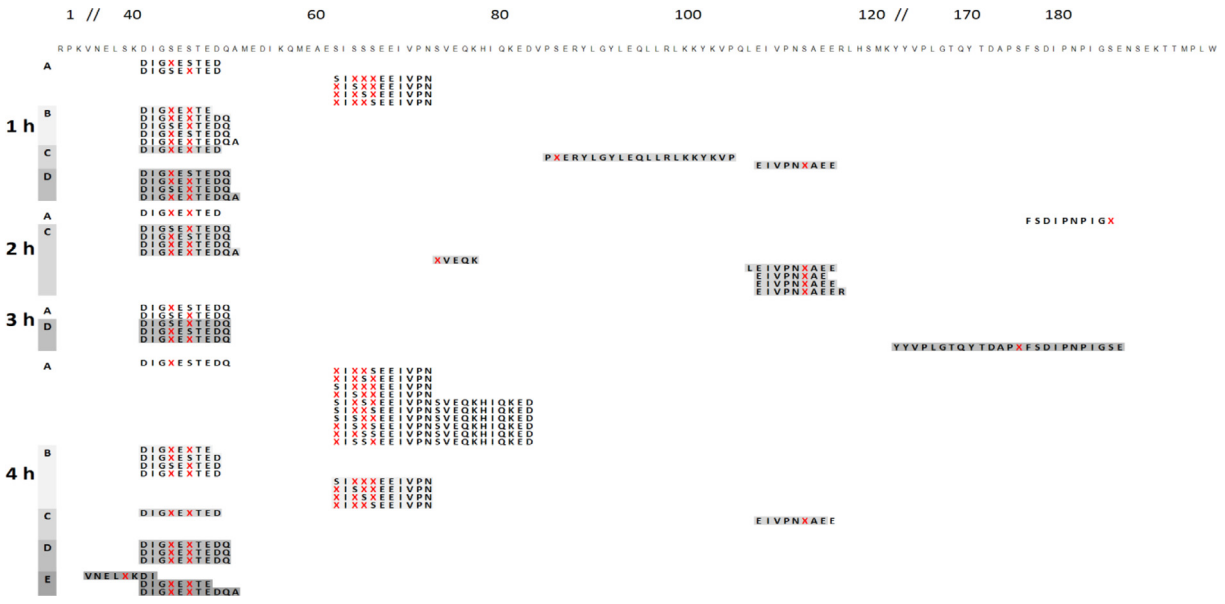

Fig. 6. Phosphorylated peptides derived from α_{s1}-casein identified in jejunal digests, at different times from ingestion. Each grey colour denotes a different subject. In peptides with multiple phosphorylation sites the position of the phosphorylated residues may vary. Phosphorylated serine residues are represented by a red coloured X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Meisel & Frister, 1989), no peptides including this domain had been previously reported in humans. By contrast, other identified sequences, namely the α_{s1}-casein f110-119, had been previously identified in human intestinal digests (Boutrou et al., 2013).

3.4. Evaluation of the peptide sequences identified in human jejunum

After casein intake, a total of 415 different peptides were recovered from human jejunal effluents, and 155 were found following the *in vitro* protocol. In the case of the whey proteins, 230 peptides were obtained *in vivo* and 80 from *in vitro* digestion. The total number of peptides identified *in vivo* was higher than *in vitro*, probably due to the inter-individual variability found. With the aim of identifying the enzymes responsible for the enzymatic cleavages, an analysis by using the online software application Enzyme Predictor was performed (Vijayakumar et al., 2012). From the comparison between *in vivo* and *in vitro* results, it was observed that several cleavages appeared exclusively in the jejunal digests. Some of them corresponded to enzymes included in the simulated juices, such as, pepsin, contained in the gastric fluids, trypsin, chymotrypsin, and elastase, contained in the pancreatic extract. The missing cleavages in the *in vitro* digests could indicate that the digestion conditions, in terms of concentration, presence of enzyme coadjuvants, or agitation, were not optimal for their occurrence.

Many studies have been focused on the resistance of certain peptide fragments during gastrointestinal digestion, and especially peptides with an attributed physiological effect. From the identified casein sequences, the percentage of peptides containing proline was 85% for β-casein, 73% for α_{s1}-casein, 45% for α_{s2}-casein, and 77% for κ-casein. Therefore, regions rich in proline residues

were resistant to gastrointestinal digestion and reached jejunum. Interestingly, the peptides whose sequences did not contain proline showed in most cases (75% on average) included aspartic or glutamic acid residues. Similarly, from the identified whey protein peptides, only a 5% did not contain proline or negatively charged residues. *In vitro*, comparable results were found, i.e., proline-containing and peptides with negatively charged residues survived to gastrointestinal digestion, pointing to a special resistance of these regions to the action of gastrointestinal enzymes.

Regarding the formation of bioactive peptides during gastrointestinal digestion, the opioid peptide β-casomorphin-7 (β-casein ^{60}YPFPGPI^{66}) and various peptides containing the same sequence were found in jejunum after consumption of casein (Supplementary Table S5), as well as in the intestinal phase of *in vitro* casein digestion. 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). It has been described that pepsin cleaves the ^{58}L-V^{59} peptide bond in β-casein, and leucine-aminopeptidase removes the valine at position 59, rendering the tyrosine residue at the *N*-terminus, characteristic of opioid peptides (Jinsmaa & Yoshikawa, 1999). At the C-terminal end of the β-casomorphin-7, the cleavage ^{66}I-X^{67} is favoured in the genetic variant A^1 of β-casein, where ^{67}X corresponds to histidine, with respect the A^2 variant, where ^{67}X corresponds to proline. The mixture of peptides released from this region is due to the presence of these two major genetic variants in the precursor casein. Most of the longer peptides containing β-casomorphin corresponded to A^2 β-casein (i.e. with proline at position 67), while free β-casomorphin-7 could likely be generated from the A^1 β-casein variant. Previous reports have shown that in hydrolysed milk with A^1 β-casein, the level of β-casomorphin-7 was 4-fold than in A^2 milk (Kamiński, Cieoelińska, & Kostyra, 2007). Similarly, neocasomorphin and various longer forms could be also identified in the jejunal digests after administration of casein. In addition to opioid peptides, these results also prove the resistance of peptide sequences with reported antihypertensive activity (Supplementary Table S5). For some of the volunteers, the reported active sequences were found in human jejunum, suggesting resistance of these regions to gastrointestinal digestion, and the possibility of these peptides being released during casein digestion. This is the case of the antihypertensive peptides β-CN ^{134}HLPLP^{138}, and α_{s1}-CN ^{143}AYFYPEL^{149}, for which the reported active forms were found in the casein jejunal digests at different sampling times. This latter peptide had been previously found in gastric human digests after milk ingestion (Chabance et al., 1998), and in addition to its antihypertensive effects in spontaneously hypertensive rats (Contreras, Carrón, Montero, Ramos, & Recio, 2009), it is also known for exerting intestinal mucin stimulatory properties in human goblet HT29-MTX cells (Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013), and opioid activity in the guinea pig ileum assay (Fernández-Tomé et al., 2016). On the contrary, for other peptides, such as, the antihypertensive tripeptides, IPP and VPP, only longer forms were found *in vivo*, which reflects the resistance of certain peptide bounds to be hydrolysed by the action of the gastrointestinal enzymes. In the region comprising the peptide ^{74}IPP^{76}, the cleavage ^{73}N-I^{74} was observed in some cases but the cleavage ^{76}P-L^{77} was not found. The difficulty of the gastrointestinal enzymes to hydrolyse the proline C-terminal peptide bonds is also illustrated in Supplementary Table S5, where different peptides from the C-terminal region the β-casein molecule, peptides comprising neocasomorphin and other antihypertensive peptides were protected from hydrolysis. It has been proposed that the post-proline cleaving protease activity is absent in human gastric and pancreatic juices which is related with the resistance of gluten-derived peptides and the inflammatory response in celiac patients (Shan et al., 2002). However, several peptide fragments found in jejunum ended by proline, resulting from the post-proline cleavage, while this cleavage seemed to be protected in other cases, which suggests the influence of the residues close to the bond to be hydrolysed.

In the case of the whey peptides identified in jejunal digests, several peptides with a proven biological activity were found. This is the case of the β-lactoglobulin ^{71}IIAEK^{75} and ^{9}GLDIQK^{14} with hypocholesterolemic activity (Nagaoka et al., 2001), and β-lactoglobulin ^{78}IPAVF^{82} with dipeptidyl peptidase-inhibitory activity (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013).

4. Conclusions

In the present work, a comparison of the standardised *in vitro* digestion model (Infogest) with *in vivo* digestion data from human jejunum is shown. Although some differences were found, the *in vitro* protocol resembles the *in vivo* intestinal digestion concerning protein degradation and peptide release. Regarding protein degradation, casein reached the jejunum in the form of degraded peptides while intact β-lactoglobulin was visible in the samples taken at 1 h in all volunteers. At the end of the *in vitro* gastrointestinal simulation a peptide pattern was comparable to that found in jejunum. It is important to highlight that the common regions which resist gastrointestinal digestion correspond to proline rich peptides or peptides containing negatively charged residues after both, casein and whey protein digestion. It was shown that the correlation found for the *in vivo/in vitro* comparison by using the frequency of appearance of each amino acid was similar to the inter-individual variability. Most of the phosphorylated peptides from β-casein found in jejunum were also identified in the *in vitro* intestinal digests, although some longer sequences were also found in the *in vitro* digests. Therefore, these results illustrate that the proposed *in vitro* digestion protocol constitutes a good approximation to the physiological gastrointestinal digestion of milk proteins.

Conflict of interest statement

The authors declare no competing financial interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.06.134.

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Protein degradation and peptide release from milk proteins in human jejunum. Peptide matching with *in vitro* gastrointestinal simulation.

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1. Western blotting analysis

The methodology is described in the manuscript, including information about the antibodies employed.

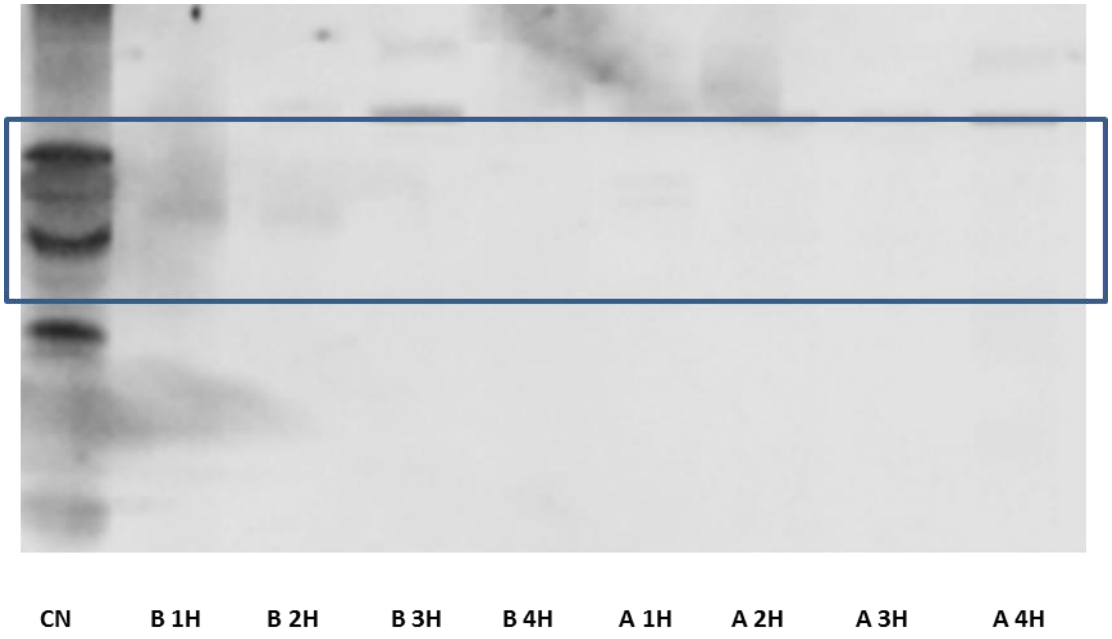

Figure S1: Western Blot analysis of jejunal casein digests. A and B corresponded to different subjects at each time of jejunal sampling (1, 2, 3 and 4 h).

2. Analysis by HPLC-tandem mass spectrometry

The equipment and the methodology are described in the manuscript.

Table S1. Identified peptides from β-casein in human jejunal digests from five different volunteers and samples taken at 1, 2, 3 and 4 h after casein ingestion

RANGE[a]		SEQUENCE[b]	RANGE		SEQUENCE
1	5	RELEE	60	67	YPFPGPIP
1	6	RELEEL	60	68	YPFPGPIPN
1	14	RELEELNVPGEIVE	60	69	YPFPGPIPNS
2	14	ELEELNVPGEIVE	62	66	FPGPI
4	12	EELNVPGEI	63	67	PGPIP
4	14	EELNVPGEIVE	63	68	PGPIPN
5	11	ELNVPGE	65	72	PIPNSLPQ
5	14	ELNVPGEIVE	68	75	NSLPQNIP
5	15	ELNVPGEIVES	69	77	SLPQNIPPL
6	13	LNVPGEIV	69	78	SLPQNIPPLT
6	14	LNVPGEIVE	69	79	SLPQNIPPLTQ
7	11	NVPGE	69	80	SLPQNIPPLTQT
7	14	NVPGEIVE	69	81	SLPQNIPPLTQTP
8	12	VPGEI	69	82	SLPQNIPPLTQTPV
9	13	PGEIV	70	77	LPQNIPPL
9	14	PGEIVE	71	77	PQNIPPL
32	38	KFQSEEQ	70	78	LPQNIPPLT
33	40	FQSEEQQQ	70	80	LPQNIPPLTQT
34	42	QSEEQQQTE	71	78	PQNIPPLT
36	42	EEQQQTE	71	79	PQNIPPLTQ
36	43	EEQQQTED	71	80	PQNIPPLTQT
36	44	EEQQQTEDE	73	77	NIPPL
36	45	EEQQQTEDEL	73	79	NIPPLTQ
36	47	EEQQQTEDELQD	73	80	NIPPLTQT
41	47	TEDELQD	73	82	NIPPLTQTPV
41	48	TEDELQDK	74	78	IPPLT
43	48	DELQDK	74	82	IPPLTQTPV
45	57	LQDKIHPFAQTQS	77	81	LTQTP
45	58	LQDKIHPFAQTQSL	77	85	LTQTPVVVP
56	62	QSLVYPF	78	82	TQTPV
56	78	QSLVYPFPGPIPNSLPQNIPPLT	79	90	QTPVVVPPFLQP
57	66	SLVYPFPGPI	80	91	TPVVVPPFLQPE
57	68	SLVYPFPGPIPN	80	92	TPVVVPPFLQPEV
58	66	LVYPFPGPI	81	88	PVVVPPFL
58	68	LVYPFPGPIPN	81	89	PVVVPPFLQ
59	66	VYPFPGPI	81	90	PVVVPPFLQP
59	67	VYPFPGPIP	81	91	PVVVPPFLQPE
59	68	VYPFPGPIPN	81	92	PVVVPPFLQPEV
59	69	VYPFPGPIPNS	81	93	PVVVPPFLQPEVM

59	70	VYPFPGPIPNSL	82	86	VVVPP
59	80	VYPFPGPIPNSLPQNIPPLTQT	82	90	VVVPPFLQP
60	66	YPFPGPI	82	91	VVVPPFLQPE
82	92	VVVPPFLQPEV	129	139	DVENLHLPLPL
83	87	VVPPF	130	138	VENLHLPLP
83	91	VVPPFLQPE	130	139	VENLHLPLPL
83	92	VVPPFLQPEV	131	138	ENLHLPLP
84	88	VPPFL	132	139	NLHLPLPL
84	91	VPPFLQPE	133	140	LHLPLPLL
85	91	PPFLQPE	134	138	HLPLP
85	92	PPFLQPEV	135	139	LPLPL
85	93	PPFLQPEVM	135	142	LPLPLLQS
88	92	LQPEV	141	156	QSWMHQPHQPLPPTVM
89	97	QPEVMGVSK	144	154	MHQPHQPLPPT
90	99	PEVMGVSKVK	145	154	HQPHQPLPPT
91	99	EVMGVSKVK	145	161	HQPHQPLPPTVMFPPQS
92	99	VMGVSKVK	146	154	QPHQPLPPT
92	100	VMGVSKVKE	147	154	PHQPLPPT
95	99	VSKVK	148	153	HQPLPP
101	105	AMAPK	148	154	HQPLPPT
110	117	PFPKYPVE	148	155	HQPLPPTV
112	118	PKYPVEP	149	161	QPLPPTVMFPPQS
114	119	YPVEPF	150	154	PLPPT
114	120	YPVEPFT	151	157	LPPTVMF
114	123	YPVEPFTESQ	155	159	VMFPP
114	124	YPVEPFTESQS	157	161	FPPQS
115	119	PVEPF	157	163	FPPQSVL
115	121	PVEPFTE	158	162	PPQSV
115	123	PVEPFTESQ	164	168	SLSQS
115	124	PVEPFTESQS	164	182	SLSQSKVLPVPQKAVPYPQ
116	120	VEPFT	164	189	SLSQSKVLPVPQKAVPYPQRDMPIQA
122	126	SQSLT	170	176	VLPVPQK
123	132	QSLTLTDVEN	171	175	LPVPQ
124	128	SLTLT	172	176	PVPQK
125	129	LTLTD	172	189	PVPQKAVPYPQRDMPIQA
125	132	LTLTDVEN	174	183	PQKAVPYPQR
126	132	TLTDVEN	175	179	QKAVP
126	133	TLTDVENL	177	189	AVPYPQRDMPIQA
126	140	TLTDVENLHLPLPLL	184	189	DMPIQA
128	132	TDVEN	185	189	MPIQA
128	138	TDVENLHLPLP	192	199	LYQEPVLG
128	139	TDVENLHLPLPL	192	201	LYQEPVLGPV
128	140	TDVENLHLPLPLL	192	209	LYQEPVLGPVRGPFPIIV
129	133	DVENL	193	199	YQEPVLG
129	135	DVENLHL	193	201	YQEPVLGPV
129	138	DVENLHLPLP	193	207	YQEPVLGPVRGPFPI

193	208	YQEPVLGPVRGPFPII
193	209	YQEPVLGPVRGPFPIIV
194	198	QEPVL
194	201	QEPVLGPV
194	209	QEPVLGPVRGPFPIIV
195	199	EPVLG
196	201	PVLGPV
196	205	PVLGPVRGPF
196	207	PVLGPVRGPFPI
198	207	LGPVRGPFPI
199	207	GPVRGPFPI
199	209	GPVRGPFPIIV
200	208	PVRGPFPII
200	209	PVRGPFPIIV
203	207	GPFPI
205	209	FPIIV

[a] Range in the mature form of the protein. Peptides corresponding to the β-casein genetic variant A^2 are shown (Uniprot accession number: P0266)

[b] One letter amino acid code is used

Table S2. Identified peptides from α_{s1}-casein in human jejunal digests from five different volunteers and samples taken at 1, 2, 3 and 4 h after casein ingestion

RANGE		SEQUENCE	RANGE		SEQUENCE
1	15	RPKHPIKHQGLPQEV	44	49	IGSEST
4	13	HPIKHQGLPQ	44	52	IGSESTEDQ
7	16	KHQGLPQEVL	53	57	AMEDI
8	12	HQGLP	53	63	AMEDIKQMEAE
8	14	HQGLPQE	57	63	IKQMEAE
8	15	HQGLPQEV	68	72	SEEIV
9	15	QGLPQEV	70	74	EIVPN
10	14	GLPQE	80	88	HIQKEDVPS
11	15	LPQEV	80	89	HIQKEDVPSE
12	19	PQEVLNEN	83	89	KEDVPSE
12	21	PQEVLNENLL	84	88	EDVPS
23	31	FFVAPFPEV	85	89	DVPSE
23	33	FFVAPFPEVFG	101	112	LKKYKVPQLEIV
24	28	FVAPF	102	107	KKYKVP
24	30	FVAPFPE	105	109	KVPQL
24	31	FVAPFPEV	105	114	KVPQLEIVPN
24	32	FVAPFPEVF	106	110	VPQLE
24	33	FVAPFPEVFG	106	114	VPQLEIVPN
25	31	VAPFPEV	110	118	EIVPNSAEE
25	32	VAPFPEVF	112	119	VPNSAEER
25	33	VAPFPEVFG	114	118	NSAEE
26	30	APFPE	120	127	LHSMKEGI
26	33	APFPEVFG	123	127	MKEGI
26	34	APFPEVFGK	125	129	EGIHA
27	31	PFPEV	125	131	EGIHAQQ
27	34	PFPEVFGK	125	137	EGIHAQQKEPMIG
29	33	PEVFG	126	130	GIHAQ
29	37	PEVFGKEKV	126	137	GIHAQQKEPMIG
29	38	PEVFGKEKVN	133	137	EPMIG
30	41	EVFGKEKVNELS	136	140	IGVNQ
35	39	EKVNE	138	145	VNQELAYF
35	41	EKVNELS	139	143	NQELA
35	44	EKVNELSKDI	143	148	AYFYPE
37	41	VNELS	143	149	AYFYPEL
38	48	NELSKDIGSES	155	161	QLDAYPS
39	47	ELSKDIGSE	155	163	QLDAYPSGA
42	46	KDIGS	157	161	DAYPS
43	47	DIGSE	157	164	DAYPSGAW
43	50	DIGSESTE	158	163	AYPSGA
43	51	DIGSESTED	159	163	YPSGA
43	52	DIGSESTEDQ	163	169	AWYYVPL
44	48	IGSES	165	171	YYVPLGT

165	172	YYVPLGTQ
165	173	YYVPLGTQY
166	170	YVPLG
167	171	VPLGT
167	177	VPLGTQYTDAP
168	172	PLGTQ
174	178	TDAPS
174	185	TDAPSFSDIPNP
174	186	TDAPSFSDIPNPI
175	186	DAPSFSDIPNPI
176	180	APSFS
179	183	FSDIP
180	184	SDIPN
180	186	SDIPNPI
180	189	SDIPNPIGSE
180	190	SDIPNPIGSEN
180	191	SDIPNPIGSENS
180	192	SDIPNPIGSENSE
180	193	SDIPNPIGSENSEK
180	196	SDIPNPIGSENSEKTTM
180	198	SDIPNPIGSENSEKTTMPL
181	185	DIPNP
181	191	DIPNPIGSENS
181	192	DIPNPIGSENSE
182	186	IPNPI
183	193	PNPIGSENSEK
185	193	PIGSENSEK
185	196	PIGSENSEKTTM
186	190	IGSEN
186	193	IGSENSEK
188	192	SENSE
194	198	TTMPL

[a] Range in the mature form of the protein. Peptides corresponding to the α_{s1}-casein genetic variant B are shown (Uniprot accession number: P02662)

[b] One letter amino acid code is used

Table S3. Identified peptides from β-lactoglobulin in human jejunal digests from four different volunteers and samples taken at 1, 2, 3 and 4 h after whey protein ingestion

RANGE		SEQUENCE	RANGE		SEQUENCE
1	5	LIVTQ	36	42	SAPLRVY
1	7	LIVTQTM	41	49	VYVEELKPT
1	8	LIVTQTMK	41	53	VYVEELKPTPEGD
1	11	LIVTQTMKGLD	41	54	VYVEELKPTPEGDL
2	6	IVTQT	41	55	VYVEELKPTPEGDLE
3	7	VTQTM	41	56	VYVEELKPTPEGDLEI
6	13	TMKGLDIQ	41	57	VYVEELKPTPEGDLEIL
9	13	GLDIQ	41	58	VYVEELKPTPEGDLEILL
9	15	GLDIQKV	41	59	VYVEELKPTPEGDLEILLQ
10	19	LDIQKVAGTW	42	48	YVEELKP
12	19	IQKVAGTW	42	49	YVEELKPT
13	17	QKVAG	42	52	YVEELKPTPEG
20	24	YSLAM	42	53	YVEELKPTPEGD
20	28	YSLAMAASD	42	54	YVEELKPTPEGDL
21	26	SLAMAA	42	55	YVEELKPTPEGDLE
22	27	LAMAAS	42	56	YVEELKPTPEGDLEI
23	28	AMAASD	42	57	YVEELKPTPEGDLEIL
24	28	MAASD	42	58	YVEELKPTPEGDLEILL
24	32	MAASDISLL	42	59	YVEELKPTPEGDLEILLQ
25	29	AASDI	43	49	VEELKPT
25	30	AASDIS	43	51	VEELKPTPE
26	30	ASDIS	43	52	VEELKPTPEG
27	33	SDISLLD	43	53	VEELKPTPEGD
27	35	SDISLLDAQ	43	54	VEELKPTPEGDL
28	32	DISLL	43	55	VEELKPTPEGDLE
28	36	DISLLDAQS	43	56	VEELKPTPEGDLEI
28	38	DISLLDAQSAP	43	57	VEELKPTPEGDLEIL
29	33	ISLLD	43	59	VEELKPTPEGDLEILLQ
30	34	SLLDA	44	51	EELKPTPE
30	39	SLLDAQSAPL	44	52	EELKPTPEG
31	35	LLDAQ	44	53	EELKPTPEGD
31	41	LLDAQSAPLRV	44	54	EELKPTPEGDL
32	41	LDAQSAPLRV	44	55	EELKPTPEGDLE
33	38	DAQSAP	45	49	ELKPT
33	40	DAQSAPLR	45	51	ELKPTPE
33	41	DAQSAPLRV	45	52	ELKPTPEG
33	42	DAQSAPLRVY	45	53	ELKPTPEGD
34	38	AQSAP	45	54	ELKPTPEGDL
34	39	AQSAPL	45	55	ELKPTPEGDLE
47	51	KPTPE	95	101	LDTDYKK

47	55	KPTPEGDLE
47	56	KPTPEGDLEI
48	52	PTPEG
48	55	PTPEGDLE
49	55	TPEGDLE
49	56	TPEGDLEI
50	54	PEGDL
50	55	PEGDLE
50	56	PEGDLEI
50	57	PEGDLEIL
60	66	KWENDEC
60	67	KWENDECA
60	68	KWENDECAQ
61	66	WENDEC
61	67	WENDECA
61	68	WENDECAQ
61	70	WENDECAQKK
62	68	ENDECAQ
67	75	AQKKIIAEK
70	74	KIIAE
71	75	IIAEK
72	78	IAEKTKI
75	79	KTKIP
76	80	TKIPA
76	82	TKIPAVF
78	82	IPAVF
80	88	AVFKIDALN
83	87	KIDAL
83	89	KIDALNE
83	91	KIDALNENK
90	94	NKVLV
91	95	KVLVL
92	96	VLVLD
92	97	VLVLDT
92	98	VLVLDTD
92	99	VLVLDTDY
93	97	LVLDT
93	99	LVLDTDY
93	100	LVLDTDYK
94	98	VLDTD
94	100	VLDTDYK
95	100	LDTDYK
96	101	DTDYKK
96	104	DTDYKKYLL
106	116	CMENSAEPEQS
107	115	MENSAEPEQ
107	116	MENSAEPEQS
108	112	ENSAE
108	116	ENSAEPEQS
108	118	ENSAEPEQSLV
109	116	NSAEPEQS
110	114	SAEPE
110	116	SAEPEQS
110	117	SAEPEQSL
110	118	SAEPEQSLV
110	119	SAEPEQSLVC
111	115	AEPEQ
112	116	EPEQS
122	131	LVRTPEVDDE
123	130	VRTPEVDD
123	131	VRTPEVDDE
123	132	VRTPEVDDEA
123	133	VRTPEVDDEAL
124	131	RTPEVDDE
125	129	TPEVD
125	130	TPEVDD
125	131	TPEVDDE
125	132	TPEVDDEA
125	133	TPEVDDEAL
125	134	TPEVDDEALE
125	135	TPEVDDEALEK
125	137	TPEVDDEALEKFD
125	138	TPEVDDEALEKFDK
126	130	PEVDD
126	135	PEVDDEALEK
127	134	EVDDEALE
127	135	EVDDEALEK
128	132	VDDEA
128	137	VDDEALEKFD
129	133	DDEAL
129	135	DDEALEK
129	136	DDEALEKF
130	134	DEALE
130	137	DEALEKFD
131	135	EALEK
132	138	ALEKFDK
133	138	LEKFDK
137	143	DKALKAL

148	154	RLSFNPT
149	154	LSFNPT
149	155	LSFNPTQ
149	156	LSFNPTQL
150	154	SFNPT
152	156	NPTQL
155	159	QLEEQ
155	160	QLEEQC
155	161	QLEEQCH
156	161	LEEQCH
157	162	EEQCHI

[a] Range in the mature form of the protein. Peptides corresponding to the β-lactoglobulin genetic variant A are shown (Uniprot accession number: P02754)

[b] One letter amino acid code is used

Table S4. Identified peptides from α-lactalbumin in human jejunal digests from four different volunteers and samples taken at 1, 2, 3 and 4 h after whey protein ingestion

RANGE		SEQUENCE	RANGE		SEQUENCE
17	25	GYGGVSLPE	82	89	DDDLTDDI
17	27	GYGGVSLPEWV	83	89	DDLTDDI
18	27	YGGVSLPEWV	83	90	DDLTDDIM
19	26	GGVSLPEW	84	88	DLTDD
19	27	GGVSLPEWV	84	89	DLTDDI
19	28	GGVSLPEWVC	84	91	DLTDDIMC
21	25	VSLPE	94	105	KILDKVGINYWL
21	27	VSLPEWV	95	99	ILDKV
21	28	VSLPEWVC	96	100	LDKVG
22	28	SLPEWVC	109	117	ALCSEKLDQ
32	39	HTSGYDTQ	111	117	CSEKLDQ
32	40	HTSGYDTQA	117	122	QWLCEK
32	41	HTSGYDTQAI			
33	41	TSGYDTQAI			
40	48	AIVQNNDST			
40	49	AIVQNNDSTE			
40	50	AIVQNNDSTEY			
40	52	AIVQNNDSTEYGL			
41	47	IVQNNDS			
41	48	IVQNNDST			
41	49	IVQNNDSTE			
41	50	IVQNNDSTEY			
41	51	IVQNNDSTEYG			
41	52	IVQNNDSTEYGL			
42	49	VQNNDSTE			
53	60	FQINNKIW			
59	67	IWCKDDQNP			
59	73	IWCKDDQNPHSSNIC			
61	68	CKDDQNPH			
63	67	DDQNP			
63	68	DDQNPH			
74	80	NISCDKF			
76	83	SCDKFLDD			
80	88	FLDDDLTDD			
80	89	FLDDDLTDDI			
81	88	LDDDLTDD			
81	89	LDDDLTDDI			
81	90	LDDDLTDDIM			
82	88	DDDLTDD			

[a] Range in the mature form of the protein. α-lactalbumin Uniprot accession number: P00711

[b] One letter amino acid code is used

Table S5: β- and α_{s1}-casein-derived peptides with antihypertensive and opioid activity and related peptides found in human jejunum. Sequences highlighted in bold correspond to the assayed peptides in animal models or ex-vivo preparations; peptides are colored in red when the reported active peptide was detected in the jejunal digests

Protein fragment in human jejunum	Peptide sequence found in human jejunum	Assayed peptide	Activity	Dose (mg/kg)	Decrease SBP (mm Hg)[a] Receptor selectivity[b]	Reference
β-CN 58-76	QS**LVYPFPGPIPNSLPQNIPP**LT	LVYPFPGPIPNSLPQNIPP	Antihypertensive	6.0	-14.9	Miguel, Recio, Ramos, Delgado, & Aleixandre, (2006); Quirós et al. (2007)
β-CN 56-78 β-CN 57-66 β-CN 57-68 β-CN 58-66 β-CN 58-68 β-CN 59-66 β-CN 59-67 β-CN 59-68 β-CN 59-69 β-CN 59-70 β-CN 59-80 β-CN 60-66	QSLV**YPFPGPI**PNSLPQNIPPLT SLV**YPFPGPI** SLV**YPFPGPI**PN LV**YPFPGPI** LV**YPFPGPI**PN V**YPFPGPI** V**YPFPGPI**P V**YPFPGPI**PN V**YPFPGPI**PNS V**YPFPGPI**PNSL V**YPFPGPI**PNSLPQNIPPLTQT **YPFPGPI**	YPFPGPI (β-casomorphin-7)	Opioid	-	μ>δ>>κ	Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, (1981)
β-CN 56-78 β-CN 57-66 β-CN 57-68 β-CN 58-66 β-CN 58-68 β-CN 59-66 β-CN 59-67 β-CN 59-68 β-CN 59-69 β-CN 59-70 β-CN 59-80	QSL**VYPFPG**PIPNSLPQNIPPLT SL**VYPFPG**PI SL**VYPFPG**PIPN L**VYPFPG**PI L**VYPFPG**PIPN **VYPFPG**PI **VYPFPG**PIP **VYPFPG**PIPN **VYPFPG**PIPNS **VYPFPG**PIPNSL **VYPFPG**PIPNSLPQNIPPLTQT	VYPFPG	Antihypertensive	8.0	-22.0	Abubakar, Saito, Kitazawa, Kawai, & Itoh, (1998)
β-CN 60-68 β-CN 60-69 β-CN 56-78	**YPFPGPIPN** **YPFPGPIPN**S QSLV**YPFPGPIPN**SLPQNIPPLT	YPFPGPIPN	Antihypertensive	7.5	-7.0	Saito, Nakamura, Kitazawa, Kawai, & Itoh, (2000)

β-CN 57-68	SLV**YPFPGPIPN**					
β-CN 58-66	LV**YPFPGPI**					
β-CN 58-68	LV**YPFPGPIPN**					
β-CN 59-68	V**YPFPGPIPN**					
β-CN 59-69	V**YPFPGPIPN**S					
β-CN 59-70	V**YPFPGPIPN**SL					
β-CN 59-80	V**YPFPGPIPN**SLPQNIPPLTQT					
β-CN 69-77	SLPQN**IPP**L	IPP	Antihypertensive	0.3	-28.3	Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, & Takano, (1995); Nakamura, Yamamoto, Sakai, & Takano (1995)
β-CN 69-78	SLPQN**IPP**LT					
β-CN 69-79	SLPQN**IPP**LTQ					
β-CN 69-80	SLPQN**IPP**LTQT					
β-CN 69-81	SLPQN**IPP**LTQTP					
β-CN 69-82	SLPQN**IPP**LTQTPV					
β-CN 70-77	LPQN**IPP**L					
β-CN 71-77	PQN**IPP**L					
β-CN 70-78	LPQN**IPP**LT					
β-CN 70-80	LPQN**IPP**LTQT					
β-CN 71-78	PQN**IPP**LT					
β-CN 71-79	PQN**IPP**LTQ					
β-CN 71-80	PQN**IPP**LTQT					
β-CN 73-77	N**IPP**L					
β-CN 73-79	N**IPP**LTQ					
β-CN 73-80	N**IPP**LTQT					
β-CN 73-82	N**IPP**LTQTPV					
β-CN 74-78	**IPP**LT					
β-CN 74-82	**IPP**LTQTPV					
β-CN 79-90	Q**TPVVVPPFLQP**	TPVVVPPFLQP	Antihypertensive	8.0	-8.0	Abubakar et al. (1998)
β-CN 80-91	**TPVVVPPFLQP**E					
β-CN 80-92	**TPVVVPPFLQP**EV					
β-CN 79-90	QTPVV**VPP**FLQP	VPP	Antihypertensive	0.6	-32.1	Nakamura et al., (1995a); Nakamura et al., (1995b)
β-CN 80-91	TPVV**VPP**FLQPE					
β-CN 80-92	TPVV**VPP**FLQPEV					
β-CN 81-88	PVV**VPP**FL					
β-CN 81-89	PVV**VPP**FLQ					
β-CN 81-90	PVV**VPP**FLQP					
β-CN 81-91	PVV**VPP**FLQPE					
β-CN 81-92	PVV**VPP**FLQPEV					
β-CN 81-93	PVV**VPP**FLQPEVM					

β-CN 82-86 β-CN 82-90 β-CN 82-91 β-CN 82-92 β-CN 83-87 β-CN 83-91 β-CN 83-92 β-CN 84-88 β-CN 84-91	VV**VPP** VV**VPP**FLQP VV**VPP**FLQPE VV**VPP**FLQPEV V**VPP**F V**VPP**FLQPE V**VPP**FLQPEV **VPP**FL **VPP**FLQPE					
β-CN 101-105	A**MAP**K	MAP	Antihypertensive	3.0	-17.0	Tonouchi, Suzuki, Uchida, & Oda, (2008)
β-CN 114-119 β-CN 114-120 β-CN 114-123 β-CN 114-124	**YPVEPF** **YPVEPF**T **YPVEPF**TESQ **YPVEPF**TESQS	YPVEPF (Neocasomorphin)	Opioid	-	μ	Jinsmaa, and Yoshikawa, (1999)
β-CN 126-140 β-CN 128-138 β-CN 128-139 β-CN 128-140 β-CN 129-139 β-CN 129-138 β-CN 130-138 β-CN 130-139 β-CN 131-138 β-CN 132-139 β-CN 133-140 β-CN 134-138	TLTDVENL**HLPLP**LL TDVENL**HLPLP** TDVENL**HLPLP**L TDVENL**HLPLP**LL DVENL**HLPLP**L DVENL**HLPLP** VENL**HLPLP** VENL**HLPLP**L ENL**HLPLP** NL**HLPLP**L L**HLPLP**LL **HLPLP**	LHLPLP HLPLP	Antihypertensive	3.0 7.0	-25.3 -23.5	Miguel et al., (2006); Quirós et al. (2007)
β-CN 164-182 β-CN 164-189	SLSQS**KVLPVP**QKAVPYPQ SLSQS**KVLPVP**QKAVPYPQRDMPIQA	KVLPVP	Antihypertensive	2.0	-32.2	Maeno, Yamamoto, & Takano, (1996)
β-CN 174-183 β-CN 177-189 β-CN 172-189 β-CN 164-189	PQK**AVPYPQR** **AVPYPQR**DMPIQA PVPQK**AVPYPQR**DMPIQA SLSQSKVLPVPQK**AVPYPQR**DMPIQA	AVPYPQR	Antihypertensive	100.0	-10.0	Karaki et al. (1990)
β-CN 192-209 β-CN 193-207 β-CN 193-208 β-CN 193-209 β-CN 194-209	LYQEP**VLGPVRGPFP**IIV YQEP**VLGPVRGPFP**I YQEP**VLGPVRGPFP**II YQEP**VLGPVRGPFP**IIV QEP**VLGPVRGPFP**IIV	VLGPVRGPFP	Antihypertensive	10.0	-16.2	Miguel et al., (2006); Quirós et al. (2007)

β-CN 196-207	P**VLGPVRGPFP**I					
β-CN 192-209	LYQEPVLGP**VRGPFPIIV**	VRGPFPIIV	Antihypertensive	10.0	-16.1	Miguel et al., (2006); Quirós et al. (2007)
β-CN 193-209	YQEPVLGP**VRGPFPIIV**					
β-CN 194-209	QEPVLGP**VRGPFPIIV**					
β-CN 199-209	GP**VRGPFPIIV**					
β-CN 200-209	P**VRGPFPIIV**					
α_{s1}-CN 23-31	**FFVAPFPEV**	FFVAPFPEVFGK	Antihypertensive	100.0	-34.0	Karaki et al. (1990)
α_{s1}-CN 23-33	**FFVAPFPEVFG**					
α_{s1}-CN 24-28	**FVAPF**					
α_{s1}-CN 24-30	**FVAPFPE**					
α_{s1}-CN 24-31	**FVAPFPEV**					
α_{s1}-CN 24-32	**FVAPFPEVF**					
α_{s1}-CN 24-33	**FVAPFPEVFG**					
α_{s1}-CN 25-31	**VAPFPEV**					
α_{s1}-CN 25-32	**VAPFPEVF**					
α_{s1}-CN 25-33	**VAPFPEVFG**					
α_{s1}-CN 26-30	**APFPE**					
α_{s1}-CN 26-33	**APFPEVFG**					
α_{s1}-CN 26-34	**APFPEVFGK**					
α_{s1}-CN 27-31	**PFPEV**					
α_{s1}-CN 27-34	**PFPEVFGK**					
α_{s1}-CN 101-112	LKK**YKVPQL**EIV	YKVPQL	Antihypertensive	2.0	-13.0	Maeno et al., (1996)
α_{s1}-CN 102-107	KK**YKVP**					
α_{s1}-CN 105-109	**KVPQL**					
α_{s1}-CN 105-114	**KVPQL**EIVPN					
α_{s1}-CN 106-110	**VPQL**E					
α_{s1}-CN 143-148	**AYFYPE**	AYFYPEL YFYPEL YFYPE YFYP	Opioid	-	μ>δ	Fernández-Tomé, Martínez-Maqueda, Girón, Goicoechea, Miralles, & Recio, (2016)
α_{s1}-CN 143-149	**AYFYPEL**					
α_{s1}-CN 143-148	**AYFYPE**	AYFYPEL	Antihypertensive	5.0	-20.0	Contreras, Carrón, Montero, Ramos, & Recio, (2009)
α_{s1}-CN 143-149	**AYFYPEL**					
α_{s1}-CN 194-198	**TTMPL**	TTMPLW	Antihypertensive	100.0	-13.6	Karaki et al., (1990)

[a] Observed decrease in systolic blood pressure in spontaneously hypertensive rats after oral administration of the indicated dose.

[b] Reported opioid receptor selectivity for opioid peptides.

Figure S2: Phosphorylated peptides derived from β-casein identified in *in vitro* digests. Each grey colour denotes a different subject. In peptides with multiple phosphorylation sites the position of the phosphorylated residues may vary. Phosphorylated serine residues are represented by a red coloured X

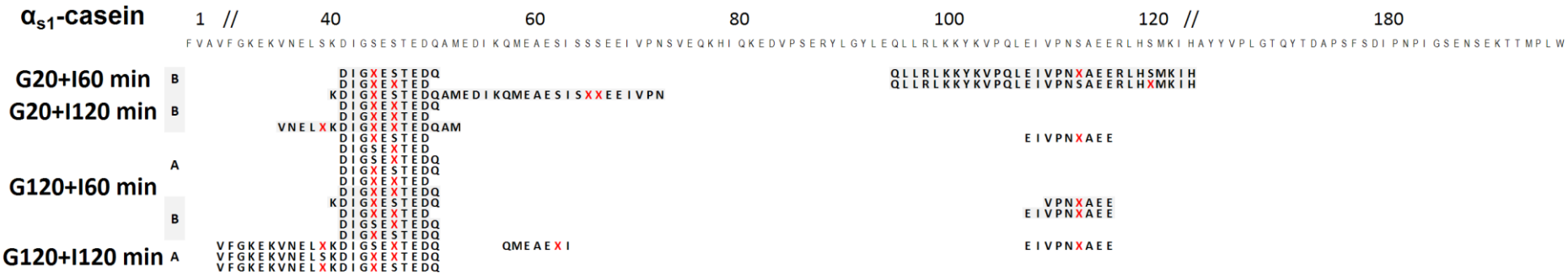

Figure S3: Phosphorylated peptides derived from α_{s1}-casein identified in *in vitro* digests. Each grey colour denotes a different subject. In peptides with multiple phosphorylation sites the position of the phosphorylated residues may vary. Phosphorylated serine residues are represented by a red coloured X.

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2.4 Publicación IV: **Milk peptidomic profile after in vitro gastrointestinal digestion including intestinal brush border membrane peptidases. Transepithelial transport of digestion resistant casein domains**

MANUSCRIPT

2.4.1 Material Suplementario Publicación IV

Milk peptidomic profile after *in vitro* gastrointestinal digestion including intestinal brush border membrane peptidases. Transepithelial transport of digestion resistant casein domains.

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Abstract

The use of enzymes from the brush border membrane (BBM) in simulated gastrointestinal digestion of milk proteins is evaluated. With this purpose, the resistant sequences from casein and milk proteins after in vitro digestion with and without BBM have been analyzed by tandem mass spectrometry. The use of BBM revealed additional cleavages to those found with pancreatic enzymes, although the number of total identified peptides decreased due to the reduction of the peptide size. These new cleavages were mainly attributed to the activity of amino- and carboxi-peptidases, and their activity was also reflected in the higher concentration of free amino acids found in the gastrointestinal digests with BBM. The peptidome of the simulated gastrointestinal digests was compared with that previously obtained in digests aspirated from human jejunum after oral administration of the same substrates. The combination of the peptides obtained with and without BBM gave a closer situation to the human situation. In addition, *in vitro* transepithelial transport of six β-casein derived peptides resistant to gastrointestinal digestion, including the opioid β-casomorphin-7, was also evaluated. It was shown the importance of the nature of the N- and C-terminal end for the transport rate through the Caco-2 cell monolayer. Therefore, the use of BBM as a complementary step after simulated pancreatic digestion can be considered in bioavailability studies since the final sequence can determine its absorption.

1. Introduction

During digestion dietary proteins are progressively hydrolyzed to amino acids and peptides in the stomach and intestine. Degradation to amino acids has a clear relevance for nutritional reasons, as it is the direct way to provide the organism the required protein constituents. The supply of particular amino acids over time can also have a metabolic role, as is the case of leucine on whole body protein anabolism (Boirie et al., 1997). A notable fraction, around 70 % of the ingested protein, remains in the form of peptides, and the surviving of specific domains of dietary proteins in the human intestine has been demonstrated (Boutrou et al., 2013; Sanchón et al., 2018). This group of resistant peptides might have an impact on many physiological responses upon their absorption or through their local effect in the intestine (Picariello et al., 2010-J. Chrom. B). After gastric and pancreatic action, the intestine content comes into contact with the villum, where multiple enzymes are responsible for further degradation prior to absorption. The enzymes from the brush border membrane (BBM) include a suite of oligopeptidases (e.g., aminopeptidases, carboxypeptidases, endopeptidases, and dipeptidases), lipases (e.g., sphingolipid hydrolyzing enzymes and phospholipases), and oligosaccharidases (e.g., α 1,4-glucosidases, α 1,6-glucosidases, α1, β2-glycosidase, β 1,4-glycosidases, and α1, α1-glucosidase) that enable nutrient hydrolysis to occur adjacent to the membrane in a pre-absorptive step (Hooton et al 2015). In the case of transport into the general circulation, additional vascular endothelial tissue peptidases and soluble plasma peptidases can yield peptide hydrolysis (Deacon et al 1995).

The determinant role of digestion on the bioavailability of food compounds is undoubted and studies with the aim to gain knowledge about the effect of the digestive process on particular nutrients are essential. Digestion *in vitro* models aim to mimetize the changes undergone by food in the digestive tract avoiding the use on in vivo trials. The Infogest model (Brodkorb et al 2019) is a consensus protocol developed in the frame of a COST action that has been validated

against in vivo models (Sanchón et al., 2018, Egger et al., 2017). The workflow comprises oral, gastric and intestinal phases where fluid compositions, pH and enzymatic activities have been set to mimic the human situation. However, in the analysis of the digested fraction, it has been claimed that the pancreatic digestion is not complete (Picariello et al 2016). When addressing the *in vivo* bioavailability of active sequences, it is crucial to elucidate in which form are ingested peptides to be found. Unexpected degradation by intestinal or blood proteases may make it difficult to determine this bioavailability (Matsui, 2018). The addition of a source of BBM during simulated digestion has been raised as the approach to reach the actual fraction ready for absorption.

On the other hand, the absorption of peptides is a controversial issue object of debate (Miner-Williams et al., 2014; Matsui, 2018). It is known that di-/tri-peptides are absorbable compounds through the action of PepT1 as transporter. Some food derived tripeptides (Gleeson et al., 2017; Fernández-Musoles et al. 2013), as well as peptides of longer size have been proved to be transported through cell models of human epithelium (Grootaert et al 2017; Regazzo et al, 2010; Contreras et al., 2012; Quirós et al., 2008; Corrochano et al., 2019). Works showing absorption *in vivo* are far fewer although pharmacokinetics studies have been reported for tripeptides (Foltz et al., 2007, van der Pilj et al., 2008) and pentapeptides (Sánchez-Rivera et al., 2014). An additional subject of discussion is the relationship between structure, stability in the physiological medium and absorption (Udenigwe and Fogliano, 2017). Whether there are structural determinants for transport is a question addressed by limited studies. The characteristics of peptides to favor their transport across epithelium monolayers point to their beta-turn structure (Knipp et al., 1997), size or hydrophobicity (Wang et al., 2020), or identity of terminal amino acid residues (Ding et al., 2017).

The objective of this work was to evaluate the inclusion in the intestinal phase of the Infogest digestion protocol of a BBM extract and to assess the effect on the resulting cleavages in

peptides by using milk whey proteins and casein. A comparison of resistant sequences and generated cleavages with those previously identified in jejunal aspirates of volunteers upon intake of the same substrates was conducted. Six structurally related sequences able to survive gastrointestinal digestion including BBM peptidases and proved to survive in human jejunum were selected to assay their *in vitro* bioavailability.

2. MATERIALS AND METHODS

2.1 Samples and reagents

Micellar casein and whey proteins separated by microfiltration and freeze-dried were used. The protein content (85% for caseins and 72% for whey proteins) was determined by Kjeldahl. A brush border membrane extract was isolated from porcine jejunum as reported by Picariello et al. (2015). Peptide synthesis of sequences VYPFPGPIPN, YPFPGPI, PGPIPN, FPGPI, VYPFPGPI, and YPF was performed by Fmoc solid-phase synthesis by Chengdu KaiJie Biopharm Co. Ldt (Chengdu, Sichuan, P.R. China). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. *In vitro* simulated gastrointestinal digestion

Casein and whey milk protein were digested according to the in vitro harmonized protocol (Brodkorb et al 2019). Thus, the products were dissolved at 6% (p/v) and subjected to the oral, gastric and intestinal phases. The oral phase included no amylase and the BBM extract was added at 15 or 45 µU of aminopeptidase activity/µg of protein in the intestinal phase. A control digestion without addition of BBM was used for each product. Digestions were performed in triplicate.

2.3 Free amino acids determination

Protein from freeze-dried digests was precipitated with 4-sulphosalicylic acid (12.5 mg/mg of protein) by keeping 1 h on ice. Centrifugation for 15 min at 15000 × g, 4ºC, was followed by filtration through 0.45 µm membranes and adjustment to pH 2.2 with 0.3 M NaOH. Amino acid

analysis was performed on a Biochrom 30 amino acid analyser (Biochrom Ltd, Cambridge, UK). Tryptophan, glutamine and asparagine were not determined under our analysis conditions. Comparison of independent averages (t-test) was performed to determine significant changes in the amino acid concentration.

2.4 Peptide identification and quantification by tandem mass spectrometry (HPLC-MS/MS)

HPLC-MS/MS analyses were performed on an Agilent 1100 series HPLC separation system (Agilent, CA, USA) coupled on line with an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). The analytical column used was a Mediterranea Sea 18 (5µm, 15cm x 0.21cm, Teknokroma, Barcelona, Spain). For peptide identification in digests, elution with a linear gradient of solvent B (acetonitrile: formic acid, 100:0.1, v/v) in solvent A (water:formic acid, 100:0.1, v/v) was used as previously reported (Sanchón et al., 2018). Data were processed with the software programs Data Analysis 4.0 and Biotools 3.2 from Bruker Daltoniks. A homemade database for milk proteins was used in MASCOT software for peptide sequencing. For peptide quantification, calibration curves with the synthetic peptides in the 1-80 µM range were conducted in duplicate. The elution gradient went from 0 to 45% eluent B in 60 min and mass spectra were acquired in the 100-1200 m/z range with target mass fixed at 750.

2.5 Transepithelial transport of peptides

Caco-2 culture

Caco-2 cells purchased from ATTC (Rockville, MD, USA) were used between passages 45 and 55. They were maintained in the Dulbecco's Modified Eagle's Medium (DMEM) with high glucose content (Biowest, Kansas City, MO, USA) supplemented with 10% FBS heat-inactivated (Biowest), 1% non-essential amino acids (Lonza group Ltd, Basel, Switzerland) and penicillin/streptomycin (Biowest) at pH 7.2-7.4. For transport studies the protocol from Hubatsch et al. (2007) was followed. Cells were seeded at a density of $2{,}6x10^5$ cells cm^{-2} in 12-

well filter support inserts (Costar®Transwell® Permeable Supports 12 mm Diameter insert 12 well 0.4 um polycarbonated Membrane, Corning, NY, USA). The integrity of the cell monolayers was periodically tested measuring the transepithelial electrical resistance (TEER) with Evom^2 Epithelial Voltammeter (World Precision Instruments). Monolayers with TEER over 400 Ω x cm^2 were considered eligible to perform the experiments.

Transport experiment

Experiments were performed 21-29 days after seeding. Medium was changed 24h before the assay. Inserts were washed with DMEM and with transport medium (HBSS) and then moved to new 12-well clusters filled with 1.5 mL of HBSS. Then 500 µl were added to the apical side and the filters were incubated at 37°C for 20 min. Peptides were dissolved in HBSS, at 1 mM concentration and filtered on 0.2 µm filters. Then, inserts were moved to new 12-well clusters with 1.5 mL of HBSS, on the apical side were added 500 µl of the sample or of HBSS for the blanks. TEER was measured immediately and, for each well, 100 µl were collected and frozen. The plate was incubated for one hour at 37°C. TEER was measured immediately and samples from the apical and basolateral sides were collected, freeze-dried and stored at -80°C. The assay was performed in triplicate. For the HPLC-MS/MS analysis samples were reconstituted in eluent A at different concentrations: the apical side collected before the incubation (t0) and after 60 min was reconstituted in 100 µl and then diluted 1:12, the basolateral samples were reconstituted in 100 µl of eluent A. The apparent permeability coefficient (P_{app}) was determined with the following equation:

$$P_{app}=\Delta Q/\Delta t \times 1/A \times 1/C_0$$

were $\Delta Q/\Delta t$ is the transport rate in µmol/s, A is the membrane surface (1,12 cm^2) y C_0 is the added concentration of peptide in the apical chamber (µmol/mL).

Lucifer yellow assay

Lucifer Yellow was used to test the integrity of the Caco-2 monolayers. A solution of 50 µM Lucifer yellow in HBSS was prepared and filtered on 0.2 µm filters. After the assay with samples, inserts were washed 3 times with HBSS and moved to another plate with 1.5 mL of HBSS in the basolateral side. On the apical side, 500 µl of the Lucifer yellow solution was added and TEER was measured. After one hour incubation at 37°C, TEER was measured again and 300 µl of the apical and basolateral side were collected and stored at -80°C. For each collected sample (both apical and basolateral side after one hour incubation and apical site at 0 min of incubation), 100 µl were used for the fluorescence measurement (Costar® Assay plate 96 well, Corning, NY, USA). A calibration curve was prepared with solutions of Lucifer Yellow at different concentrations: 200 µM, 100 µM, 50 µM, 10 µM, 5 µM, 1 µM, 0,5 µM, 0,25 µM, 0,1 µM. Fluorescence was measured using a Fluostar Optima microplater reader (BMG Labtech, Ortenberg, Germany) setting 480 nm of excitation and 520 nm of emission. The analysis was performed in duplicate. The values were used to confirm the integrity of the monolayer during the experiment when less than 1% of the added amount in the apical chamber was found in the basolateral (Broeders, van Eijkeren, Blaauboer, & Hermens, 2012).

3. Results

3.1. Comparative peptide analysis of gastrointestinal digests

Both casein and whey proteins were digested following the INFOGEST protocol, as it is, and with the addition of a porcine BBM extract at 15 or 45 µU/µg of protein in the intestinal phase. The number of irredundant identified peptides in control digestion of casein (210) or whey (111) was reduced with the addition of BBM at both concentrations. After casein digestion, 127 and 88 unique sequences were identified when 15 or 45 µU/µg of BBM were used, respectively. In whey, 68 and 67 sequences were correspondingly identified for the 15 or 45 µU/µg BBM addition. The decreasing figures evidence the progression in the hydrolysis to lower size peptides and amino acids. Among the identified sequences, the peptide size ranged from 5 to

13 amino acid residues for casein, but was narrowed to 5 to 9 amino acids in the case of 45 µU/µg BBM addition (Figure 1). The relative proportion of five residues peptides increased from 39 to nearly 50% with the inclusion of BBM. In contrast to casein, the peptide size range in whey digests remained at all conditions in 5 to 11 amino acids, despite effective reduction in the number of identified peptides after BBM addition. The relative portion of five residues peptides did not change greatly but reduced percentage of eight and nine-residues peptides were noted when using 45 µU/µg BBM. This points to a different effect on the compared substrates, with higher resistance of medium-size sequences from whey proteins α-lactalbumin and β-lactoglobulin to degradation by the microvillus enzymes.

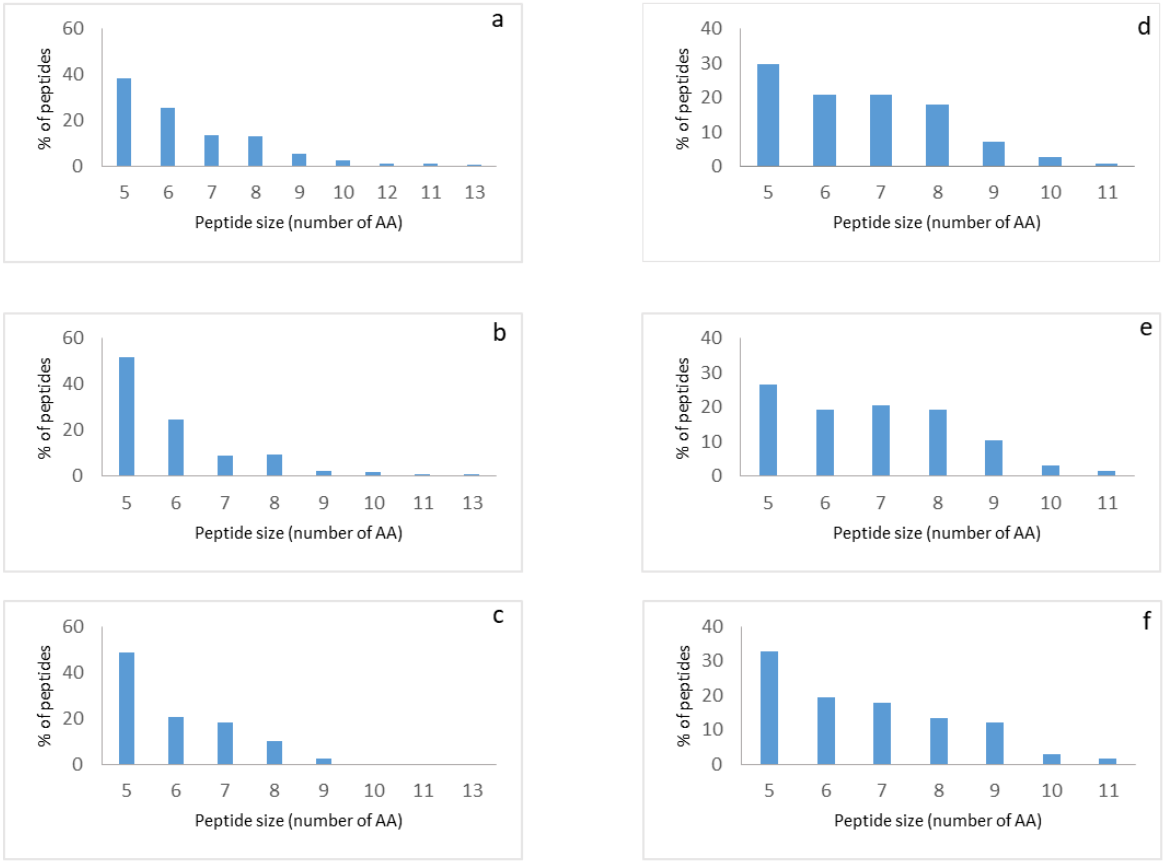

Figure 1. Percentage and size of peptides identified in casein (left side) and whey (right side) digestion by the use of the Infogest protocol (a), (d), Infogest-BBM15 (b), (e) and Infogest-BBM45 µU/µg of protein (c), (f).

Peptides released from proteins with highest coverage, β- and α_{s1}-casein, or β-lactoglobulin and α-lactalbumin, are shown in Supplementary Figures 1 and 2 (Supplementary Material). Their alignment in the primary sequence of the parent protein allowed to comprehensively detect the differing cleavages between conditions. In β-casein N-terminal region, ^{1}RELEEL^{6}, the generated sequence when BBM were added at any concentration, presented a Glu at the N-terminal position while the Arg-Glu cleavage was not observed without the enzymatic extract. Exclusive cleavages generated by BBM were also detected in the final Pro-Asn in ^{59}VYPFPGPIPN^{68}, or the Pro-Gln cleavage in the ^{170}VLPVPQ^{175} fragment of β-casein. Similar examples were observed as well in α_{s1}-casein, β-lactoglobulin and α-lactalbumin and the resulting peptides are shown in Table 1. Many of these new cleavages corresponded to the activity of aminopeptidases on fragments previously generated by pancreatic enzymes. The aminopeptidase family of proteases is involved in the proteolytic processing of precursor proteins to produce biologically active peptides and hormones. These include dipeptidyl-peptidase, prolyl-oligopeptidase, aminopeptidase N and aminopeptidase B (Hooton et al 2015). The activity of other oligopeptidases present in the BBM extract, such as carboxypeptidases and endopeptidases was also evidenced in the new cleavages observed. By contrast, the activity of dipeptidases should be tackled with an alternative analytical approach suited to determine dipeptides. The observed results illustrate the ability of the present simulation to mimic the combined action of a suite of different enzymes on the digestion products of proteins.

Table 1. Peptide sequences exclusively generated by BBM after digestion of casein and whey with the Infogest protocol with addition of BBM at 15 or 45 μU/μg in comparison with the corresponding sequences in control digestions.

CONTROL		**Exclusively generated by BBM**	
Protein (range)	**Sequence**	**Protein (range)**	**Sequence**
β-CN (1-6)	**RELEEL**	β-CN (1-5)	ELEEL
β-CN (6-12)	**LNVPGEI**	β-CN (6-11) β-CN (7-11)	**LNVPGE** **NVPGE**
β-CN (36-44)	**EEQQQTEDE**	β-CN (35-40)	SEEQQQ
β-CN (39-44) β-CN (39-45) β-CN (39-47)	QQTEDE QQTEDEL QQTEDELQD	β-CN (40-45) β-CN (41-45)	QTEDEL TEDEL
β-CN (58-66)	**LVYPFPGPI**	β-CN (60-65)	**YPFPGP**
β-CN (67-72)	HNSLPQ	β-CN (67-71)	HNSLP
β-CN (108-113)	**EMPFPK**	β-CN (109-113)	**MPFPK**
β-CN (137-143)	LPLLQSW	β-CN (137-142)	LPLLQS
β-CN (170-175)	**VLPVPQ**	β-CN (170-174)	**VLPVP**

β-CN (193-201)	**YQEPVLGPV**	β-CN (195-201)	**EPVLGPV**
α_{S1}-CN (8-13)	**HQGLPQ**	α_{S1}-CN (8-12)	**HQGLP**
α_{S1}-CN (25-30)	**VAPFPE**	α_{S1}-CN (26-30)	**APFPE**
α_{S1}-CN (173-178)	YTDAPS	α_{S1}-CN (174-178)	**TDAPS**
κ-CN (106-111)	MAIPPK	κ-CN (107-111)	AIPPK
κ-CN (125-131)	IASGEPT	κ-CN (126-131)	ASGEPT
κ-CN (142-148)	TVATLED	κ-CN (142-146)	TVATL
α-lactalbumin (48-55)	TEYGLFQI	α-lactalbumin (49-55)	EYGLFQI
β-lactoglobulin (110-116)	**SAEPEQS**	β-lactoglobulin (111-116) β-lactoglobulin (112-116)	AEPEQS **EPEQS**
β-lactoglobulin (127-135)	**EVDDEALEK**	β-lactoglobulin (130-134) β-lactoglobulin (130-135) β-lactoglobulin (131-135)	**DEALE** DEALEK **EALEK**

3.2. Free amino acid profile

The fraction of free amino acids was isolated in all digests. Figure 2 shows the concentration of each amino acid residue in control digests of the assayed substrates. Whey digests, presented higher overall concentration of free amino acids than casein digests, with prominent differences in Leu, Ile, Val, and Lys, with values nearly two-fold higher in whey than in casein digests. One possible explanation could be the higher abundance of these amino acids in whey proteins in comparison to casein. However, this is not the case for Val and Ile (Rasmussen, Greenwood, Kalman, & Antonio, 2008). On the other hand, whey presents 20 and 30% more Lys and Leu that casein, respectively. The observed differences between substrates confirm the selective effect of pancreatic enzymes on the release of particular amino acids, with the resultant impact on the rate of digestion and absorption of the different dietary proteins. The superior availability of amino acids in whey, in particular some branched chain amino acids, have been claimed to produce an anabolic response in humans driven by a stimulation of muscle protein synthesis that might counteract the loss of skeletal muscle mass in ageing (Bulkhari et al 2015) or maximize performance in athletes (Rasmussen, 2008; Wolfe, 2017). However, the digestion rate is a crucial parameter also involved and the postprandial leucine balance, an index of protein deposition, is better with casein as a slow protein class (Dangin et al., 2001).

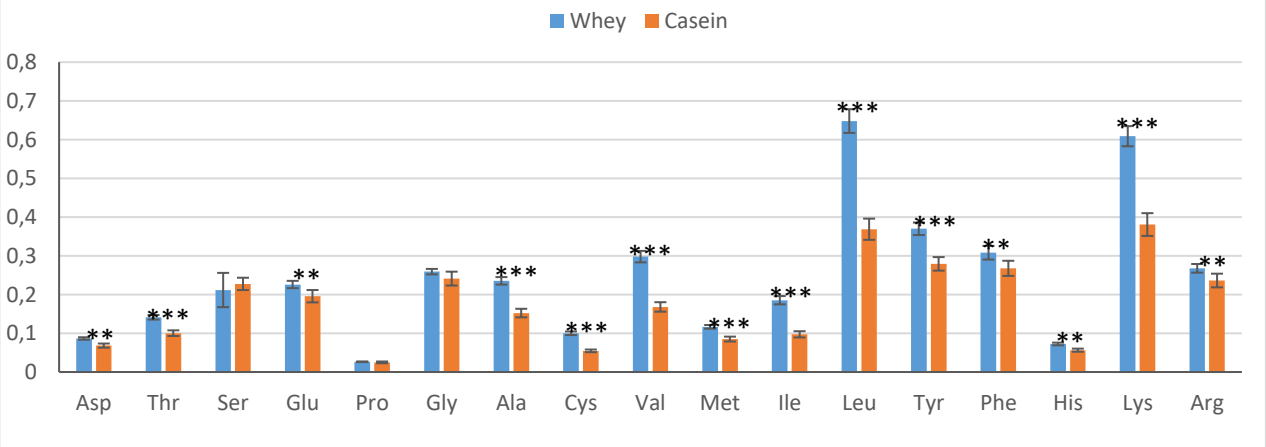

Figure 2. Free amino acid concentration (nmol/µL) in control whey and casein digests.

Table 2 shows the determined amino acid values for each BBM concentration versus the values in the control digestion. All amino acids except Gly, Phe and Lys raised their amount in digests at both BBM concentrations, and those showing no significant change tended to increase or kept their concentration. These results support the important contribution of amino- and carboxypeptidases to the amino acid release in a complex mixture of peptides during the hydrolysis progression. In both substrates the greatest increase corresponded to Ser, and to a lesser extent to Pro, Ala, Val, and Ile. This will have implications both in the final forms reached by peptides in terms of the terminal residues at N or C positions, as well as in the supply of these particular amino acids and should be contrasted with values *in vivo*. Indeed, the postprandial blood levels of amino acids appear to be mediated, at least in part, by the gastrointestinal behavior (Luiking et al., 2016).

Table 2. Free amino acid concentration (nmol/mg of protein) in casein and whey digests

Casein	CONTROL	BBM 15		BBM45	
Asp	1,36	1,65	**	1,73	***
Thr	2,01	2,47	***	2,44	***
Ser	4,55	7,30	***	7,33	***
Glu	3,91	4,51	**	4,41	**
Pro	0,50	0,93	***	1,48	***
Gly	4,82	5,27	ns	4,80	ns
Ala	3,04	4,07	***	4,18	***
Cys	1,09	1,19	*	1,06	ns
Val	3,36	4,56	***	4,64	***
Met	1,70	2,18	***	2,14	***
Ile	1,95	2,76	***	2,92	***
Leu	7,37	8,66	**	8,20	**
Tyr	5,59	6,18	**	5,72	ns
Phe	5,35	5,82	*	5,20	ns
His	1,13	1,51	***	1,59	***
Lys	7,62	8,31	*	7,72	ns
Arg	4,72	5,15	*	4,75	ns

Whey	CONTROL	BBM 15		BBM45	
Asp	1,73	1,91	***	2,31	***
Thr	2,83	3,07	***	3,39	***
Ser	4,23	5,49	***	8,63	***

Glu	4,52	4,79	**	5,32	***
Pro	0,53	0,77	***	1,27	***
Gly	5,18	5,36	ns	5,41	ns
Ala	4,71	6,01	***	7,13	***
Cys	2,00	2,17	**	2,31	***
Val	5,96	6,96	***	7,76	***
Met	2,33	2,69	***	2,86	***
Ile	3,71	4,51	***	5,56	***
Leu	12,96	14,31	***	14,60	***
Tyr	7,40	7,62	*	6,96	**
Phe	6,16	6,53	ns	6,52	ns
His	1,45	1,70	***	1,89	***
Lys	12,18	12,48	ns	12,20	ns
Arg	5,35	5,63	**	5,42	ns

3.3. Comparison with peptide profile in human jejunal aspirates

A comparison of sequences identified in the simulated digests with those previously reported in human jejunum was performed (Sanchón et al 2018). In casein, only the identified sequences from β- and α_{s1}-casein were considered for comparison as they represent nearly 90% from the total number of peptides identified both in human jejunum and in the simulated digests. Figure 3 shows Venn diagrams with sequences in human jejunum and those found in our study with or without the addition of BBM to the INFOGEST protocol of digestion. In the case of casein, shared sequences between the INFOGEST protocol and human jejunum represented 14.5% of the total identified peptides while the INFOGEST protocol with addition of BBM resulted in 12.2% of common sequences. In the case of whey proteins β-lactoglobulin and α-lactalbumin, shared sequences between the INFOGEST protocol and human jejunum represented 14.9% and with addition of BBM this percentage resulted in 13.9%. Each simulation allowed the identification of a number of exclusive sequences identical to those found in human jejunum, which evidences their complementary power. However, the addition of the microvilli enzymes under the present conditions is not leading to a closer approximation to the peptide profile found *in vivo*. The number of identified sequences is notably higher in the case of the *in vivo* digest. This can be due to the inter-individual human variability but also digestion conditions *in vitro* might not be

finest in terms of concentration or agitation for the release of all possible fragments, as it was previously observed (Sanchón et al., 2018). In Table 1, peptides previously identified in human jejunum identical to those found in the present study are marked in bold. Biological activity has previously shown for some of these relevant sequences. Thus, fragment EMPFPK presents antibacterial properties (Sedaghati et al., 2016), fragment LVYPFPGPI includes the opioid β-casein fragment 60-66, β-casomorphin (Brantl et al., 1981), and sequence VLPVP greatly overlaps with the antihypertensive β-casein fragment 169-174 (Maeno et al., 1996).

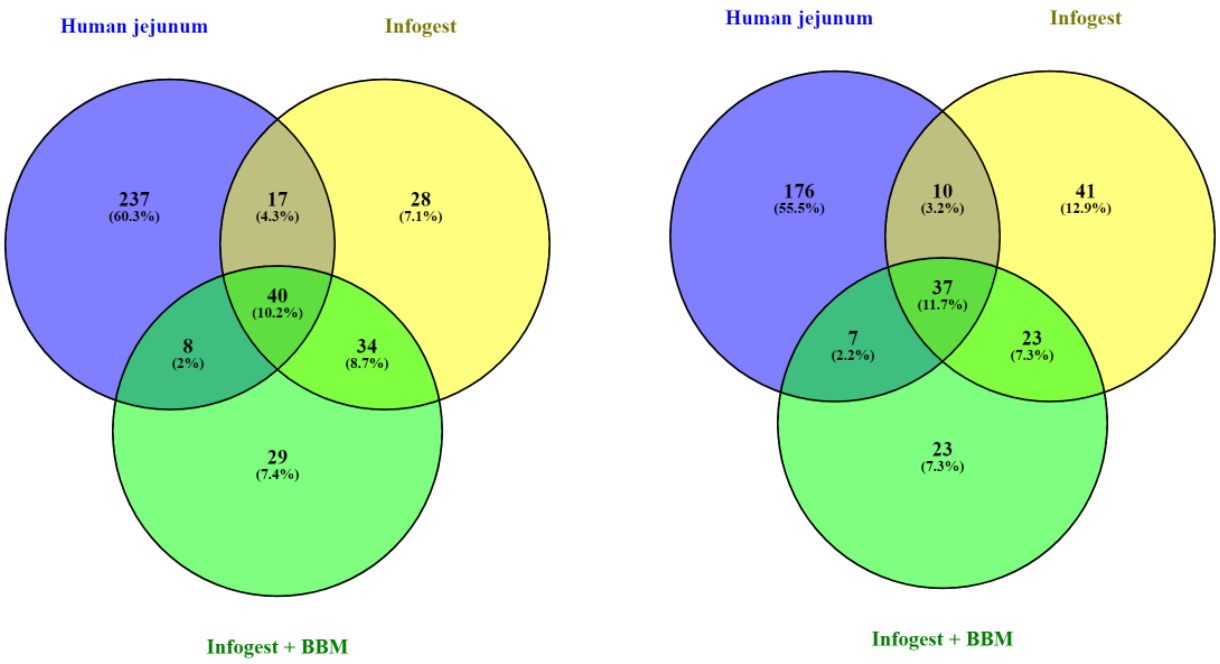

Figure 3. Venn diagram of peptide sequences identified in human jejunum (Sanchón et al., 2018), and in vitro digestion of casein (A) and whey (B).

3.4 Transport of selected sequences

The β-casein region between residues 58 and 66 is one of the recurrent domains in the resistant peptides from casein, and is the source of many related sequences in the simulated digestions. In the present study, at least seven peptides could be identified belonging to this domain, with and without the addition of BBM. More importantly, many peptides comprised in this region have been identified the human jejunum after casein ingestion, both in our previous study

(Sanchón et al., 2018) as in an earlier report (Boutrou et al., 2013). This region includes the opioid peptide β-casomorphin-7, corresponding to fragment ^{60}YPFPGPI^{66} (Brantl et al. 1981). The *in vitro* transepithelial transport of this sequence and five longer and shorter related forms identified as well in the human jejunum was performed, with the aim to evaluate the transport ability of sequences with proven resistance to enzymatic degradation. These sequences had been preliminarily assayed both at 1 mM and 5 mM in order to confirm that no effect on the integrity of the monolayer could be ascribed to their application.

The quantification of peptides both in the apical and basolateral solution was possible by the analysis by HPLC-MS/MS of calibration curves prepared with five-point dilutions of the synthetic peptides. Figure 4 shows the apparent permeability values (P_{app}) calculated for the apical-to-basolateral transport. All assayed sequences were absorbed through the cell monolayer, although a wide variety in the P_{app} was observed, with values in the range 0.126 to 4.98 x 10^{-6} cm/s. P_{app} values above 4 x 10^{-6} cm/s were reached by sequences with the motif IPN at terminal position, which raises the question on the capacity of such C-terminal end to favor the transference through the Caco-2 cell monolayer. Some authors have reported the importance of specific amino acids to the transport of oligopeptides (Ding et al. 2017). In C-terminal position, Tyr, Lys, Asn or Arg were reported as beneficial for the transport of tetra- and pentapeptides. Thus, the presence of Asn in the assayed sequences, despite the higher number of amino acids, might have favored their passage to the basolateral chamber. Val and Pro at N-terminal position were also suggested by these authors as important for a good permeability, related to their nonpolar hydrophobic character, and are the N-terminal end residues shown by the three best transported peptides, i.e., fragments VYPFPGPIPN, PGPIPN, and VYPFPGPI. The susceptibility of the assayed sequences to the action of epithelial peptidases is assumed to play a role in observed results. The concentration of peptide in the apical medium after 60 min indicated resistance of 39, 23, and 33% for fragments YPFPGPI, FPGPI, and YPF, respectively, as opposed to 75 and 50% for sequences VYPFPGPIPN and PGPIPN, respectively. Survival of fragment

VYPFPGPI as intact peptide (100% resistance) along the incubation was observed and well-suited to its previously reported prolyl-peptidase inhibitory activity (Asano et al., 1992). In view of the different endurance of the assayed sequences, lower transport was clearly linked to higher susceptibility to cell proteases of peptides YPFPGPI, FPGPI, and YPF, as compared to the rest of the sequences. A report on the *in vitro* transport of β-casomorphin-7 detected this peptide when a concentration of 2 mM was used, in contrast to lower concentrations, with a permeability of 0.13×10^{-6} cm s^{-1} (Osborne et al., 2014). In our study, the P_{app} reached 0.65×10^{-6} cm s^{-1} with a 1 mM concentration, but the application time was 60 min instead of the time of 30 min used in the previous study. The low permeability rates observed in the former and present study confirm the impaired transport of this opioid peptide. To date, intact β-casomorphin-7 has not been detected in adult human plasma following ingestion of dairy products containing casein (EFSA Report). Only the presence of this peptide in infant plasma following ingestion of formula containing bovine milk was reported (Kost et al., 2009). On the other hand, the notably transported hexapeptide ^{63}PGPIPN^{68} has been described as immunomodulatory and might play a role in the proliferation and maturation of T cells and natural killer cells of the neonate against a wide range of bacteria (Migliore-Samour and Jollès, 1987). This peptide has been shown to be

present in infant formula and survives digestion (Wada and Lönnerdal, 2015). Therefore, the absorption of this peptide in the newborn might have a protective role.

Conclusions

The inclusion of a BBM extract in the intestinal phase of the Infogest protocol shows a reduction in the number of peptides, and relative size decrease, with a greater effect on caseins compared to whey proteins. However, although exclusive sequences are found with the inclusion of BBM, no improvement in the similarity to peptides found in the human jejunum is observed. However, the use of BBM in the digestion protocol can be complementary to the use of pancreatic enzymes, and the sum of the pancreatic generated peptides together with those released by the action of BBM gave a closer situation to the human digestion. In addition, the use of BBM can be recommended when the study of the survival of certain sequences is the goal, for instance, prior to absorption studies or in the evaluation of protein allergenicity. The progressed hydrolysis to small peptides and amino acids in the BBM digests has been evidenced in the increase in the free amino acid fraction with the addition of the BBM extract, being Ser, and to a lesser extent to Pro, Ala, Val, and Ile the most enriched residues. *In vitro* transepitelial transport of six β-casein related sequences, including the opioid β-casomorphin-7, with proved

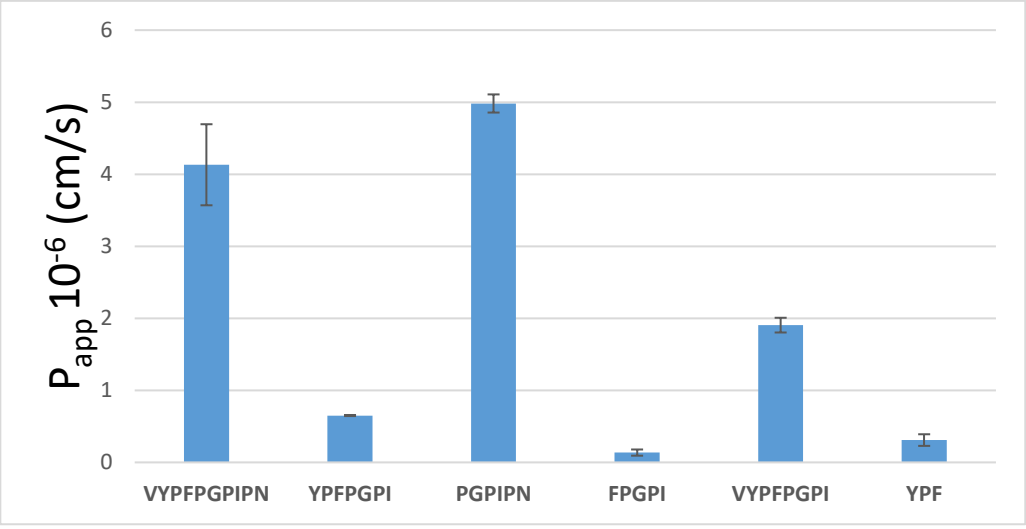

Figure 4. Apparent permeability values calculated for the apical-to-basolateral transport of the assayed peptides in the Caco-2 monolayer (n=3).

digestion resistance and previously found in human jejunum was assayed. From the relationship between the structure of the studied sequences and their transport rate, amino acid residues Asn at C-terminal and Val and Pro at N-terminal, were shown to impart higher resistance to brush-border peptidases and can be considered important features for these sequences to be absorbed.

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Milk peptidomic profile after *in vitro* gastrointestinal digestion including intestinal brush border membrane peptidases. Transepithelial transport of digestion resistant casein domains.

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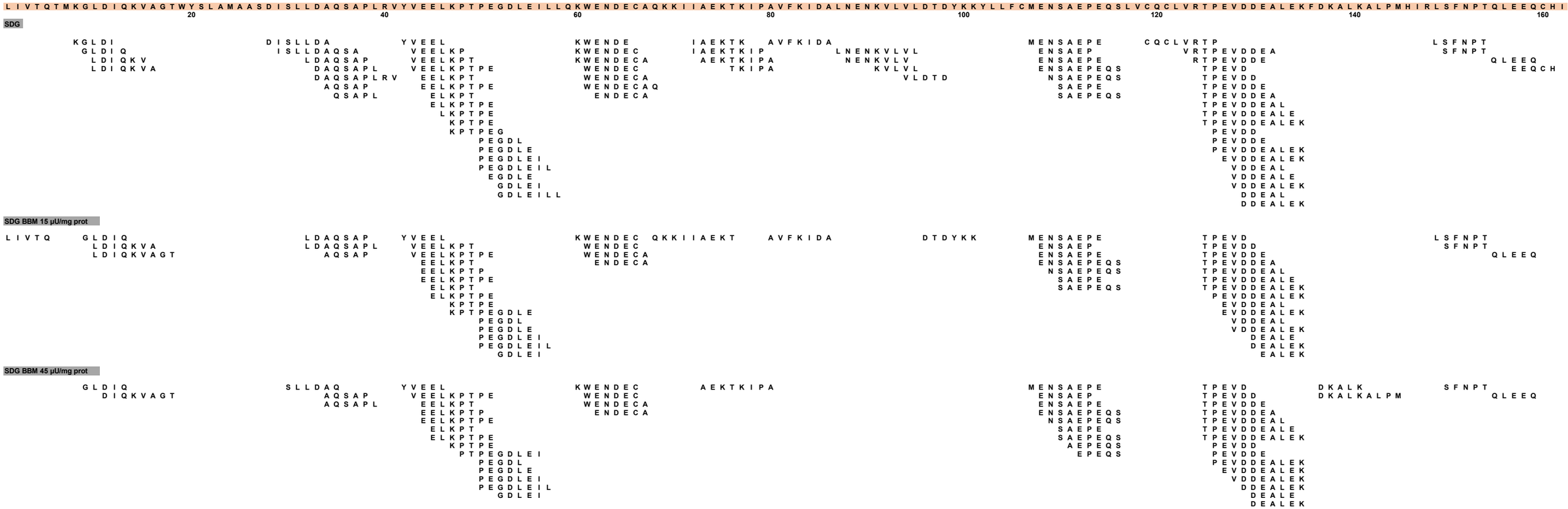
β-casein

α_{s1}-casein

Figure S1. Identified peptides from β-casein and α_{S1}-casein after casein digestion in different conditions: “SDG” a control digestion using the harmonized protocol; “SDG BBM 15 µU/mg prot” the SDG digestion adding to the intestinal fluid a brush border membrane extract, BBM (15 µU/mg prot); “SDG BBM 45 µU/mg prot” the SDG digestion adding to the intestinal fluid BBM (45 µU/mg prot).

β-Lactoglobulin



SDG BBM 15 µU/mg prot

SDG BBM 45 µU/mg prot

α-Lactalbumin

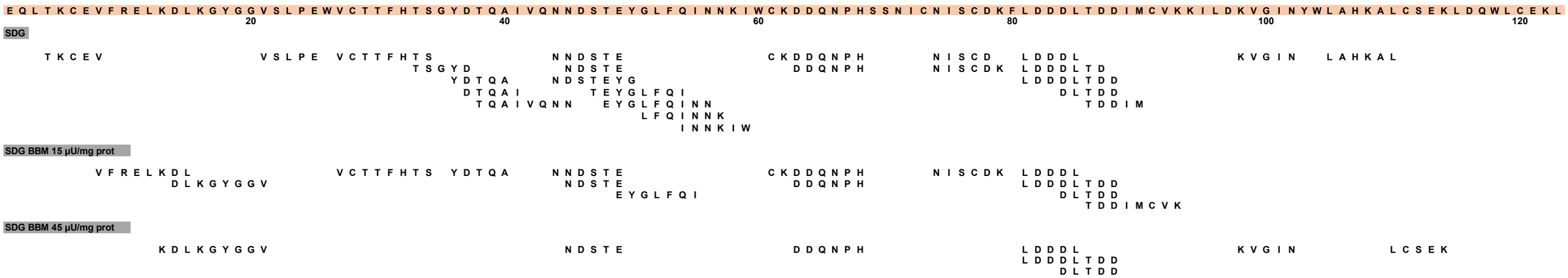


Figure S2. Identified peptides from β-lactoglobulin and α-lactalbumin after whey digestion in different conditions: “SDG” a control digestion using the harmonized protocol; “SDG BBM 15 µU/mg prot” the SDG digestion adding to the intestinal fluid BBM (15 µU/mg prot); “SDG BBM 45 µU/mg prot” the SDG digestion adding to the intestinal fluid BBM (45 µU/mg prot).

2.5 Publicación V: **Transepithelial transport of lunasin and derived peptides: Inhibitory effects on the gastrointestinal cancer cells viability.**

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Original research article

Transepithelial transport of lunasin and derived peptides: Inhibitory effects on the gastrointestinal cancer cells viability

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ABSTRACT

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 assay suggested that the cell line type was determinant in the behavior of lunasin added to the culture medium, and therefore in the anti-proliferative activity of released fragments.

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 (Shimizu and Hachimura, 2011). 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 (Segura-Campos et al., 2011). 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 (Sánchez-Rivera et al., 2014). 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 (Deferme et al., 2008). Among the several routes described for the transport of peptides in the gut, the main mechanisms include the PepT1, a proton-coupled membrane transporter (Brodin et al., 2002), the paracellular passive pathway through intercellular TJs, the transcellular passive diffusion, and the vesicle-mediated transcytosis (Ziv and Bendayan, 2000). For oligopeptides, susceptibility to brush-border peptidases has been recognised 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 (Shimizu et al., 1997).

Lunasin is a 43-amino acid peptide naturally present in soybean, with a variety of biological functions demonstrated by cell cultures and animal models (Fernández-Tomé and Hernández-Ledesma, 2016). *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 (Jeong et al., 2007; Park et al., 2007). Moreover, Hsieh et al. (2010) showed that lunasin was bioavailable

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0889-1575/

when orally administered to mice and rats, and Dia et al. (2009) 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 (Picariello et al., 2015), 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 iso-inhibitor 1 (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 (Cruz-Huerta et al., 2015). Thus, the aims of the present study are i) to investigate whether lunasin and selected lunasin-derived fragments arising from its digestion are resistant to brush-border peptidases and susceptible to intestinal transepithelial transport in Caco-2 monolayers, identifying the potential mechanism involved in the intestinal absorption by using selective inhibitors. and ii) to evaluate the anti-proliferative effect of lunasin and released peptides on human adenocarcinoma gastric (AGS) and colorectal (HT-29 and Caco-2) cells.

2. Materials and methods

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. The purity of synthetic peptides was determined by HPLC–MS analysis through peptide peak area integration.

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 °C 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. (2007). 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 (García-Nebot et al., 2014).

2.3.2. Transepithelial transport of peptides

Transport experiments were performed as described by Quirós et al. (2008), with some modifications. Cell monolayers were rinsed with DMEM and transferred into new 12-well plates (Costar) containing HBSS (1.5 mL) in the basolateral side to remove interferences with medium components. HBSS (0.5 mL) was carefully added to the apical side, and monolayers were equilibrated at 37 °C 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 °C for 60 min, and apical and basolateral samples were withdrawn, freeze-dried, and kept at − 20 °C 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 °C for 30 min. Afterwards, Lucifer Yelow in HBSS (50 μM) was added to the apical compartment and incubated at 37 °C 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

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. (2015).

Lucifer Yellow transport (<1% added to apical chamber) were used as parameters to confirm the Caco-2 monolayer integrity during experiments (Broeders et al., 2012). 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. (2012) 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 (Quirós et al., 2008). 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 (Cruz-Huerta et al., 2015), 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) (Wang et al., 2016), and 5×10^4 cells/well (Caco-2 and HT-29) (Dia and de Mejia, 2010). 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 10× magnification, coupled to a camera Leica DFC420C (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 analysed 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 (Cruz-Huerta et al., 2015). 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 proven resistance to the gastrointestinal enzymes. In order to evaluate a possible structure/activity relationship, peptide lunasin and these four fragments integrating the complete 43-amino acid sequence were assessed for their resistance to brush-border peptidases and their intestinal transport by using Caco-2 monolayers.

The integrity of the 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 while lunasin at 50–1000 μM resulted in a reduction of the TJs strength indicating altered integrity of the monolayers. However, 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 viability during the period of incubation of this peptide with differentiated human

Caco-2 (García-Nebot et al., 2014), and liver HepG2 cells (Fernández-Tomé et al., 2014). Thus, 10 μM was the lunasin's concentration selected to carry out the following transport studies. As illustrated in Fig. 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, 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 (Cruz-Huerta et al., 2015). Similarly, some food-derived antihypertensive peptides from milk caseins such as VPP and IPP (Ohsawa et al., 2008), and HLPLP (Quirós et al., 2008), and from egg white QIGLF (Ding et al., 2014), and the immunomodulatory peptide β-casein f(193–209) (Regazzo et al., 2010) have shown remarkable resistance against intestinal brush-border peptidases. As shown in Fig. 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-stage hydrolysis process simulating gastrointestinal digestion in absence of IBB1, but enclosed and protected from the enzymatic action when the protease inhibitor was present (Cruz-Huerta et al., 2015).

In the case of the lunasin-derived peptides, the fragments found were mostly formed as consequence of cleavages at the N-terminus (Fig. 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 activity, especially when Caco-2 cells are completely differentiated (Howell et al., 1992). 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 (Mentlein, 1999). In our study, 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

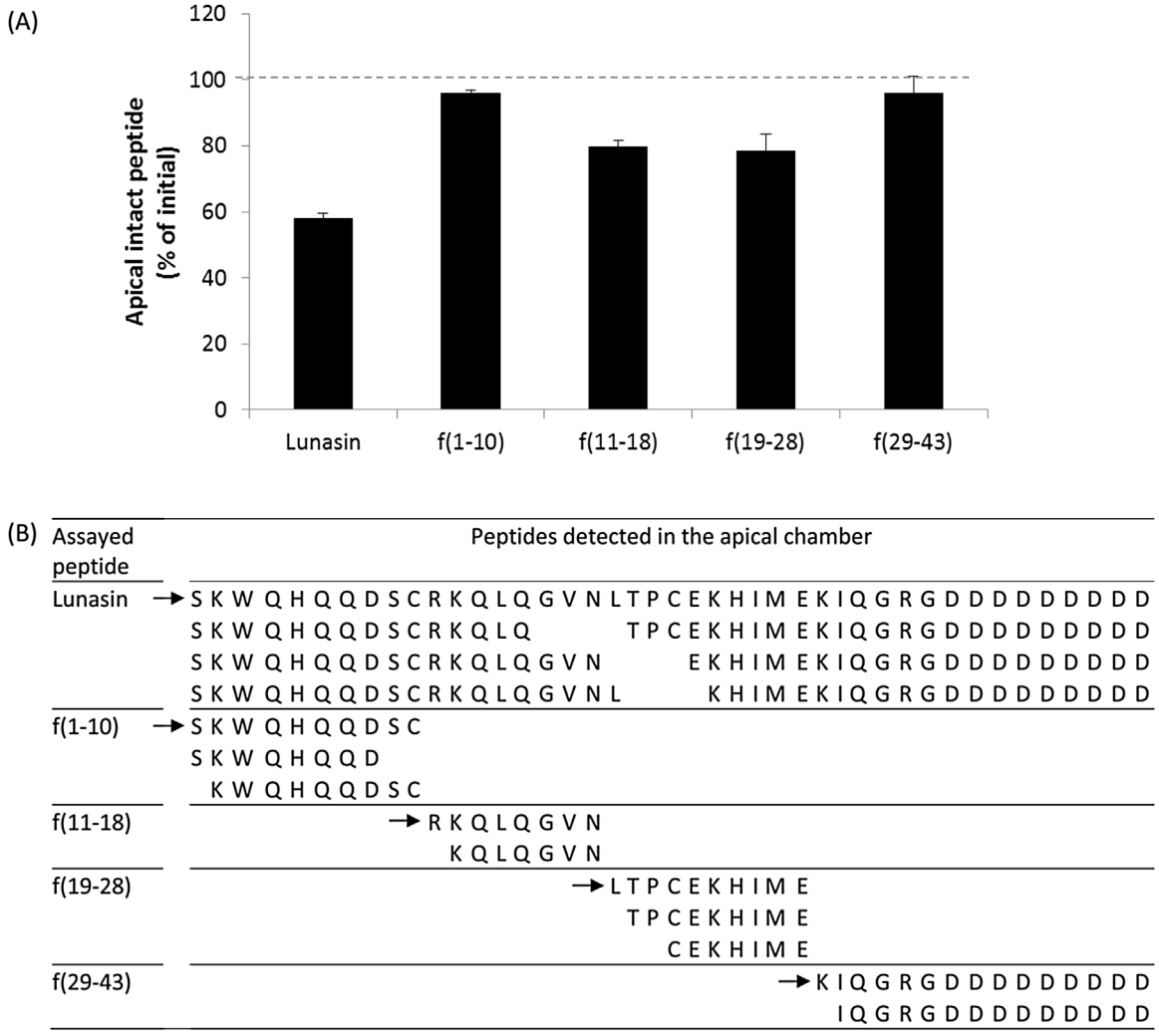

Fig. 1. Behaviour of lunasin and lunasin-fragments added to Caco-2 monolayers. Analysis by HPLC–MS/MS of peptides in apical solutions after incubation of lunasin 10 μM and lunasin-fragments 1000 μM with Caco-2 monolayers for 60 min. (A) Remaining intact peptide in apical solutions was evaluated by the 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.

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 (Quirós et al., 2008), FRADHPFL (Miguel et al., 2008), RYLGY and AYFYPEL (Contreras et al., 2012), and RVPSL (Ding et al., 2015) in Caco-2 monolayers.

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 (Sienkiewicz-Szlapka et al., 2009), and human (Iwan et al., 2008) origins that nearly disappeared after incubation in the apical side of Caco-2 monolayers. These results imply the relevance of studying the resistance of bioactive peptides to the epithelial brush-border membrane as first attempt to evaluate their *in vivo* bioavailability.

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 lunasin and derived-peptide 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 (Fig. 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) (Contreras et al., 2012), and VLPVP (2.78×10^{-7} cm/s) (Lei et al., 2008). 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) (Foltz et al., 2008), RVPSL (6.97×10^{-6} cm/s) (Ding et al., 2015), and SRYPSY (9.21×10^{-6} cm/s) (Sienkiewicz-Szlapka et al., 2009).

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 (Shimizu and Son, 2007). Peptides longer than three amino acids residues have been described as not substrates for the PepT1 transporter (Vig et al., 2006). 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 (Quirós et al., 2008). As illustrated in Fig. 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) (Regazzo et al., 2010). 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 (Regazzo et al., 2010). The paracellular route is characterised by the passive diffusion of molecules between adjacent cells, and is regulated by the intercellular TJs forming a

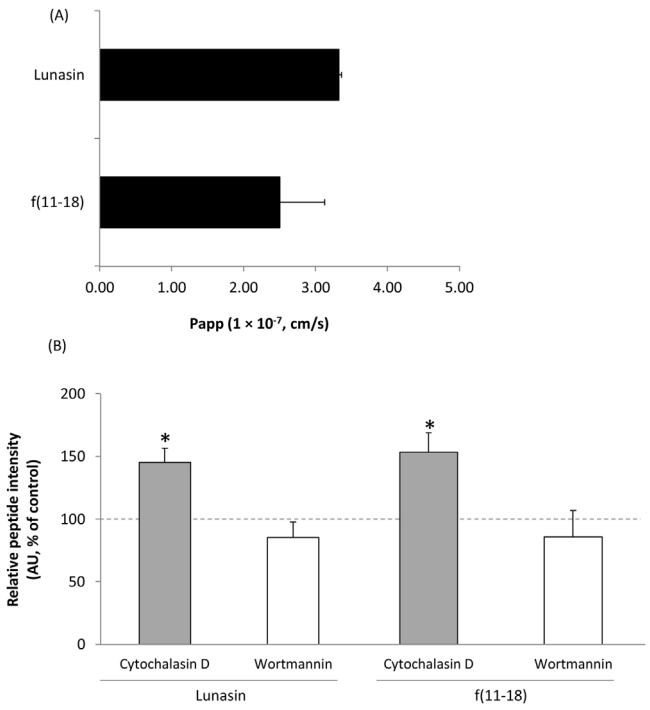

Fig. 2. Transepithelial transport of peptides lunasin and f(11–18), RKQLQGVN. (A) Peptides were analysed by HPLC–MS/MS in the basolateral solutions after incubation of 10 μM lunasin and 1000 μM lunasin-fragment f(11–18). The apparent permeability coefficient (P_{app}) was calculated as described in Materials 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.

biological barrier with selective permeation function (Segura-Campos et al., 2011). The aqueous nature of this pathway makes it favourable for the absorption of water-soluble substances including oligopeptides (Salamat-Miller and Johnston, 2005), 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 (Satake et al., 2002), GGYR (Shimizu et al., 1997), HLPLP (Quirós et al., 2008), QIGLF (Ding et al., 2014), RVPSL (Ding et al., 2015), TNGIIR (Ding et al., 2016), and VLPLP (Lei et al., 2008). 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 (Antunes et al., 2013), 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.

This is the first study that evaluates the behaviour of lunasin and some digestion-fragments on the Caco-2 monolayer. Artursson and Karlsson (1991) 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 (Cheng et al., 2008; Press and Di Grandi, 2008). In this sense, Dia et al. (2009) 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 (Antunes et al., 2013). This is similar to the flux found for some dietary whey protein-derived peptides in the β-lactoglobulin sequence f(114–146) (Picariello et al., 2013). 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 (Galvez et al., 2001). 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 (Dia and de Mejia, 2011). Nevertheless, in this study, the RGD-containing peptide f(29–43) was not transported across the Caco-2 monolayer. The peptide f(11–18) was the only fragment resembling the transepithelial behaviour of the parent peptide, thus it may be hypothesised whether this peptide is 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 (Hernández-Ledesma and Hsieh, 2015). Peptide lunasin has demonstrated anti-proliferative activity in colorectal cancer HCT-116, HT-29, KM12L4, and RKO cells (Dia and de Mejia, 2010, 2011). Cruz-Huerta et al. (2015) recently showed an inhibitory effect for the gastrointestinal digests of lunasin:IBB1 mixtures in colorectal cancer HT-29 and Caco-2 cells. Since the amount of lunasin in these digests was low (ranged between 0.1% and 5.3% from initial lunasin), peptides released during digestion of lunasin might contribute on the anti-proliferative effects observed. Thus, in this study, lunasin and some new derived fragments (Table 1) were evaluated for their potential anti-proliferative effect in the gastrointestinal tract by the MTT protocol.

As shown in Fig. 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% significant induction of non-viable cells at all concentrations assessed (Fig. 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 (2010) 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 (Fig. 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, any 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 grow at the same ratio as control cells after 72 h. As shown in Fig. 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. Fig. 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. Together these findings indicated the higher susceptibility of HT-29 cells than both Caco-2 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 (Cruz-Huerta et al., 2015). 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 (Dia and de Mejia, 2011) to 181 μM in breast cancer MDA-MB-231 cells (Hernández-Ledesma et al., 2011).

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 (Fig. 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

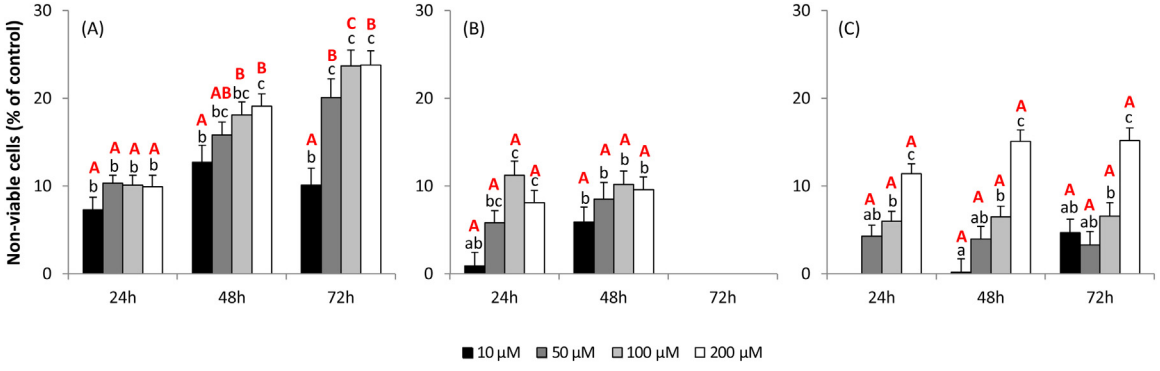

Fig. 3. Effects of lunasin on cancer cells viability. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after treatment of human adenocarcinoma colorectal (A) HT-29 and (B) Caco-2 cells and (C) gastric cancer AGS cells with lunasin (10–200 μM, 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.

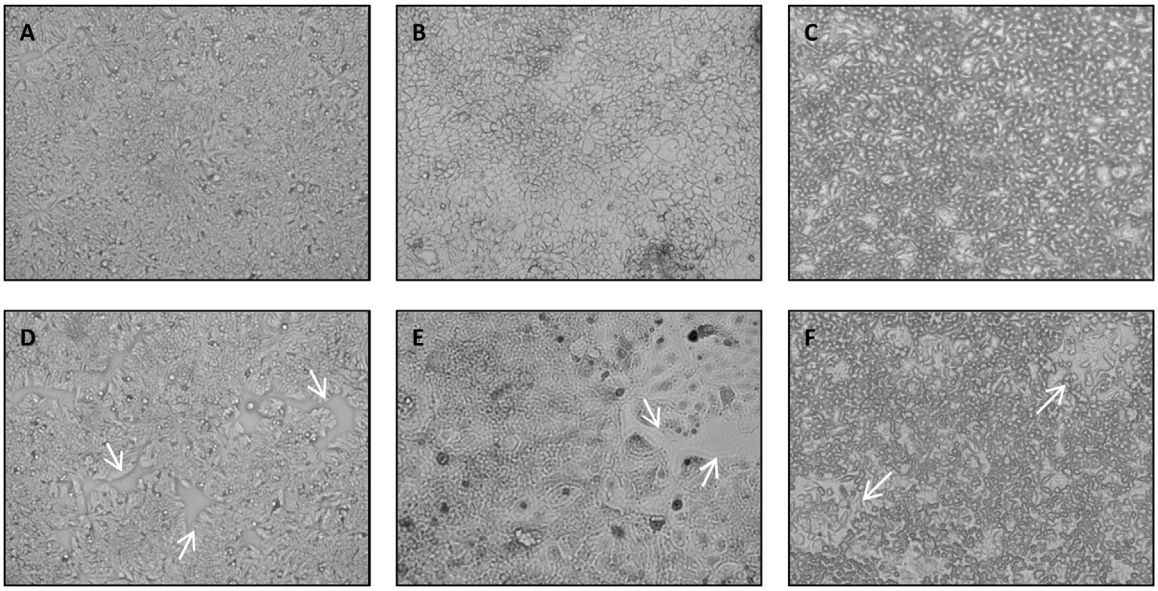

Fig. 4. Morphological analysis. Representative images, taken by using an optical microscope at 10× magnification, of HT-29, Caco-2, and AGS (A–C, respectively) non-treated cells, and (D-F, respectively) lunasin-treated cells (200 μM, 48 h). Lunasin-treated cells showed areas with minor cellular density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

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 Fig. 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%) (Fig. 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 (Fig. 5D). As it was shown for lunasin treatment on Caco-2 cells, the activity of both fragments was notably decreased after 48 h, with 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 (Galvez et al., 2001). 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 (Hernández-Ledesma et al., 2011). 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 EKHIMEKIQGRG f(23–34), and EKHIMEKIQGRGDDDDDDDDDD f(23–43), respectively, while the fragment SKWQHQQDSCRKQLQGVNLTPC f (1–22) 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. (2014) 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 (Cruz-Huerta et al., 2015). 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 (McConnell et al., 2015). 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. 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

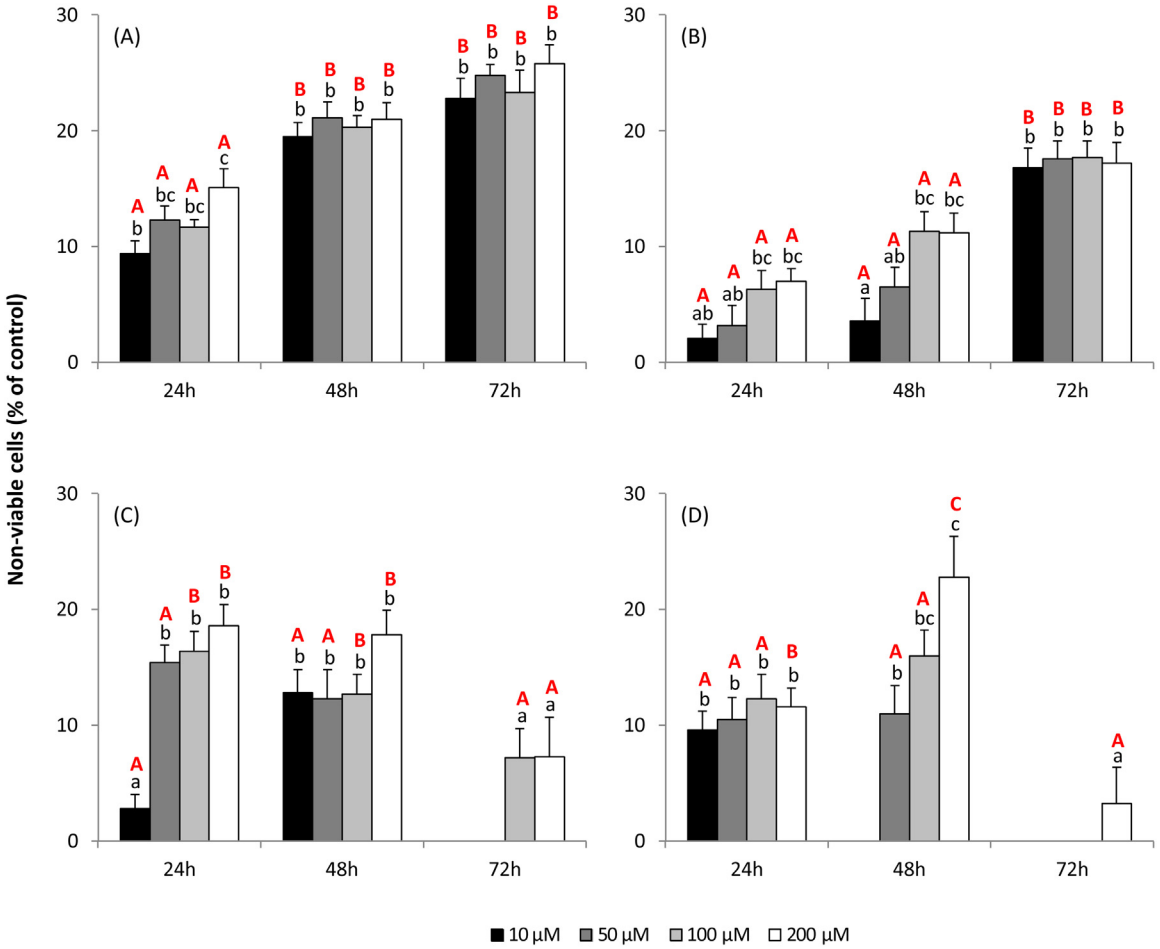

Fig. 5. Effects of lunasin-derived fragments on cancer cells viability. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after treatment of human adenocarcinoma colorectal (A and B) HT-29 and (C and D) Caco-2 cells with (A and C) peptide SKWQHQQDSC, 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.

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			
	2 h	24 h	48 h	72 h	2 h	24 h	48 h	72 h
% residual lunasin	100.0	100.0	86.8	73.0	97.3	93.1	77.9	68.0
^{23}EKHIMEKIQGRGDDDDDDDDD^{43}	–	–	+	+	–	+	+	+
^{25}HIMEKIQGRGDDDDDDDDD^{43}	–	–	+	+	–	+	+	+
^{29}KIQGRGDDDDDDDDD^{43}	–	–	+	+	–	+	+	+
^{32}GRGDDDDDDDDD^{43}	–	–	–	–	–	–	–	+

Limit of detection (LOD): 0.05 µM.
[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).

fragments EKHIMEKIQGRGDDDDDDDDD f(23–43), HIMEKIQGRGDDDDDDDDD f(25–43), and KIQGRGDDDDDDDDD f(29–43) 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 GRGDDDDDDDDD f(32–43) 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 (Galvez et al., 2001), and human THP-1 macrophages (Cam et al., 2013), colorectal cancer KM12L4 cells (Dia and de Mejia, 2011), and prostate epithelial RWPE-2 cells (Galvez et al., 2011). Four lunasin-derived fragments were formed corresponding to the C-terminal region of the sequence from the amino acid residue 23. Among them, fragment f(29–43) 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 (Cruz-Huerta et al., 2015). In this sense, it is worthy to mention that the lunasin-derived peptide SKWQHQQDSC f(1–10) 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 f(25–43), f(29–43), and f (32–43) had been already identified in a previous study of lunasin's stability in human liver HepG2 cells (Fernández-Tomé et al., 2014), which suggested that these cultures might share enzymatic activities upon incubation with lunasin peptide. In contrast, neither lunasin decrease nor derived fragments were demonstrated in Caco-2 cells along the incubation time (data not shown). Lunasin degradation in this culture was thus not the cause of the anti-proliferative activity loss showed after 72 h treatment. 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 intestinal epithelium. 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 of lunasin and derived-fragments, suggesting potential to exert bioactive effects both locally in the gastrointestinal tract as well as at a systemic level. Studies focused on detecting lunasin-derived peptides in plasma and target tissues should be needed to confirm their demonstrated *in vitro* bioavailability. 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.

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3. DISCUSIÓN

DISCUSIÓN

La digestión gastrointestinal es un complejo proceso que ocurre en el tracto digestivo, el cual es considerado como el órgano endocrino más amplio del organismo. En su interior, una gran variedad de compuestos se degradan para permitir que el organismo pueda asimilar los distintos nutrientes. En este proceso, las proteínas alimentarias dan lugar a péptidos y aminoácidos. Los aminoácidos tienen una importancia primordial por su función nutritiva. Sin embargo, en los últimos años, los péptidos han cobrado un gran interés científico, ya que se ha demostrado la funcionalidad de una gran cantidad de ellos (Xu, Yan, Zhang, & Wu, 2019). Por otro lado, el interés por conocer los cambios que tienen lugar en los nutrientes durante la digestión, unido al hecho de las dificultades que entraña realizar ensayos en animales, ha provocado que nazca la necesidad de utilizar métodos de simulación de la digestión *in vitro* efectivos y eficaces que sean capaces de reproducir al detalle lo que ocurre en las digestiones *in vivo* (Minekus et al., 2014). Las observaciones experimentales recogidas en esta Tesis Doctoral arrojan nuevos datos sobre la degradación de las proteínas lácteas durante la digestión, tanto en su llegada a duodeno como a lo largo de su estancia en el yeyuno. La comparación con estos datos ha permitido la validación de un protocolo armonizado de la digestión *in vitro*. En ambos casos, pese a encontrar ciertas diferencias entre los resultados obtenidos *in vivo* e *in vitro,* el protocolo de digestión *in vitro* se asemeja a la digestión duodenal e intestinal *in vivo* en lo que respecta a la degradación proteica y liberación de péptidos y aminoácidos. En base a estos resultados iniciales y, tras existir estudios que sugerían la necesidad de añadir un complejo enzimático formado por las enzimas presentes en el borde en cepillo del epitelio intestinal (BBM) (G. Picariello et al., 2016), se decidió estudiar el efecto que este grupo de enzimas podrían ejercer en la degradación de caseína y suero lácteo, utilizando el modelo de digestión *in vitro* consensuado. Se estudiaron diferentes concentraciones enzimáticas añadidas a la fase intestinal de la digestión y los resultados fueron comparados con los obtenidos previamente en las muestras de digeridos humanos recogidas en el yeyuno, mostrando que la opción de adicionar este paso al

modelo *in vitro* pudiera mejorar la similitud de los mismos. Por otro lado, se identificaron una serie de péptidos resistentes a la digestión *in vitro*, a la digestión *in vitro* con la adición de las BBM y a la digestión *in vivo* en humanos y que, además, han mostrado en otros estudios previos actividad biológica relevante en el cuerpo humano. Además, se evaluó la biodisponibilidad de algunos de estos péptidos bioactivos en un modelo celular del epitelio intestinal. Este modelo de transporte transepitelial también ha sido aplicado a un grupo de péptidos derivados de un fragmento funcional de la soja con propiedades quimiopreventivas: la lunasina.

3.1 Degradación de las proteínas lácteas en el tracto gastrointestinal y comparativa de las digestiones in vivo vs in vitro

Se estudió la degradación durante la digestión gástrica de la caseína. Inicialmente, se observaron resultados similares a los obtenidos por (Chiang, Croom, Chuang, Chiou, & Yu, 2008) donde la fase líquida permanecía en el estómago durante 300 min tras la ingesta del alimento. Durante este proceso de digestión de la caseína, se observó la degradación de la misma generando fragmentos más pequeños de la misma, pero también se observaron pequeñas cantidades de caseínas no degradadas a distintos tiempos de digestión, tal y como había sido descrito previamente (Barbé et al., 2014), aunque otros estudios no detectaron esta caseína intacta al analizar el contenido duodenal tras la ingesta de un preparado de leche en polvo desnatada (Egger et al., 2017). Esto podría deberse no solo a la forma de obtener la muestra, sino también a la naturaleza y características del alimento.

Una gran cantidad de péptidos cuyo tamaño oscilaba entre 5 y 34 aminoácidos fueron identificados en los efluentes duodenales de los cerdos alimentados con caseína, incluyendo péptidos que se encontraban fosforilados. La mayoría de los péptidos fosforilados fueron identificados por primera vez en este estudio *in vivo*, aunque también se encontraron péptidos fosforilados reportados previamente en otros trabajos

(Chabance et al., 1998). Por otro lado, muchos de los péptidos encontrados en este estudio, habían sido previamente descritos como péptidos con actividades opioide, antibacteriana, antioxidante, antihipertensiva o inmunomodulante (Fernández-Tomé et al., 2016; Jinsmaa & Yoshikawa, 1999; Yoshikawa et al., 1994), algunos de los cuales podrían ejercer su actividad a nivel intestinal, mientras que otros podrían ser absorbidos y ejercer su función en otros órganos. En cuanto al perfil de aminoácidos libres encontrado, se determinó que representaban entre un 0,3 – 1% sobre el total del nitrógeno presente en las muestras, de manera similar a lo obtenido en otros estudios (Egger et al., 2017) y siendo esta cantidad mucho menor que la que ha sido descrita al final del intestino (30%) (Goodman, 2010).

Como se ha comentado anteriormente, investigadores de 35 países europeos habían consensuado a nivel europeo un protocolo para la digestión gastrointestinal *in vitro* de diferentes matrices alimentarias (Minekus et al., 2014). Por esta razón, se planteó un estudio donde se compararon digeridos *in vivo* obtenidos a través de la canulación duodenal de cerdos que fueron alimentados con caseína frente a digeridos *in vitro* obtenidos mediante la digestión gástrica utilizando el protocolo consensuado de digestión, aunque se tomaron puntos intermedios de la digestión *in vitro*, modificando ligeramente el protocolo, con el objetivo de obtener una visión más detallada de lo que ocurre en cada momento del proceso. La caseína se degrada progresivamente y acaban desapareciendo las bandas de proteína intacta al finalizar el proceso de la digestión gástrica *in vitro,* tal y como había sido reportado en otros estudios realizados en cerdos (Barbé et al., 2014) e *in vitro* (Egger et al., 2016), mientras que en el análisis electroforético del contenido duodenal aparecen unas bandas ligeras, lo que parece indicar la presencia de caseína sin digerir en algunos tiempos de digestión, pudiendo alcanzar cierta cantidad de la misma el intestino delgado. Esto indica, por un lado, que el contenido del estómago que llega a duodeno es de características similares. Otros autores han estudiado recientemente la degradación de proteínas lácteas en el tracto

gastrointestinal *in vivo* con la administración de leche en polvo en un modelo porcino. En este caso se empleó el contenido total del duodeno y no el material que llega a este compartimento a lo largo del tiempo (Egger et al 2017).

Para seguir estudiando las similitudes y diferencias entre ambas digestiones y poder cuantificar lo que afecta la rápida digestibilidad de las caseínas *in vitro* frente al proceso más gradual observado in *vivo,* se realizaron análisis de identificación de los péptidos generados mediante HPLC-MS/MS. Para observar los resultados obtenidos en este caso se utilizó el software *Peptigram (Manguy et al., 2017),* el cual facilita la comparación. Se observan regiones muy representadas tanto *in vivo* como *in vitro* en N- y C-terminal para β-caseína y α_{S1}-caseína, así como las regiones f(41-59) y f(122-144) de la β-caseína y f(20-40) de la α_{S1}-caseína, donde se encuentran muchos péptidos idénticos entre ambas digestiones. Se utilizó, en este estudio, la frecuencia de aparición de cada aminoácido formando parte de un péptido provenientes de la β-caseína y α_{S1}-caseína para analizar la correlación de Spearman entre los digeridos de los distintos animales y entre los digeridos animales frente a los digeridos *in vitro.* Los resultados obtenidos mostraron una buena correlación *in vivo/in vitro,* comparable con los resultados mostrados para la correlación interindividual. Estos datos ofrecen una valoración positiva sobre la efectividad del protocolo de digestión *in vitro* utilizado, pese a que, inicialmente, la degradación proteica ocurra a tiempos diferentes. Parece que los péptidos generados tras ambas digestiones acaban coincidiendo en un alto porcentaje, lo que hace sugerir que la fracción de péptidos que se genera en la digestión *in vitro* refleja bien las características del contenido del estómago que llega a duodeno. Sin embargo, este material recogido en duodeno es relativamente homogéneo, mientras que los tiempos de digestión in vitro están reflejando el proceso de degradación que ocurre en el estómago a lo largo del tiempo.

Se realizó, también, un estudio de los aminoácidos presentes en el digerido *in vitro* en el último tiempo de la digestión estudiado (120 min) y en los digeridos

duodenales. Se observó que la concentración de aminoácidos libres en la muestra *in vitro* se encontraba en el límite inferior de los valores observados en las muestras *in vivo.* Esto podría tener su explicación debido a la falta de aminoácidos endógenos en las muestras *in vitro* que sí que están presentes *in vivo,* o bien, debido a una falta de actividad enzimática que pueda generar estos aminoácidos. En cuanto a las proporciones de aminoácidos encontradas, los más abundantes fueron fenilalanina y tirosina, tanto en los digeridos *in vivo* como en los digeridos *in vitro,* mientras que en el caso de lisina, leucina y arginina se encontraron en mayor concentración *in vivo.* En estudios previos se ha reportado que los aminoácidos arginina, lisina, tirosina y fenilalanina se encontraban en mayor concentración que la prolina y la cisteína cuando se trata de digestiones de caseína, proteína de bacalao, soja y gluten (Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005). Por tanto, los resultados obtenidos, en cuanto a la proporción de aminoácidos liberados, coinciden con lo previamente reportado en otros estudios.

Una vez obtenida una validación de la fase gástrica, se pasó a estudiar cual era la fiabilidad del método al realizar también la fase intestinal de la digestión. Por ello, se planteó el primer estudio comparativo de muestras obtenidas del yeyuno humano en varios voluntarios tras la ingesta de caseína y suero de leche y muestras digeridas en el laboratorio de los mismos sustratos, aunque combinando tiempos de digestión gástrica e intestinal. Se comprobó, inicialmente, mediante electroforesis en gel, el perfil proteico que tenían las muestras y su degradación a lo largo del tiempo, observándose, por un lado, que la caseína se degradaba totalmente durante la fase gástrica de la digestión ya fuera *in vivo* o *in vitro.* Por otro lado, en cuanto a las proteínas de suero, se observa la resistencia a la digestión gástrica de la β-lactoglobulina, mientras que queda totalmente digerida tras el inicio de la fase intestinal. La resistencia de esta proteína a la acción de la pepsina está claramente documentada tanto *in vivo (Bouzerzour et al., 2012)* como *in vitro (Dupont, Mandalari, Molle, et al., 2010; Kopf-Bolanz et al., 2012),* mientras que en

el caso de la α-lactoalbúmina, se observa cierta resistencia a la digestión de la pepsina, aunque a las dos horas aparece en gran parte degradada por la misma. Sin embargo, se observaron bandas de β-lactoglobulina intacta en yeyuno, lo que supone que esta proteína puede alcanzar intacta el intestino de adultos sanos, aunque su concentración iba disminuyendo a lo largo de los tiempos tomados.

Todas las muestras a distintos tiempos de digestión gástrica y gastrointestinal fueron analizadas mediante HPLC-MS/MS y los resultados se analizaron, de nuevo, en base a la frecuencia de aparición de cada aminoácido de cada proteína formando parte de un péptido. Estos resultados ofrecieron una visión cualitativa de la similitud entre las digestiones realizadas *in vitro* e *in vivo.* En los digeridos *in vivo*, se encontraron patrones de resistencia a la digestión similares a los diferentes tiempos de digestión para los distintos voluntarios, aunque se pudieron observar ciertas regiones en las que la variabilidad inter-individual fue mayor. Además, estos digeridos *in vivo* fueron similares a los obtenidos en las digestiones gastrointestinales *in vitro.* De hecho, la región N-terminal de la β-caseína y la región f(57-99) resultaron altamente representadas en todos los digeridos. Sin embargo, los péptidos de la región central de la proteína y la región f(115-175) fueron más abundantes en los digeridos obtenidos de los voluntarios humanos. En el caso de la α_{s1}-caseína se obtuvo un patrón más completo de los digeridos *in vivo* en comparación con el obtenido *in vitro.* Existían antecedentes de péptidos fosforilados identificados en el tracto digestivo de ratas y cerdos. En cambio, en el caso de humanos, únicamente se habían descrito algunas secuencias monofosforiladas correspondientes a algunas regiones de β-caseína, α_{s1}-caseína y α_{s2}-caseína. Todas las muestras obtenidas a partir de la digestión de la caseína fueron tratadas con un método de precipitación selectiva con $CaCl_2$ con el objetivo de identificar las secuencias fosforiladas en las fracciones enriquecidas obtenidas. Se identificaron varios caseín-fosfopéptidos, entre ellos el f(15-24) de la β-caseína conocido por contener la secuencia SerP-SerP-SerP-Glu-Glu, principal responsable de la unión y

biodisponibilidad de ciertos minerales (Zidane et al., 2012) y el péptido de la α_{s1}-caseína f(66-70) encontrado por primera vez en digeridos humanos, aunque había sido previamente identificado el contenido intestinal de ratas (Hirayama et al., 1992) y en cerdos enanos (Barbé et al., 2014; H. Meisel & H. Frister, 1989). Posiblemente, este hallazgo ha sido posible gracias al método de precipitación selectiva que no utilizaron otros autores. Otras secuencias ya reportadas en otros estudios *in vivo* fueron identificadas en este ensayo tanto en los digeridos *in vivo* como en los *in vitro* y en general mostraron una gran similitud entre ambas digestiones.

La mayoría de los péptidos generados para la β-lactoglobulina se encontraron en la fase intestinal de la digestión, aunque aparecieron varios péptidos a los 30 minutos de digestión gástrica *in vitro*, de manera similar a lo que otros autores han reportado en digestiones *in vitro* de este mismo sustrato *(Benedé et al., 2014; Egger et al., 2016)*. Se identificaron varias regiones resistentes *in vivo*, que coinciden con regiones también identificadas *in vitro*, aunque se observaron un mayor número de péptidos, en concreto de las regiones centrales de la proteína, en los digeridos *in vivo*. Por su parte la α-lactoalbúmina se comportó de manera similar en cuanto a la digestibilidad generada por parte de la pepsina, mostrando varios péptidos al final de la fase gástrica, coincidiendo con la resistencia exhibida en el ensayo de electroforesis. Además, varias regiones fueron coincidentes entre los digeridos *in vivo* e *in vitro* lo que indica que el método *in vitro* parece asemejarse a lo que ocurre *in vivo.*

Para obtener valores cuantitativos, se realizaron análisis estadísticos basados en la correlación de Spearman para comparar la variabilidad interindividual y la variabilidad *in vivo/in vitro,* basándose en la frecuencia de aparición de los aminoácidos de cada péptido en la cadena de proteína. El coeficiente de correlación entre las muestras *in vivo* y los resultados obtenidos para los digeridos *in vitro* intestinales para las proteínas β-caseína y α_{s1}-caseína fue de 0,71 ± 0,08; valores que se encuentran dentro del rango en el que fluctúan las muestras *in vivo* (0,58-0,73) y similares a los que

fueron observados en los ensayos duodenales expuestos anteriormente, donde se alcanzó una correlación *in vivo/in vitro* de 0,66-0,79 y una variabilidad interindividual de 0,51-0,75. En el caso de las proteínas β-lactoglobulina y α-lactoalbúmina los valores de la correlación de Spearman fueron de 0,74 ± 0,16; siendo los valores obtenidos entre los diferentes individuos de entre 0,43 y 0,76, aunque para estos análisis de las proteínas de suero algunas muestras fueron descartadas debido a su bajo contenido en péptidos identificados. Estos datos se complementaron con un análisis de componentes principales realizado entre las muestras correspondientes a los péptidos obtenidos de las proteínas β-caseína y α_{s1}-caseína por un lado y β-lactoglobulina y α-lactoalbúmina, por otro. Los resultados mostraron dos grupos claramente diferenciados: los datos correspondientes a la fase gástrica de la digestión *in vitro* formaban un grupo, mientras que los correspondientes a la fase gastrointestinal de la digestión *in vitro* se agrupaban con los obtenidos en digeridos del yeyuno humano, confirmando de este modo la similitud existente a nivel peptídico entre el modelo gastrointestinal *in vitro* y las muestras *in vivo.*

Por último, se realizó un estudio de las secuencias peptídicas encontradas. Se identificaron un mayor número de péptidos en los digeridos *in vivo* en la digestión *in vitro* tanto para las caseínas como para las proteínas séricas. Esta diferencia entre el número de péptidos identificados puede deberse a la variabilidad interindividual encontrada. Sin embargo, cuando se estudiaron los puntos de corte enzimáticos existentes en todos los digeridos mediante el software *Enzyme Predictor (Vijayakumar et al., 2012),* se observaron varios puntos de corte presentes únicamente en los péptidos obtenidos de los digeridos *in vivo.* Aunque las enzimas responsables de los mismos estaban presentes en los fluidos simulados *in vitro,* por lo que todo indica a que esta actividad enzimática se ve atenuada o disminuida posiblemente por las condiciones en las que se desarrolla la propia digestión en cuanto a concentraciones, presencia de coadyuvantes o agitación. De los péptidos encontrados provenientes de la digestión de caseína *in vivo*

un gran porcentaje contenían prolina (85% β-caseína; 73% α_{s1}-caseína; 45% α_{s2}-caseína; 77% κ-caseína), que ha sido previamente estudiada como posible causante de la resistencia a la digestión gastrointestinal. Por otro lado, de los péptidos identificados que no contenían prolina, el 75% de ellos contenía residuos de ácido aspártico o glutámico. Resultados similares se obtuvieron para las digestiones séricas donde sólo un 5% de los péptidos identificados no contenía ni prolina, glutámico o aspártico. Los resultados *in vitro* muestran un perfil similar al encontrado *in vivo* confirmando la resistencia de los péptidos que contienen prolina o con residuos cargados negativamente a la digestión gastrointestinal.

Una gran cantidad de péptidos bioactivos y de precursores de los mismos fueron identificados tanto *in vivo* como *in* vitro durante las digestiones de caseína y suero. Uno de ellos, la β-casomorfina-7 fue identificada en ambos digeridos, aunque en las digestiones *in vitro* se ha identificado formando parte de otros péptidos que contenían esa secuencia y se detectó, previamente, *in vivo* (Boutrou et al., 2013). Por otro lado, los péptidos antihipertensivos de la β-caseína: HLPLP, f(133-138) y de la α_{s1}-caseína: AYFYPEL, f(143-149) también han sido identificados en los digeridos yeyunales y fueron identificados previamente en digeridos gástricos humanos tras la ingesta de leche (Chabance et al., 1998), además de generar actividad antihipertensiva en ratas espontáneamente hipertensas (Contreras et al., 2009) y estimular la generación de mucinas intestinales (D. Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013). Esto da pie a sugerir que ambos péptidos podrían llegar a estar bioaccesibles para su absorción y ejercer su función antihipertensiva en humanos. En el caso de los péptidos provenientes de las digestiones de suero, se identificaron varios péptidos bioactivos provenientes de la β-lactoglobulina con actividad hipocolesterolémica como IIAEK y GLDIQN (Nagaoka et al., 2001) y con actividad inhibitoria de la DPP-IV como IPAVF (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013).

Los resultados obtenidos en esta comparativa entre los digeridos yeyunales humanos y las digestiones gastrointestinales *in vitro* mostraron una gran similitud entre ellos, sobre todo a la hora de comparar la fase intestinal de los digeridos *in vitro*. La fase gástrica del protocolo de digestión consensuado no se pudo comparar adecuadamente, ya que los digeridos *in vivo* provenían de la zona yeyunal del intestino delgado de los voluntarios humanos.

En general, los ensayos realizados muestran que existen diferencias entre los resultados obtenidos de las muestras de digestión *in vitro* e *in vivo* tanto en el caso de los digeridos duodenales de cerdo comparados con la fase gástrica del protocolo *in vitro,* como en el caso de los digeridos yeyunales humanos comparados con la fase gastrointestinal del mismo protocolo. No obstante, pese a estas diferencias, se ha demostrado que este modelo de digestión *in vitro* podría ser utilizado para reproducir el proceso de la digestión *in vivo*, ya que simula bien las características de lo observado *in vivo* en cada compartimento. Sin embargo, no pueden reproducir los cambios que se están produciendo en el espacio, para esto se necesitan modelos dinámicos.

3.2 Incorporación de peptidasas en la digestión in vitro y biodisponibilidad de péptidos

Existen estudios relacionados con la posible implicación que tienen las BBM en el proceso de la digestión y biodisponibilidad de los péptidos (Picariello et al., 2015). Por tanto, se decidió realizar digestiones de caseína y suero de leche *in vitro* mediante el método de digestión armonizado adicionando estas enzimas en la fase intestinal de la digestión a dos diferentes concentraciones y comparar los resultados tanto con los obtenidos previamente *in vitro* como con los digeridos yeyunales humanos.

Inicialmente se realizó una comparativa del número de péptidos obtenidos en las digestiones *in vitro* control, donde se seguía el protocolo armonizado frente a la misma digestión adicionando dos concentraciones diferentes de BBM: 15 µU/ µg de proteína y

45 µU/µg de proteína. Los resultados mostraron un mayor número de péptidos identificados en el caso de la digestión control, frente a los que se pudieron detectar con BBM a ambas concentraciones, tanto para los digeridos de caseína, como para los de suero. Para los digeridos de caseína, además, se pudieron detectar mayor cantidad de péptidos cuando se digirió a la concentración de 15 µU/ µg de proteína frente a 45 µU/ µg de proteína, generándose, además mayor número de péptidos únicos para esta misma concentración. Cabe destacar, que para las digestiones de caseína con la adición de BBM se genera un mayor porcentaje de péptidos de cinco aminoácidos que para la digestión control. No ocurre igual, sin embargo, en el caso de los digeridos de suero, donde se observan resultados similares a ambas concentraciones de BBM. El hecho de encontrar menor número de péptidos en los digeridos con BBM, pero observar un crecimiento en el número de péptidos de menor tamaño identificados en los digeridos con BBM sugerían que esto era debido a la acción de estas peptidasas, que podrían generar, péptidos menores de cinco aminoácidos que no se pueden detectar en las condiciones de espectrometría de masas utilizadas, e incluso liberar más aminoácidos al tracto intestinal.

Por esta razón se plantearon los siguientes ensayos, donde se quiso comparar el perfil de aminoácidos libres que tenía cada uno de los distintos digeridos. Se observó que para muchos aminoácidos había una cantidad estadísticamente significativa mayor en los digeridos con BBM que en la digestión control como, por ejemplo: Ser, Pro, Ala, Val, Ile e His. Esto ocurrió tanto en el caso de los digeridos de caseínas como de suero, aunque no se observaron diferencias relevantes entre los digeridos de suero a distintas concentraciones de BBM. Estos datos reafirman la hipótesis de que debido a la acción de estas proteasas los péptidos se degradan mucho más, generándose mayor cantidad de aminoácidos libres, una de las razones por las cuales se observan menor cantidad de péptidos en estas digestiones, ya que esta liberación de más aminoácidos hace que

se generen péptidos menores a cinco aminoácidos, los cuales no podemos detectar en las condiciones analíticas en las que se han realizado estos experimentos.

En cuanto a los péptidos identificados, se encuentran una gran cantidad de péptidos nuevos que solo son identificados en los digeridos realizados con BBM y muchos de ellos, además, habían sido identificados previamente en otros ensayos *in vivo*, incluyendo en nuestros ensayos en muestras de digeridos yeyunales (Boutrou et al., 2013; Sanchón et al., 2018). Pero, además, se identificaron ciertas regiones de las proteínas que permanecían intactas aún después de haber sido digeridas con las proteasas del borde en cepillo del epitelio intestinal. Estas regiones generaron ciertos péptidos que son de gran interés puesto que son capaces de resistir al proceso completo de la digestión, pudiendo ejercer, tal vez alguna funcionalidad, al estar accesibles para ser absorbidos y alcanzar el torrente sanguíneo. Una de estas regiones es el fragmento f(58-66) procedente de la β-caseína. En este estudio al menos han sido identificadas siete secuencias relacionadas con este dominio que, además, habían sido descritos en digeridos humanos (Boutrou et al., 2013; Sanchón et al., 2018), incluyendo el péptido β-casomorfina-7 f(60-66). Este péptido y otros cinco más largos y más cortos se seleccionaron para estudiar su posible transporte a través de una monocapa de células Caco-2. Aquellos péptidos que poseían el motivo IPN en posición terminal exhibían mayor tasa de transporte que aquellos que no lo tenían manifestando la posible importancia de contar con una secuencia específica de aminoácidos para ser transportado (Ding et al., 2017). La presencia de Tyr, Lys, Asn o Arg en posición C-terminal ha sido descrita como factor beneficioso para el transporte de tetra- y pentapéptidos, por tanto, la presencia de Asn en los péptidos estudiados podría haber sido determinante en el proceso de transporte. Además, Val y Pro en la posición N-terminal también había sido identificada como factor positivo para el transporte de péptidos, coincidiendo con los tres péptidos que mejor se transportan en nuestro ensayo: VYPFPGPIPN, PGPIPN, y VYPFPGPI.

Por otro lado, las peptidasas presentes en las células de la monocapa también jugaron un papel fundamental en los resultados observados. La resistencia de los mismos al transporte rondó desde el 23 al 75%, salvo para el péptido VYPFPGPI, el cual resistió al 100% coincidiendo con que ya había sido reportada su actividad inhibitoria de la prolilpeptidasa (Asano et al., 1992). Los péptidos que tenían la menor tasa de transporte se correspondían con aquellos que mostraban una mayor susceptibilidad a las proteasas celulares.

Por último, se realizaron los mismos ensayos de transporte en otro péptido alimentario proveniente de la soja, la lunasina, y fragmentos derivados de su secuencia. Mostraron una resistencia a las enzimas presentes en las células Caco-2 de entre un 58 y un 80 %, mientras que los fragmentos f(1-10) y f(29-43) permanecieron intactos. Se observó que, al ensayar la lunasina sintética frente a las enzimas presentes en estas células, se produjeron varias roturas por la zona central de este péptido, dejando intactas las regiones terminales. En el caso de los fragmentos, la mayoría de las roturas se produjeron en los extremos N-terminal y son producidas, especialmente, por la DPPIV serin-proteasa, que tiene una gran afinidad por las regiones N-terminal y su mayor actividad se produce cuando las células están completamente diferenciadas. Además, los aminoácidos básicos o hidrofóbicos son su sustrato diana, mientras que la prolina muestra resistencia a la enzima. A pesar de ello, los péptidos fueron bastante resistentes al ataque enzimático, especialmente los fragmentos f(1-10) y f(29-43), más aún si se comparan con otros péptidos como los péptidos receptores agonistas μ-opioides β-casomorfina-5 y β-casomorfina-7 tanto bovinos (Sienkiewicz-Szłapka et al., 2009) como humanos (Iwan et al., 2008), que prácticamente desaparecen tras la incubación con la monocapa de Caco-2.

Tras ello, se observó que, tanto la lunasina como el fragmento f(11-18), eran transportados a través de la monocapa de células Caco-2 con unos coeficientes aparentes de transporte similares a los que habían sido determinados para otros

péptidos bioactivos utilizando este mismo modelo como AYFYPEL, RYLGY (Contreras et al., 2012) y VLPVP (Lei et al., 2008). Se estudió, posteriormente, el mecanismo por el cual estos péptidos eran transportados, descartándose la posibilidad de que se produjera mediante la ruta mediada por PepT1, ya que a través de ella no se transportan péptidos más largos de tres aminoácidos (Vig et al., 2006). Se utilizaron inhibidores de la transcitosis y disruptores de las uniones estrechas resultando ser la ruta paracelular a través de las uniones estrechas la vía de transporte de ambos péptidos, coincidiendo con lo que ocurre para otros muchos péptidos como VPP (Satake et al., 2002), GGYR (Shimizu et al., 1997), HLPLP (Quirós et al., 2008), QIGLF (Ding et al., 2014) y VLPLP (Lei et al., 2008). Además, las células Caco-2 poseen unas mayores uniones celulares debido a su naturaleza tumoral colónica, lo que implicaría que el transporte de estos péptidos podría llegar a ser aún mayor en células epiteliales de intestino delgado humano o animal sano (Antunes, Andrade, Ferreira, Nielsen, & Sarmento, 2013).

Por último, se estudió el efecto que ejercían el péptido y los fragmentos en la viabilidad celular en células cancerígenas gástricas y colorrectales, ya que otros péptidos provenientes de alimentos habían mostrado esta funcionalidad (B. Hernández-Ledesma & Hsieh, 2015). El péptido lunasina ya había mostrado capacidad antiproliferativa en varios tipos de cultivos celulares y se observó, además, que este posible efecto podían llevarlo a cabo los péptidos generados tras la degradación de la propia lunasina. Los resultados obtenidos en este estudio mostraron que la lunasina afectaba la viabilidad de las tres líneas celulares ensayadas (Caco-2, HT-29 y AGS). Se observó una reducción del porcentaje de células vivas de entre el 10 y el 24% en el caso de las células HT-29, cuando otros estudios realizados con lunasina natural alcanzaban unos valores de hasta un 63%, por lo que se sugiere que la lunasina sintética podría tener diferentes estructuras secundarias y terciarias que pudieran afectar a estos rendimientos. En el caso de la actividad exhibida para las otras dos líneas celulares, se puso de manifiesto que fueron mucho menos susceptibles al efecto del péptido, tal y

como había ocurrido en estudios similares (Cruz-Huerta et al., 2015). Posteriormente, se estudió la misma actividad proliferativa, pero en este caso de siete fragmentos sintéticos diferentes provenientes de la lunasina para poder determinar qué región o motivos de la lunasina podrían ser considerados como los responsables de esta actividad citotóxica. Hasta el momento, la actividad antiproliferativa de la lunasina había sido atribuida a la región C-terminal de la misma (Galvez, Chen, Macasieb, & de Lumen, 2001). Sin embargo, los resultados mostraron una mayor capacidad antiproliferativa del fragmento f(1-10), siendo este el primer estudio que sugiere que la zona N-terminal del péptido tenga un papel protector antitumoral.

Los resultados presentados en esta Tesis Doctoral han pretendido evaluar el modelo de digestión como una herramienta básica y eficaz que, junto con los ensayos de transporte pretenden mimetizar lo que ocurre en el tubo digestivo y en la barrera gastrointestinal hasta llegar a la absorción. Toda la parte digestiva se ha validado para las proteínas lácteas con modelos *in vivo*. La absorción de los péptidos es un tema controvertido, pero se aportan nuevos datos sobre las secuencias más resistentes a la digestión y se evalúan las posibles características que pueden hacer que unos péptidos se transporten más fácilmente que otros. Llegar a establecer una relación entre transporte transepitelial en modelo celular y biodisponibilidad *in vivo* requerirá de futuros estudios.

4. CONCLUSIONES

CONCLUSIONES

1. El análisis de los efluentes de duodeno porcino tras la administración oral de las caseínas refleja la coagulación de la caseína en el estómago. Los aminoácidos libres suponen un 1% del contenido de nitrógeno total y el resto correspondió a péptidos y proteína intacta. Además de proteínas de suero, se detectaron trazas de caseína intacta a nivel duodenal. Se identificaron aproximadamente 4000 péptidos mediante espectrometría de masas en tándem, y presentando la mayor parte de los mismos una longitud entre 5 y 10 aminoácidos y puntos isoeléctricos entre 3 y 4, con predominio de prolina y ácido glutámico en sus secuencias. Se identificaron péptidos fosforilados de entre 5 y 16 aminoácidos.

2. El punto final de la digestión gástrica simulada mediante el protocolo *in vitro* consensuado de la digestión ofrece una buena aproximación a la degradación proteica a nivel duodenal *in vivo* en base a las similitudes exhibidas a nivel de resistencia proteica y liberación de péptidos y aminoácidos. El coeficiente de correlación de Spearman calculado con la frecuencia de aparición de cada aminoácido en las secuencias identificadas tras la digestión gástrica simulada y en duodeno osciló entre 0,66 y 0,79, y este mismo coeficiente entre distintos animales estuvo entre 0,51 y 0,75.

3. En los digeridos obtenidos en yeyuno humano tras la administración oral de caseína no se detectó caseína intacta y la fracción nitrogenada se encontró en forma de péptidos y aminoácidos libres. Se identificaron más de 400 péptidos procedentes de las caseínas, siendo especialmente abundantes los péptidos con prolina en su secuencia, seguidos por péptidos con ácido aspártico o glutámico. Además, se identificaron péptidos fosforilados de 5 a 31 aminoácidos de longitud.

4. Tras la administración oral de proteínas de suero, se detectó β-lactoglobulina intacta tras 1 hora en todos los voluntarios, encontrándose presente en algunos de ellos en la muestra tomada a las 2 horas. Se identificaron más de 200 péptidos procedentes de β-lactoglobulina y α-lactoalbúmina en yeyuno humano, aunque el número de péptidos disminuyó con el tiempo de muestreo indicando su degradación a péptidos pequeños y amino ácidos libres. Al igual que para las caseínas, las regiones resistentes a la digestión gastrointestinal son ricos en aminoácidos cargados negativamente (ácido glutámico y aspártico)

5. La simulación de la digestión gastrointestinal con el protocolo consensuado ofrece resultados similares y comparables a nivel de degradación proteica y de liberación de péptidos con los obtenidos en yeyuno humano. El coeficiente de correlación de Spearman al comparar las secuencias encontradas in vitro e in vivo fue de 0,74, mientras que entre distintos voluntarios osciló entre 0,43 y 0,76.

6. La incorporación de las peptidasas del borde en cepillo como paso adicional al protocolo consensuado de la digestión *in vitro* permite la generación de una serie de péptidos nuevos formados principalmente por la acción de amino- y carboxi-peptidasas. Además, muchos de los péptidos nuevos fueron identificados en yeyuno humano, por lo que este paso podría suponer una mejora en la eficacia del método de digestión *in vitro.*

7. Los fragmentos procedentes de la β-caseína f(59-68) VYPFPGPIPN, f(63-68) PGPIPN y f(59-67) VYPFPGPI identificados en yeyuno humano y los digeridos gastrointestinales simulados mostraron capacidad de transporte a través de monocapas de células Caco-2 con coeficientes aparentes de

transporte de 3,6 x 10^{-6}, 4,4 x 10^{-6} y 1,9 x 10^{-6} (cm/s), respectivamente. Los péptidos f(60-66) YPFPGPI, f(62-66) FPGPI y f(60-62) YPF mostraron una capacidad de transporte más limitada con coeficientes aparentes de transporte inferiores a 0,8 x 10^{-6} cm/s.

8. Los fragmentos generados a partir del péptido lunasina de soja durante la digestión gastrointestinal f(1-10) SKWQHQQDSC y f(29-43) KIQGRGDDDDDDDDD mostraron una notable resistencia a la acción de las peptidasas expresadas en el cultivo de células Caco-2. Lunasina y el péptido f(11-18) RKQLQGVN se transportaron a través de la monocapa de células Caco-2 con coeficientes aparentes de absorción de entre 2-4 × 10^{-7} cm/s y se identificó el transporte paracelular como principal ruta de transporte.

9. Lunasina y el fragmento (1-10) con secuencia SKWQHQQDSC son los péptidos que mostraron mayor actividad citotóxica frente a las células HT-29 de cáncer colorectal. Por tanto, la región N-terminal de la lunasina podría ser relevante a la hora de explicar su actividad antiproliferativa. La actividad antiproliferativa de la lunasina y los fragmentos generados durante la digestión gastrointestinal simulada dependen del cultivo celular empleado, siendo más susceptibles los cultivos de células AGS de adenocarcinoma gástrico y HT-29 que las células Caco-2, aunque estas dos últimas provienen de adenocarcinoma colorectal humano.

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