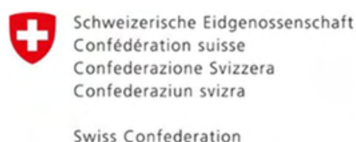
UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Development of an *in vitro* digestion protocol for assessing protein quality and functionality

Desarrollo de un protocolo de digestión *in vitro* para evaluar la calidad y funcionalidad de las proteínas



Federal Department of Economic Affairs,
Education and Research EAER
Agroscope

ANA RAQUEL MARQUES DE SOUSA

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FACULTAD DE CIENCIAS

Development of an *in vitro* digestion protocol for assessing protein quality and functionality

Desarrollo de un protocolo de digestión *in vitro* para evaluar la calidad y funcionalidad de las proteínas

Memoria presentada por:

Ana Raquel Marques de Sousa

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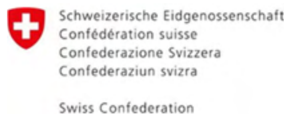

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Trabajo realizado bajo la dirección de:

Dra. Charlotte Egger

Dr. Reto Portmann

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Economic Affairs,
Education and Research EAER

Agroscope
Methods Development and Analysis (MEA)

CHARLOTTE EGGER DEPUTY HEAD OF RESEARCH GROUP "BIOCHEMISTRY OF MILK & MICROORGANISMS" AND RETO PORTMANN HEAD OF TEAM "PROTEINS & METABOLITES", SCIENTISTS AT SWISS FEDERAL STATION AGROSCOPE

Inform:

The present work entitled "**Development of an *in vitro* digestion protocol for assessing protein quality and functionality**", which constitutes the Report presented by the Graduate in Biochemistry Ana Raquel Marques de Sousa to opt for the degree of Doctor with International Doctorate Mention, was carried out under our direction at Agroscope.

We hereby sign this report in Bern May 20th, 2022.

Fdo. Charlotte Egger

Fdo. Reto Portmann

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If I was brave enough to start, I am strong enough to finish!

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To Grandma and Maria.

‘Out of clutter, find simplicity. In the middle of difficulty, lies opportunity.’

Albert Einstein

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Abstract

In this thesis an *in vitro* method for the determination of protein digestibility and assessment of Digestible Indispensable Amino Acid Score (DIAAS) in foods was established, based on the INFOGEST static protocol. Moreover, the newly developed method was compared to *in vivo* digestibilities, for the exact same protein sources, which were selected based on expected differences in their digestibility rates.

The eight protein sources, consisting either of isolated proteins, namely whey protein isolate, zein and collagen or complex foods, namely All-Bran®, black beans (*Phaseolus vulgaris*), pigeon peas (*Cajanus cajan*), sorghum (*Sorghum spp*.), and peanuts (*Arachis hypogaea*), were first characterized for their proteins, amino acids profiles, fat, and carbohydrates contents. The proteins of all the substrates were analysed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and identified after tryptic digest using Liquid Chromatography with tandem Mass Spectrometry (HPLC-MS/MS). Individual amino acid composition of each substrate was analysed by ultra-high-performance liquid chromatography (UHPLC).

Thereafter, the protein sources were *in vitro* digested according to the static INFOGEST protocol and the digesta were qualitatively analysed. No intact protein from the substrates was visually detected by SDS-PAGE after the intestinal phase of *in vitro* digestion. However, digestion-resistant peptides were detected in all substrates after the intestinal digestion phase.

To allow the analysis of *in vitro* protein digestibility and calculation of DIAAS however, the INFOGEST digestion protocol was slightly modified in the pancreatin solubilization procedure and more importantly, an analytical workflow allowing the quantification of bioavailable and non-bioavailable fractions, needed to be developed. Therefore, after *in vitro* digestion according to the INFOGEST protocol, the digests were immediately precipitated with MeOH, to separate bioavailable- from non-bioavailable fraction. The peptide length was analysed in both fractions, using size exclusion chromatography, previously calibrated with a set of compounds of known molecular weights. It could be observed that most of the peaks from the supernatant (bioavailable fraction) were eluting after 40 min, corresponding to peptides of 8-10 amino acids in length and most of the peaks from the pellet (non-bioavailable fraction) were eluting earlier and were therefore longer than 10 amino acids in length. The 8-10 amino acid long peptides present in the supernatant are longer than the peptides absorbed *in vivo* conditions. However, due to the lack in the brush border enzymes *in vitro*, which would further hydrolyse these peptides, this cut-off size was acceptable. It was assumed that the *in vitro* non-bioavailable fraction corresponds to the ileal digesta *in vivo* and the bioavailable fraction to the absorbed fraction *in vivo*. After *in vitro* digestion and precipitation, supernatants

and pellets were analysed with three different methods: total nitrogen by Kjeldahl, primary amines by OPA and individual amino acids by UHPLC. Protein digestibility was calculated in a similar way for the three methods. In addition, it was necessary to run in parallel a protein-free food matrix (protein-free cookie) as an enzyme blank. For OPA and UHPLC, all samples were previously hydrolysed to allow the quantification of each individual amino acid. Finally, the *in vitro* DIAAR values of the indispensable amino acids were calculated by dividing the $DIAA_{measured}$ by the $DIAA_{reference}$ for growing children as provided by FAO. The comparison between *in vitro* digestibility and *in vitro* DIAAR values with *in vivo* values showed a high agreement between both methods. The *in vitro* digestibility slightly overestimated by 1.2 % the *in vivo* situation, and the correlation between *in vitro* and *in vivo* DIAAR had a slope of 0.96 and a mean bias between the two methods of 0.1 %, according to a Bland-Altman statistical evaluation. These results suggest that the *in vitro* protocol is a powerful tool to predict the DIAAR values of the different tested foods. However, the validation of this protocol was performed so far only for seven different protein sources and needs to be further validated with a higher number of protein sources.

As next, the method described above was tested in highly processed foods from plant origin. Three different plant-based products (soy burger, pea-faba burger and soy meat analogue), together with their isolated ingredients, were digested using the INFOGEST *in vitro* digestion protocol and their digestibility and DIAAR values were calculated as previously described. A beef burger was digested in parallel, to compare the results from the plant-based products with a meat burger. Comparison between *in vivo* DIAAR values available in literature and the *in vitro* DIAAR determined with the new method showed a very good correlation. Thus, the method proved to be suitable to assess digestibility and DIAAR values in highly processed foods, and as expected, the animal protein presented higher values for digestibility and DIAAR. So far, no significant impact of the extrusion and texturizing processes was found on protein digestibility and DIAAR values in the tested foods, by comparison with the ingredients.

At last, the effect of the *in vitro* digests from zein, collagen, sorghum, black beans, pigeon peas and peanuts on hormonal secretion in STC-1 cell line. The secretion and gene expression of cholecystokinin (CCK) and glucagon like peptide 1 (GLP-1) were evaluated in SCT-1 cells in response to gastric and gastrointestinal digests from zein, collagen, sorghum, black beans, pigeon peas and peanuts. Hormone secretion was measured by ELISA and CCK and GLP-1 mRNA levels by RT-PCR. After 2 h incubation, gastric and intestinal soluble fractions of the different digests induced significant secretion of GLP-1 and a moderate CCK secretion in a dose-dependent manner. CCK and GLP-1 secretion was maximal with black bean, sorghum, All-Bran® and zein intestinal digests. For gastric fractions, GLP-1 and CCK secretion was higher for pigeon peas, sorghum and All-Bran®. A significant correlation between the protein content of the digested fractions and the secretion of CCK was found, confirming

earlier results. Moreover, as predictable, GLP-1 secretion was directly and significantly correlated with the carbohydrate content of the digested fractions. With the aim to investigate the effect of digestion-resistant peptides from protein isolates that are generated during *in vitro* digestion, the most abundant digestion-resistant peptides present in the digests of zein and colllagen were synthetized. The evaluation of the intracellular calcium concentration revealed the activation of the enteroendocrine cells in response to some of these sequences.

Resumen

En esta Tesis se ha desarrollado un método *in vitro* para calcular la digestibilidad y la calidad nutricional de las proteínas alimentarias mediante el cálculo del Índice de Aminoácidos Indispensables Digestibles (DIAAS), basándose en el protocolo de digestión gastrointestinal estático propuesto por la red internacional INFOGEST. Además, la digestibilidad calculada mediante el nuevo método se comparó con la obtenida *in vivo* para los mismos sustratos, que fueron seleccionadas por tratarse de fuentes proteicas en las que se esperaba una diferente digestibilidad.

Para ello, ocho fuentes proteicas, en concreto, aislados de proteínas de suero, zeína y colágeno bovino y de alimentos completos, como, cereales integrales (All-Bran®), alubias negras (*Phaseolus vulgaris*), gandul o guandú (*Cajanus cajan*), sorgo (*Sorghum spp.*) y cacahuetes (*Arachis hypogaea*), se caracterizaron en cuanto a su perfil proteico y aminoacídico y se determinó el contenido en grasa y carbohidratos. Las proteínas mayoritarias de cada sustrato se identificaron mediante electroforesis en gel en presencia de dodecilsulfato sódico PAGE-SDS en combinación con hidrólisis tríptica y análisis mediante cromatografía líquida acoplada a espectrometría de masas en tándem (HPLC-MS/MS). La composición aminoacídica de los sustratos se determinó mediante cromatografía líquida de ultra-alta resolución (UHPLC).

A continuación, los sustratos se sometieron a un proceso de digestión gastrointestinal simulada siguiendo el protocolo INFOGEST y los digeridos se analizaron cuantitativamente. no se detectaron proteínas intactas al final de la fase intestinal del protocolo de digestión mediante PAGE-SDS. Sin embargo, se identificaron péptidos resistentes a la digestión gastrointestinal a partir de todos los alimentos e ingredientes estudiados.

Con el fin de determinar la digestibilidad proteica *in vitro* y el cálculo del DIAAS, el protocolo de digestión INFOGEST fue modificado en la etapa de solubilización de la pancreatina y especialmente en el flujo de trabajo que permitió la estimación de la fracción biodisponible y no biodisponible. Tras la digestión *in vitro*, los digeridos se trataron con metanol con el fin de separar la fracción biodisponibles y no biodisponible. Ambas fracciones se caracterizaron mediante cromatografía de exclusión molecular. En el método cromatográfico, se empleó una calibración previa, empleando distintos compuestos de peso molecular conocido. Se observó que la mayor parte del material contenido en el sobrenadante de metanol (fracción biodisponible), eluyó tras los 40 min lo que correspondía a un tamaño de corte de 1000 Da, equivalente a 8-10 aminoácidos. Dado que el protocolo de digestión *in vitro* empleado carece de la actividad peptidásica presente en la membrana de borde en cepillo, se consideró que los péptidos de hasta 10 aminoácidos serían degradados y absorbidos en la situación *in vivo*. Por tanto, la fracción complementaria, el precipitado, correspondería al

contenido ileal *in vivo*, es decir, la fracción no biodisponible. Tras la digestión *in vitro* y la precipitación, todas las fracciones (soluble e insoluble) se analizaron mediante tres métodos diferentes: nitrógeno total mediante Kjeldahl, grupos amino libres mediante OPA y aminoácidos totales mediante UHPLC. La digestibilidad proteica se calculó de forma similar por los distintos métodos. Además, fue necesario llevar a cabo en paralelo la digestión de una matriz alimentaria libre de proteínas (protein-free cookie) como blanco de enzimas. Ambas fracciones, sobrenadante y precipitado, se sometieron a hidrólisis ácida previa a la evaluación mediante OPA y HPLC. Una vez calculada la digestibilidad *in vitro*, y conociendo el contenido de aminoácidos totales por gramo de alimento, se pudo calcular el Índice de Aminoácidos Indispensables Digestibles ($DIAA_{calculado}$) multiplicando estos dos valores. El valor de $DIAA_{calculado}$ para cada uno de los aminoácidos esenciales dividido por el valor de $DIAA_{refencia}$ para niños en crecimiento, establecido por la FAO, permite obtener el DIAAS. La digestibilidad *in vitro* y los valores de DIAAR *in vitro* se compararon con los obtenidos *in vivo*. Se obtuvo una buena correlación entre el DIAAR *in vitro* e *in vivo*. Se observó que el protocolo *in vitro* resulta en una ligera sobreestimación de la digestibilidad (con una diferencia entre *in vivo* e *in vitro* del 1,2%) y que la correlación entre DIAAR in vivo e in vitro fue 0,96 y la desviación media entre los dos métodos del 0,1%, según el test Bland-Altman. Estos resultados sugieren que el protocolo *in vitro* es una herramienta eficaz para predecir los valores DIAAR de los diferentes alimentos estudiados. Sin embargo, en esta Tesis este protocolo *in vitro* ha sido validado con valores *in vivo* en 7 fuentes proteicas distintas, por lo que para asegurar su aplicabilidad, sería conveniente su validación en un mayor número de alimentos y matrices de distinta naturaleza.

Como continuación, el método optimizado anteriormente, se aplicó a alimentos de origen vegetal altamente procesados. Tres productos: hamburguesa de soja, hamburguesa de guisante y haba y un análogo cárnico elaborado con soja, junto con sus ingredientes de partida, fueron digeridos empleando el protocolo de digestión estático INFOGEST y se calculó su digestibilidad y los valores DIAAR. En paralelo, se empleó una hamburguesa de carne de vacuno como referencia. La comparación entre los valores DIAAR *in vivo* descritos en la bibliografía y los valores *in vitro* obtenidos, mostraron una buena correlación. Por tanto, el protocolo optimizado sería también aplicable a alimentos altamente procesados de origen vegetal. Como era de esperar, la hamburguesa de origen animal presentó valores más elevados tanto de digestibilidad como de DIAAR. No se observó un impacto negativo sobre la digestibilidad o DIAAR debido a los procesos de extrusión o texturización al comparar con los ingredientes de partida.

Para finalizar, se evaluó el efecto de los productos de digestión de los sustratos estudiados, zeína, colágeno bovino, cereales integrales, alubias negras, gandul, sorgo y cacahuetes sobre la secreción hormonal en la línea celular STC-1. En concreto, se determinó la secreción hormonal y la expresión de los genes que codifican para colecistoquinina (CCK)

y el péptido similar al glucagón (GLP-1) en respuesta a los digeridos gástricos y gastrointestinales. La secreción de estas dos hormonas se determinó mediante ensayos ELISAs y la expresión génica, mediante RT-PCR. Tras dos horas de incubación, la fracción soluble de los digeridos gástricos e intestinales inducen la secreción de forma dosis-dependiente de GLP-1 y de CCK aunque en este caso de forma más moderada. La secreción de GLP-1 y CCK se indujo notablemente en presencia de los digeridos gástricos de gandul, sorgo y All-Bran. Sin embargo, los digeridos intestinales de alubias negras, sorgo, All-Bran y zeína son los que indujeron una mayor secreción de ambas hormonas. Se encontró una correlación significativa entre el contenido en proteína y la secreción hormonal. Además, como era de esperar, la secreción de GLP-1 correlacionó significativamente con el contenido en carbohidratos de los digeridos. Con el fin de conocer el efecto de algunos de los péptidos generados durante la digestión de los aislados proteicos, zeína y colágeno, se sintetizaron los dominios más representativos resistentes a la digestión gastrointestinal *in vitro*. Mediante la determinación de la concentración de calcio intracelular, se demostró la capacidad de algunas de estas secuencias para activar las células enteroendocrinas.

Abbreviations list

AA	Amino Acid
AAbA	α-aminobutyric acid
ARCOL	Artificial Colon
BCAA	Branched-Chain Amino Acid
cAMP	Cyclic Adenosine Monophosphate
CaSR	Calcium-Sensing Receptor
CCK	Cholecystokinin
DGM	Dynamic Gastric Model
DIAA	Digestible Indispensable Amino Acid
DIAAR	Digestible Indispensable Amino Acid Ratio
DIAAS	Digestible Indispensable Amino Acid Score
EAA	Essential Amino Acids
EECs	Enteroendocrine cells
ELISA	Enzyme-Linked Immunosorbent Assay
ESIN	Engineered Stomach and small Intestinal
FAO	Food and Agriculture Organization of the United Nations
GE	Gastric Emptying
GLP-1	Glucagon-like peptide-1
GLU	Glutamic acid
GPCRs	G protein-coupled receptors
HGS	Human Gastric Simulator
HPLC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
IAA	Individual Indispensable Amino Acids
IAAO	Indicator Amino Acid Oxidation
IP3	Inositol 1,4,5-triphosphate
IVDIAAS	*in vitro* DIAAS
IVD	*in vitro* digestion
MA	Metabolic Availability
mRNA	Messenger RNA
NEAA	Nonessential Amino Acids
NPU	Net Protein Utilization
OPA	*o*-phthalaldehyde assay
PAMPA	Parallel Artificial Membrane Permeation Assay
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PEG	Polyethylene glycol

PEPT1	Human peptide transporter 1
PER	Protein Efficiency Ratio
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SGF	Simulated Gastric Fluid
SHIME®	Simulator or the Human Intestinal Microbial Ecosystem
SIF	Simulated Intestinal Fluid
simgi®	Simulator of the Gastro-Intestinal tract
SSF	Simulated Salivary Fluid
TAAD	True Amino Acid Digestibility
TID	True Ileal Digestibility
UHPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet
WHO	World Health Organization

Introduction

1. Digestion

All the metabolic processes and energy-consuming activities that occur in our bodies are derived from the foods we ingest. The gastrointestinal system allows us to use food from diverse sources and utilizes them as an energy source to nourish our body. It also allows us to absorb substances that the human body cannot produce itself, such as essential amino acids or fatty acids, directly from food to use them directly in the body. The gastrointestinal system consists of the oral cavity, esophagus, stomach, small intestine (duodenum, jejunum and ileum), large intestine, and the assisting organs such as salivary glands, liver, gallbladder, and pancreas (figure 1). The digestive system has a wide range of functions, including ingestion (the reception, mastication, and softening of the food), transport of the ingested food, secretion of digestive enzymes, acid, mucus and bile, absorption of the end products of digestion, movement of undigested material, and elimination of waste products. In short, the fundamental function of the human digestive tract is to mechanically, chemically, and enzymatically disintegrate the food matrix and its constituents to release the nutrients, thereby becoming accessible and available for uptake into the body and excrete the waste products.

1.1 Oral Phase

Although it starts in the mouth, the majority of digestion processes take place in the stomach and small intestine and absorption occurs only in the small and large intestine. To make the nutrients in the food accessible to the digestive processes, the bounds connecting the nutrients together must firstly to be broken down. This process of breaking down the food into smaller units and finally into absorbable nutrients comprehends both chemical and physical activities. Physical activity includes mastication and the movement of muscles along the gastrointestinal tract that break down food into smaller pieces and mix it with digestive secretions. Chemical digestion is the breaking of covalent chemical bonds in nutrients, to produce smaller units thanks to enzymatic activity. In addition to enzymes, there are other chemicals supporting the digestive process, such as the acid in the stomach, a neutralizing base in the small intestine, bile that prepares fat for digestion, and mucus secreted along the gastrointestinal tract.

As soon as food enters our mouth, the digestive process begins. By chewing, the food is mixed with saliva and broken down into smaller particles suitable for swallowing, increasing the surface available for enzymatic activity. Textural and rheological characteristics of the foods as well as age, gender, and eating ability of individuals affect the oral food processing (Sensoy, 2021).

Saliva is a pH- neutral fluid secreted by the salivary glands with a flow rate of ~0.3 mL/min in unstimulated healthy adults and 1 - 2 mL/min when stimulated (Sreebny, 2000). It lubricates and moistens the inside of the mouth to aid speech and food processing, giving it viscosity, cohesion and lubrication, transforming it into a semi-solid mass (bolus) that facilitates swallowing (Boland, 2016). Saliva is composed by 99 % of water, containing sodium, potassium, calcium, bicarbonate, mucins, antibodies, enzymes (amylase, lingual lipase), and waste products (Ogobuiro et al., 2021).

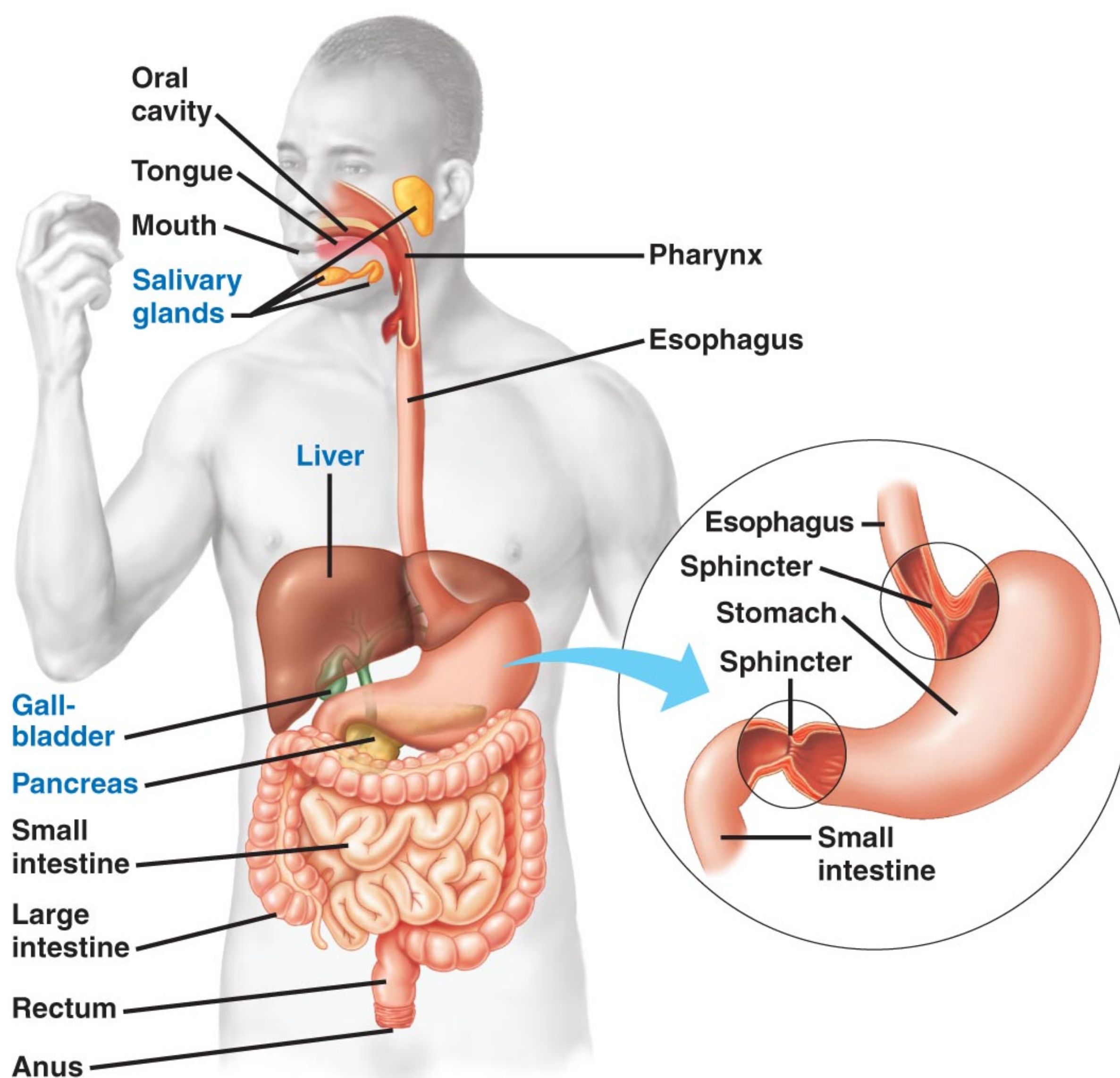


Figure 1. Anatomical organization of the human gastrointestinal system (Gafacom, 2019).

Salivary amylase is responsible for the hydrolysis of starch into maltose, maltotriose and dextrins (Feher, 2017). The amylase content of the saliva is variable but seems unaffected by stimulation and is typically ~45 U/mL (Neyraud et al., 2012). This enzyme is active at neutral

pH, and once the bolus enters the stomach, the acidic pH from the stomach deactivates it. Therefore, when carbohydrates reach the stomach, no further chemical breakdown occurs, nevertheless mechanical breakdown is ongoing. Due to the short time the food remains in the mouth, only 5 % of the starch is hydrolysed at this stage (Kibble & Halsey, 2014). Smaller carbohydrates, such as tri-saccharides and disaccharides, are not enzymatically digested until they reach the small intestine (Sanders, 2016).

Lingual lipase, unlike salivary amylase, which works best in non-acidic environments, lingual lipase is stable and more active at lower pH values. Thus, the process begins in the mouth and it continues in the stomach, where triglycerides are broken down into diglycerides (Wahbeh & Christie, 2011).

1.2 Gastric Phase

The digestion process in the stomach involves physical and chemical processes. When the bolus arrives in the stomach, the gastric mucosa and the stomach muscles are responsible for gastric secretions to degrade and dissolve the food and for the contractions that grind and push the food towards the pyloric sphincter. Gastric contractions mix the bolus with digestive juice and reduce particle sizes (<1 - 2 mm) by a grinding action to form a fluidized mixture called chyme. Between 1.2 to 1.5 L of digestive juice is produced per day. This fluid is a mixture of water, hydrochloric acid, electrolytes (sodium, potassium, calcium, phosphate, sulfate, and bicarbonate), mucus, enzymes (pepsin and gastric lipase), hormones (gastrin, serotonin) and the intrinsic factor (Hightower et al., 2020).

In fasting state, the stomach has a very acidic milieu, with a pH between 1-3. Nevertheless, the stomach walls are protected from this highly acidic juice by the membrane adjacent to the stomach lumen where the pH is almost neutral (pH 7) due to the bicarbonate secreted from the mucosa, while the acidity on the lumen side is very high (pH 2). However, after food ingestion, the pH rises to 5.5 - 7 due to the neutral pH of the bolus, depending on the buffering capacity of the food. As the digestive process progresses and the stomach empties, the pH decreases.

The gastric mucosa is covered by different cell types, which are mucus-secreting epithelial cells, mucoid cells, chief cells, gastrin cells, parietal cells, and other endocrine cells. Gastrin cells secrete gastrin as a response to meal intake. This hormone is responsible for the expansion of the gastric wall and stimulates the production of acid leading to a decrease in pH. Parietal cells produce an intrinsic factor (glycoprotein) that is essential for the absorption of vitamin B12 in the small intestine as well as the appetite-regulating hormone ghrelin. Mucoid cells secrete gastric mucus, and endocrine cells secrete serotonin, a hormone that stimulates the contraction of stomach muscles (Sensoy, 2021). Chief cells secrete gastric lipase and

pepsinogen, the latter being converted to the active digestive enzyme pepsin by the acidic conditions of the stomach (Prozialeck & Wershil, 2017).

Dietary proteins are affected by the gastric acid, which destroys their three-dimensional shape and exposing their peptide bonds, thus increasing their vulnerability to enzymatic attack. Pepsin is an endopeptidase that hydrolyses dietary proteins into smaller polypeptides with a high cleaving specificity for peptide bonds having aromatic amino acids such as tyrosine, phenylalanine, tryptophan, and leucine (Goodman, 2010). In middle-aged humans, basal pepsin output was shown to be about 1900 IU per 15 min while maximum pepsin output after stimulation with pentagastrin was 4600 IU per 15 min (Feldman et al., 1996). Pepsins are responsible for about 10 to 15 percent of protein digestion and their ability to break down protein is conditioned by the need for an acidic environment with a pH between 1.8 and 3.5.

Gastric lipase is responsible for 10 % to 30 % hydrolysis of ingested triglycerides and the remaining fat digestion is dependent on pancreatic lipase. Unlike pepsin, gastric lipase is not dependent on the acid pH and remains active in the small intestine (Martin & Freedman, 2019).

The stomach muscles mix and shear food into a thick creamy fluid, called chime. This semiliquid is slowly released into the small intestine through the pyloric sphincter, which acts as a sieve and a pump for the selective emptying of small particles. When food particles are sufficiently reduced in size and are nearly soluble (<1 mm), the emptying of the stomach starts. Particles larger than 1 - 2 mm are not cleared from the stomach until it is completely empty. This is important because the stomach is the last stage in the gastrointestinal tract with a mechanical function capable of breaking down particles (Boland, 2016). The emptying rate of the stomach is controlled by different factors, such as the physical and chemical composition of the meal and the particle size. Liquids are emptied faster than solids, carbohydrates faster than proteins, and proteins faster than fats. In addition, meals with high fibre and fat contents can delay gastric emptying. When enters the duodenum, the acidic chyme coming from the stomach triggers the secretion of hormones that slow or inhibit gastric secretion in order to prevent more acidic chyme from entering the small intestine (Hightower et al., 2020).

1.3 Intestinal Phase

Once in the intestine, the chyme is mixed and separated by segmenting contractions. A short segment of the intestinal wall contracts and constricts the lumen creating a pressure difference between the adjacent segments, which divides the intestinal contents promoting its movement (Sensoy, 2021). As the chyme enters the small intestine, pancreatic enzymes (a complex mixture of proteases, amylases, and lipases) are released through the hepatopancreatic sphincter into the duodenum (first segment of the small intestine) together

with pancreatic bicarbonate and bile. The bicarbonate neutralizes the acidity of the gastric juice, increasing the pH to a more favourable environment for enzymatic activity.

Pancreatic α-amylase is released to continue the digestion of starch that began in the mouth. Here, starch is split into the disaccharide maltose. Disaccharides such as maltose, sucrose and lactose cannot be broken down into their monomers yet. The end products of starch hydrolysis, α-dextrin, maltotriose, and maltose are further hydrolysed by enzymes present in the microvilli of the small intestine, the so called brush border enzymes. These enzymes include glycosidases such as maltase-glucoamylases, sucrase-isomaltases and lactases. Therefore, here sucrose and lactose can be hydrolysed. The end products of carbohydrate digestion are monosaccharides, which are quickly absorbed by the cells of the small intestine (enterocytes). However, any carbohydrates that is not digested and/or absorbed in the small intestine, such as dietary fiber, pass into the large intestine where they are then fermented by microbes residing in the intestine (Hornbuckle et al., 2008; Sanders, 2016).

Pancreatic proteases (trypsin, chymotrypsin, elastase, and carboxypeptidases) are synthesized in their inactive form, as zymogens, to not become active while inside the pancreas and causing pancreatitis. The enzyme enterokinase, a jejunal brush-border enzyme whose activity is enhanced by the bile salts, cleaves the zymogen trypsinogen into its active form, trypsin. In turn, trypsin activates the other zymogens into their active forms, chymotrypsin, elastase and carboxypeptidase (figure 2). Trypsin, chymotrypsin, and elastase are serine proteases that act as endopeptidases by cleaving peptide bonds within a polypeptide or protein being responsible for the breakdown of the protein chains into smaller peptides or free amino acids. Trypsin is very specific and cleaves peptide bonds next to lysine or arginine, chymotrypsin is less specific and cleaves peptide bonds adjacent to hydrophobic amino acids, while elastase cleaves elastin and peptide bonds adjacent to alanine, glycine, and serine. After the action of the endopeptidases, the remaining peptides are then attacked by exopeptidases, such as carboxypeptidase, which cleave one or two amino acids at the time at the carboxy-terminal of the polypeptide.

Most protein digestion takes place in the duodenum and upper jejunum (the two first segments of the small intestine) due to a more powerful activity of trypsin compared to pepsin. Consequently, and despite pepsins are responsible for 10 to 15 % of protein digestion, this process is commonly not impaired after the total removal of the stomach (Goodman, 2010; Hightower et al., 2020). Almost all the aminopeptidase activity is present in the brush border where the digestion of proteins is complete. At this level, hexapeptides or smaller chains are hydrolysed into tetra-, tri- and dipeptides or free amino acids that are transported through the luminal enterocyte membrane (Wahbeh & Christie, 2011). Amino acids, peptides and proteins that have not been absorbed and/or digested in the small intestine end up in the large intestine where they will be fermented by the gut microbiota (Joye, 2019).

In the stomach, gastric lipase cleaves 15 to 20 % of the fatty acids and the remaining fat is hydrolysed in the duodenum by pancreatic lipases. Although these enzymes are secreted in their active form, they need colipase to help with the digestive process. Lipase activity is enhanced by bile salts, which increase the surface area of oil-water interfaces at which water-soluble lipase is effective. Pancreatic lipase products are glycerol, 2- monoglycerides and free fatty acids. Monoglycerides and fatty acids can accumulate at the oil-water interface and inhibit lipase activity. HCO_3^- secreted by the pancreas and bile salts remove these molecules from the interface to the aqueous phase avoiding lipase enzymatic inactivity (Goodman, 2010; Hornbuckle et al., 2008).

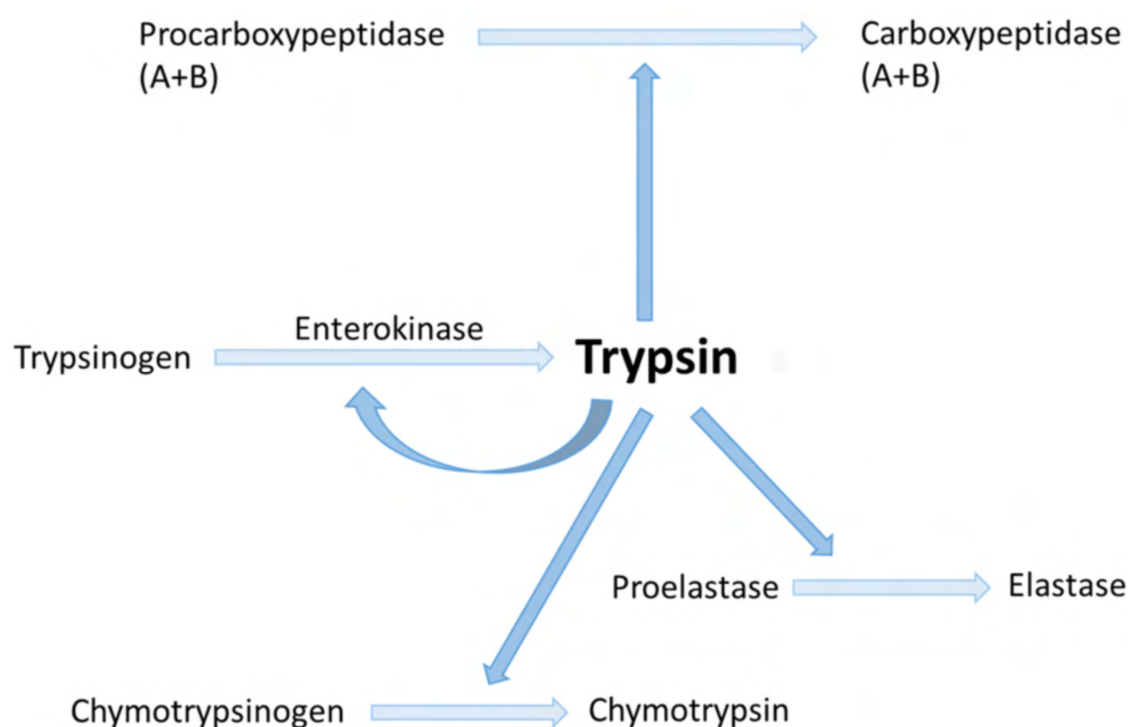


Figure 2. Trypsin enzymatic activity (Wahbeh & Christie, 2011).

Bile salts are synthesised in the liver and stored in the gallbladder. During digestion, bile is secreted into the duodenum where it facilitates the emulsification, solubilisation and hydrolysis of the dietary lipids by pancreatic lipase (Pitt & Gadacz, 2013). Bile is a complex mixture of bile salts, bile pigments, phospholipids, cholesterol, inorganic electrolytes, and end products of metabolism. The surfactant properties of bile play a key role in intestinal fat absorption by solubilizing dietary fats in the hydrophilic environment of the intestine due to the detergent action of its major components, the bile salts and phospholipids. In the gut, bile salts form water-soluble aggregates together with the fatty acids originated from the dietary fats, the so-called micelles. Bile salt micelles consist of a central nonpolar core, where the fatty acids, monoglycerides, and other lipids are solubilized, and an external polar region.

These micelles are formed when the critical micellar concentration of 2 mMol/L of the bile salt-monoglyceride-fatty acid-water is achieved. The micelles are highly soluble and favour the transport of the products of digested fats to the absorptive surfaces of the intestinal brush border. Fats diffuse across the membrane and the bile salts remain in the intestinal lumen. Later, they are absorbed in the terminal ileum and returned to the liver via the portal vein to be recycled via the enterohepatic circulation (Bodewes et al., 2015; Hornbuckle et al., 2008; Pitt & Gadacz, 2013).

A summary of the physical and chemical processes and digestion conditions occurring in the human digestive system is present in table 1.

Table 1. Summary of the physical and chemical processes occurring in the human digestive system (Ekmekcioglu, 2002; Sensoy, 2021).

Section	Physical process	Chemical process	Conditions (adult)
Mouth	**Mastication** Food breakdown Mixing	**Enzymatic hydrolysis** Starch breakdown (α-amylase) Lipid breakdown (lingual lipase)	pH: 5 - 7 Transit time: 5 s - 2 min Saliva flow rate: 0.042 - 1.83 mL/min (unstimulated) 0.77 - 4.15 mL/min (stimulated) Biting force: 100 - 400 N
Esophagus	**Peristalsis** Bolus transport	-	Transit time: 8 - 10 s solids; 1 - 2 s liquids
Stomach	**Contractions/Peristalsis** Food breakdown Mixing Gastric transport Gastric sieving	**Enzymatic hydrolysis** Protein breakdown (pepsin) Lipid breakdown (gastric lipase) Acid hydrolysis Food softening and dissolution (gastric acid)	pH: 1 - 3 Transit time: 15 min - 4 h Gastric juice secretion: 1 - 3 L/day Basal pepsin output ≈ 1900 IU /15 min Maximum pepsin output ≈ 4600 IU /15 min Contraction frequency: 3 cycles/min
Small intestine	**Peristalsis** Chyme transport **Segmentation** Mixing **Absorption** Monosaccharides	**Enzymatic hydrolysis** Starch breakdown (pancreatic amylase, dextrinase, sucrose,	pH ≈ 5.7 - 6.4 in the duodenum, up to ≈ 7.4 in the jejunum, up to ≈ 7.7 in the ileum Transit time: 1 - 5 h Pancreatic juice secretion: ~1.5 L/day Luminal fluid volume: ~9 L entering duodenum per 24 h

	(glucose, galactose, fructose) Amino acids, peptides Monoacylglycerides, glycerol, free fatty acids Phosphates, nitrogenous bases, pentose sugars Minerals and vitamins	maltase, lactase, amyloglucosidase) Protein breakdown (trypsin, chymotrypsin, carboxypeptidase, elastase) Lipid breakdown (pancreatic lipases, phospholipase) Nucleic acid breakdown (nucleases, nucleosidases and phosphatases)	Transport area (without the microvilli): 100 000 cm^2 Effective pore radius of tight junctions ≈ 6.7 - 8.8 Å in jejunum Peak amylase output ≈ 39 kU /h Peak trypsin output ≈ 5 - 10 kU /30min Food stimulated lipase output ≈ 4 kU /min Food stimulated bile salt output ≈ 20 μM /min Peristaltic frequency ≈ 11.7 /min in the duodenum, 8.9 – 9.8 /min in the jejunum and ileum
Large intestine	**Peristalsis** Chyme transport **Segmentation** Mixing **Absorption** Water, ions, minerals, vitamins, fats and organic molecules	**Fermentation** Production of short chain fatty acids and other by products	pH: 5 - 7 Transit time: 12 - 24 h Microbiota: ~10^{11}- 10^{12} (>1000 different species)

2. Protein absorption

Dietary proteins and peptides are subjected to complex changes during ingestion, digestion and absorption. In adults, essentially all protein is absorbed as tripeptides, dipeptides or amino acids and this process occurs in the duodenum or proximal jejunum of the small intestine.

The small intestine has a diameter of 3 - 4 cm, but its total absorptive area is approximately 4500 square meters due to the numerous concentric folds of the mucosa, which provide a large absorptive surface (figure 3) (Sensoy, 2021). The intestinal mucosa is composed by different types of cells: the enterocytes or brush border cells (the normal absorptive cells and the most abundant cell type in the intestinal mucosa), the Goblet cells (responsible for secreting mucin), the endocrine cells (responsible for the nutrients detection

and the secretion of gastrointestinal hormones) and the Paneth cells (secretion of digestive enzymes, growth factors and antimicrobial peptides (Lea, 2015).

The intestinal mucosa layer is selectively permeable to bacterial metabolites and digested nutrients. Permeability is strictly dependent on the size, charge, and surface properties of the particles, but also on the pore size of the network through which it is transported (Mackie et al., 2012).

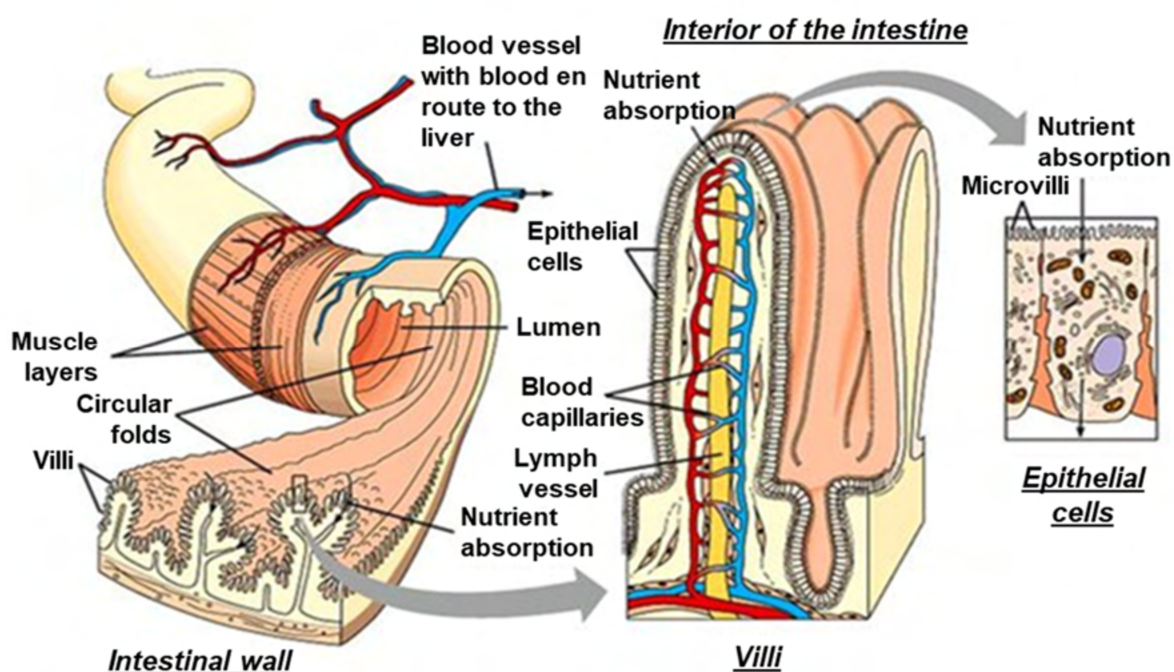


Figure 3. The inner wall of the small intestine is covered by numerous folds of mucous membrane. The surface of these folds contains tiny projections called villi and microvilli, which further increase the total area for absorption. Absorbed nutrients are moved into circulation by blood capillaries or lymph channels (Soffar, 2017).

Nutrients are transported by active diffusion and/or active transport. The active transport pathway requires energy and it is mediated by transporters once the absorption of the particles occurs against an electrical or chemical gradient and it is carrier-mediated. Passive diffusion is the most common mechanism of absorption across the intestinal membrane and depends mainly on the concentration gradient between the intestinal lumen and the interstitium. It can be divided into two pathways: the paracellular pathway, where the particles cross the epithelium through the aqueous pores at the tight junctions between the intestinal enterocytes; and the transcellular pathway, which requires particles diffusion across the enterocyte cell membrane. Paracellular transport (1 in figure 4) consists in the transport of soluble molecules through the paracellular space between the epithelial cells and it is carried out by passive diffusion that depends mainly on the concentration gradient between the

intestinal lumen and the interstitium and on the pore radius of the intercellular tight junction structures (Ekmekcioglu, 2002). Transcellular transport can be distinguished in passive diffusion through the lipid enterocyte membrane (2 in figure 4); endocytosis (eventually via membrane receptors) allowing vesicle-mediated uptake, transport and release at the basolateral side in a process called transcytosis (3 in figure 4); and carrier-mediated uptake (4 in figure 4), which contribute to internalize luminal molecules and diffusion through the epithelial cell layerEach of these transport mechanisms depends on the physicochemical properties of the compound, its ability to interact with and pass the plasma membrane, its molecular weight and size, stability, and charge distribution (Lea, 2015).

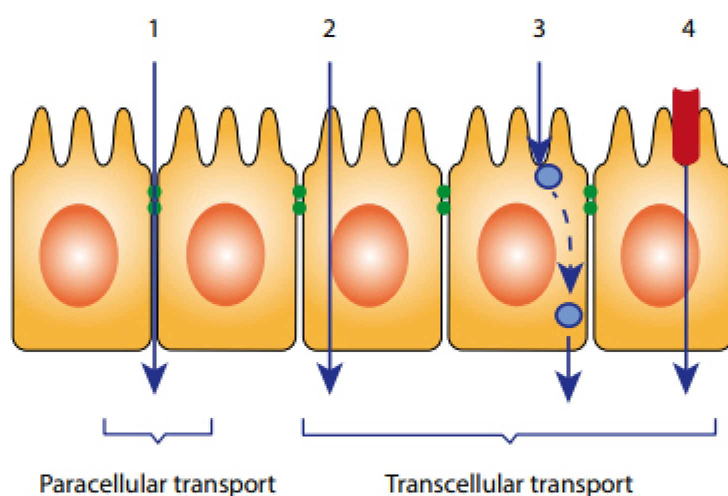


Figure 4. The figure illustrates the different modes of absorption and transport through the intestinal epithelium: (1) paracellular transport, (2) passive diffusion, (3) vesicle-mediated transcytosis and (4) carrier-mediated uptake (Lea, 2015).

The transport of amino acids, electrolytes, short-chain fatty acids, and sugars occurs typically via transcellular permeability and is regulated by selective transporters (Groschwitz & Hogan, 2009). Despite most of the ingested dietary protein being absorbed as free amino acids and di- and tripeptides, the intestinal mucosa is not fully impermeable to large polypeptides and the absorption of insulin (MW 5700 Da; (Laskowski et al., 1958), ribonuclease (MW 13700 Da; (Alpers & Isselbacher, 1967), ferritin, and horseradish peroxidase (Warshaw et al., 1971) has been previously demonstrated. Different proteolytic enzymes are necessary to hydrolyse dietary proteins into small peptides and amino acids due to their specificity for different types of peptide bonds. First, hydrolysis of proteins by gastric and pancreatic endopeptidases results in a pool of polypeptides. Then, exopeptidases cut one amino acid at a time either from the carboxy-terminal (carboxypeptidases) or from the amino-terminal (aminopeptidases) of the polypeptides, producing small peptides or free amino acids. Protein hydrolysis yields 30 % free amino acids and 70 % oligopeptides (2 - 8 amino acids).

In the small intestine, the brush border membrane is composed by numerous microvilli housing the so-called brush border digestive enzymes. These enzymes (including aminopeptidases, carboxypeptidases, endopeptidases, and dipeptidases) are responsible for the final stage of peptide digestion (before their absorption into the enterocytes) by reducing poly- and oligopeptides to free amino acids, and di- and tripeptides (Hooton et al., 2015). Some of the tri- and dipeptides are absorbed into the enterocytes and hydrolysed by cytosolic peptidases within the enterocytes cytoplasm, while others are absorbed into the blood stream intact. The absorptive capacity for di- and tripeptides is higher in the proximal small intestine than in the distal small intestine, whereas in the case of free amino acids the absorptive capacity is higher in the distal small intestine (Ganapathy et al., 2006).

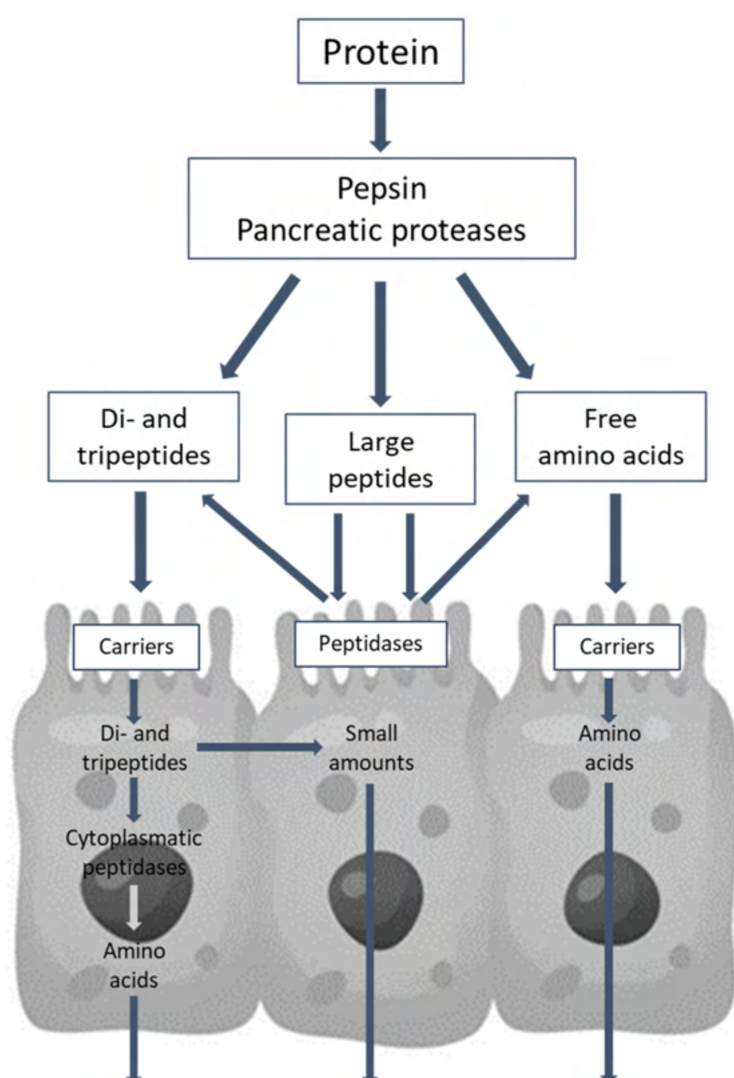


Figure 5. Protein digestion and absorption scheme adapted from (Wahbeh & Christie, 2011).

Despite more than 90 % of the proteins and oligopeptides are hydrolysed intracellularly, some proteins and oligopeptides can be transported by transcytosis (vesicle-mediated

transcellular transport), with the absorption rate of intact proteins and peptides depending on the maturity and health status of the individual (Jahan-Mihan et al., 2011)

3. Satiety

The human digestive system consists of the digestive tract and the accessory organs controlled by the neural network and hormones.

Enteroendocrine cells (EECs) scattered along the epithelial layer of the gastrointestinal tract and constitute <1 % of the cell population in the intestinal epithelium. Despite their low abundance, taken together these cells constitute one of the largest endocrine systems in the body, representing an important component of the gut–brain axis and playing critical physiological roles. Enteroendocrine cells are specialized for secretion. They sense luminal contents, particularly nutrients, and secrete a variety of hormones such as glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), somatostatin, ghrelin, and serotonin that regulate digestion, gastric acid secretion, gut motility and food intake (Gribble et al., 2018; Shea-Donohue, 2018).

CCK and GLP-1 are secreted from enteroendocrine I and L cells, respectively. Each enteroendocrine cell type exhibits a characteristic distribution along the length of the gastrointestinal tract, with I-cells typically more abundant in the duodenum and jejunum and L-cells in the jejunum, ileum and colon (Gribble et al., 2018). The time courses of gut hormones that appear in the bloodstream after a meal are mirrored by the location of their respective secretory cell types along the gastrointestinal tract length. Thus, it is generally reported that CCK appear in the bloodstream as soon as nutrients enter the duodenum, whereas GLP-1 release is delayed until the food has been shifted lower down along the gastrointestinal tract (Pilichiewicz, Chaikomin, et al., 2007; Pilichiewicz, Papadopoulos, et al., 2007).

Interest in gut peptides has increased in recent decades, with the finding that they have profound and sustained physiological effects on appetite and insulin release. GLP-1, is an incretin hormone that stimulates glucose-stimulated insulin secretion, somatostatin secretion, and inhibits glucagon secretion (figure 6). Additionally, GLP-1 induces an ‘ileal break' by inhibiting gastric emptying and decelerating ileal transit, thereby decreasing food intake and preventing malabsorption and postprandial metabolic disturbances (Müller et al., 2019). This hormone has been very successfully exploited for the treatment of type-2 diabetes (Onge et al., 2017). GLP-1 release is particularly strongly stimulated by glucose and fat, nevertheless, dietary protein is also a very strong stimulus for GLP-1 release by the small intestine (Bowen et al., 2006; Gribble et al., 2018). The amino acids glutamine (Tolhurst et al., 2011) and glycine (Gameiro et al., 2005) are the stronger protein-derived inducers of GLP-1 release. Amino acids

stimulate GLP-1 secretion via one G protein–coupled receptor (GPRC6A) and the heterodimer TAS1R1-TAS1R3 (Steensels & Depoortere, 2018).

Cholecystokinin is a peptide hormone produced by the I-cells in the proximal small intestine and in response to dietary fat and protein. Aromatic amino acids (L-phenylalanine and L-tryptophan) have proven to be strong stimulants of CCK release (Wang et al., 2011). CCK plays a key role in gallbladder contraction having a big impact on stimulating postprandial pancreatic enzyme secretion. Furthermore, CCK inhibits gastric emptying and acid secretion, promotes intestinal motility, satiety, and reduces food intake (figure 7). The mechanisms by which digested proteins stimulate CCK secretion include activation of PEPT1 and the calcium-sensing receptor (CaSR) (Gribble et al., 2018).

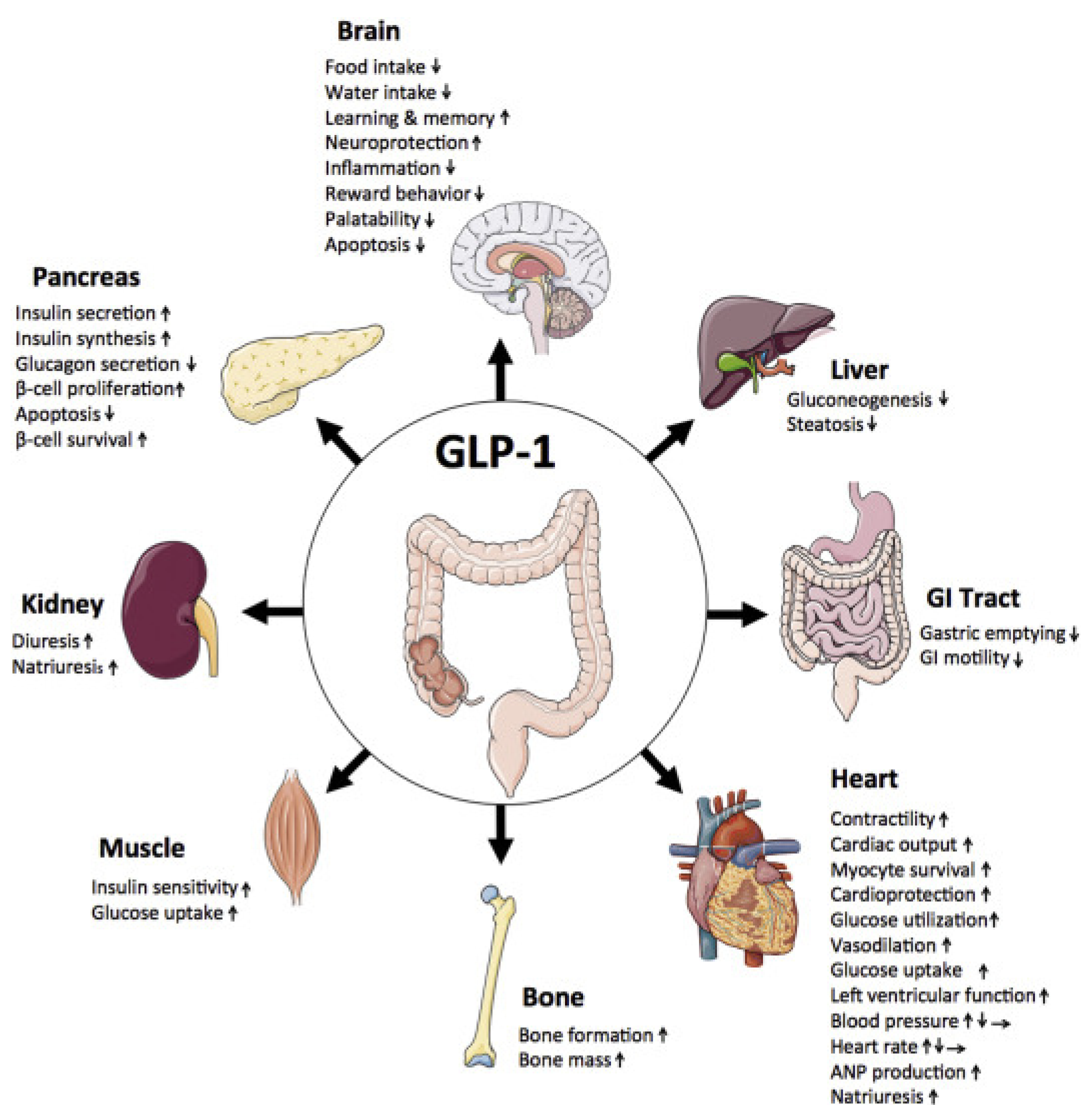


Figure 6. Metabolic effects of GLP-1 (Müller et al., 2019).

3.1 Satiety studies

The present obesity pandemic is a big global health issue, which has arisen due to the abundance of highly palatable, calorie-dense food combined with reduced levels of physical activity. However, currently available pharmacological weight loss agents are only modestly effective. Therefore, formulating foods, which contain targeted nutraceuticals to exploit the various nutrient detection systems present in EECs, represents another possible approach to the treatment of obesity (Spreckley & Murphy, 2015).

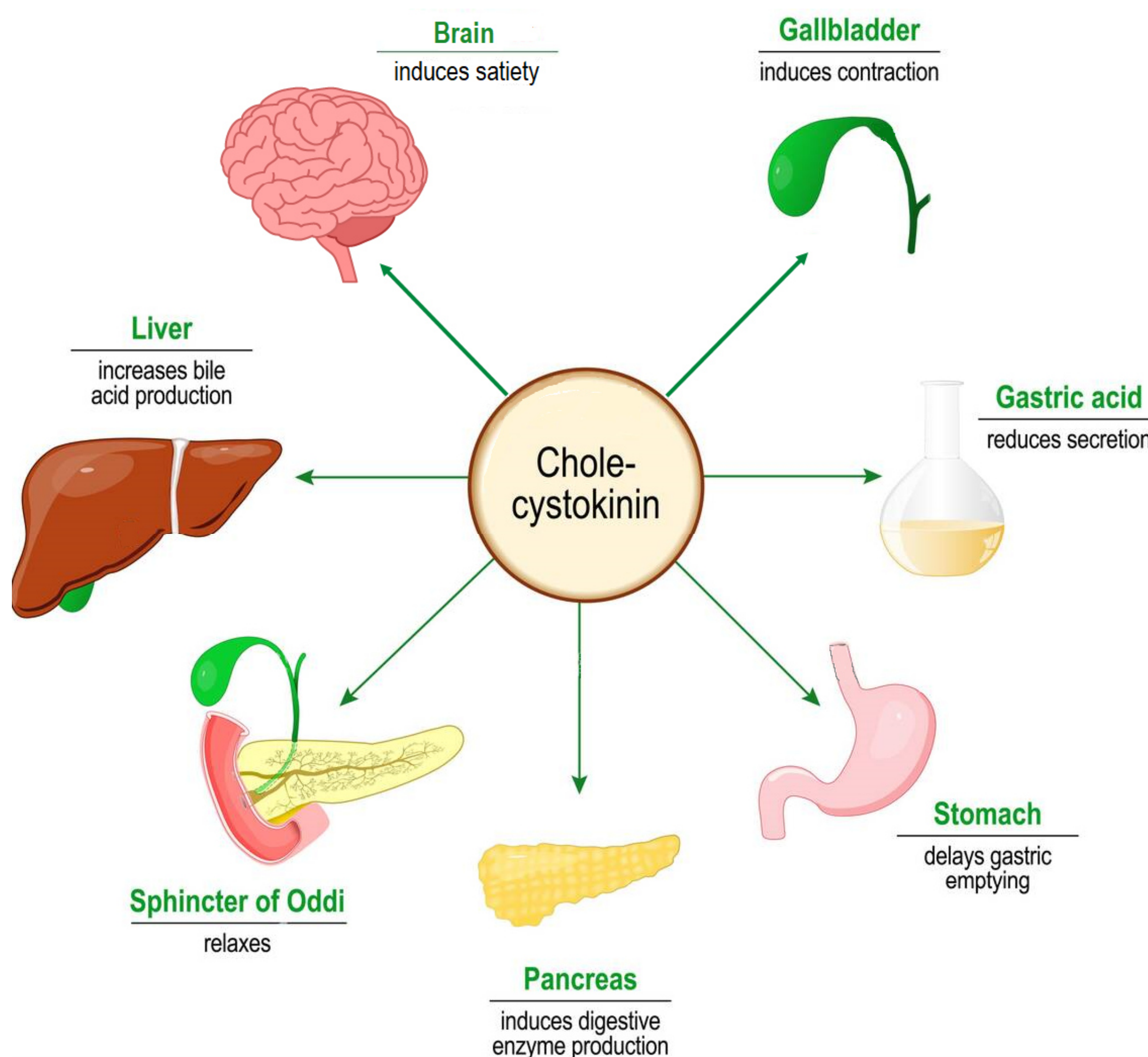


Figure 7. Metabolic effects of CCK (Zhabska, 2020).

Isolated mammalian enteroendocrine cells would be the best screening model to measure gut hormonal responses to food. However, isolation of native intestinal cells is a tedious process with very low yields because enteroendocrine cells are sparsely dispersed throughout the gastro-intestinal tract and are co-localized with abundant enterocytes.

Therefore, *in vitro* models of enteroendocrine cell lines are difficult to develop (Evans et al., 1994).

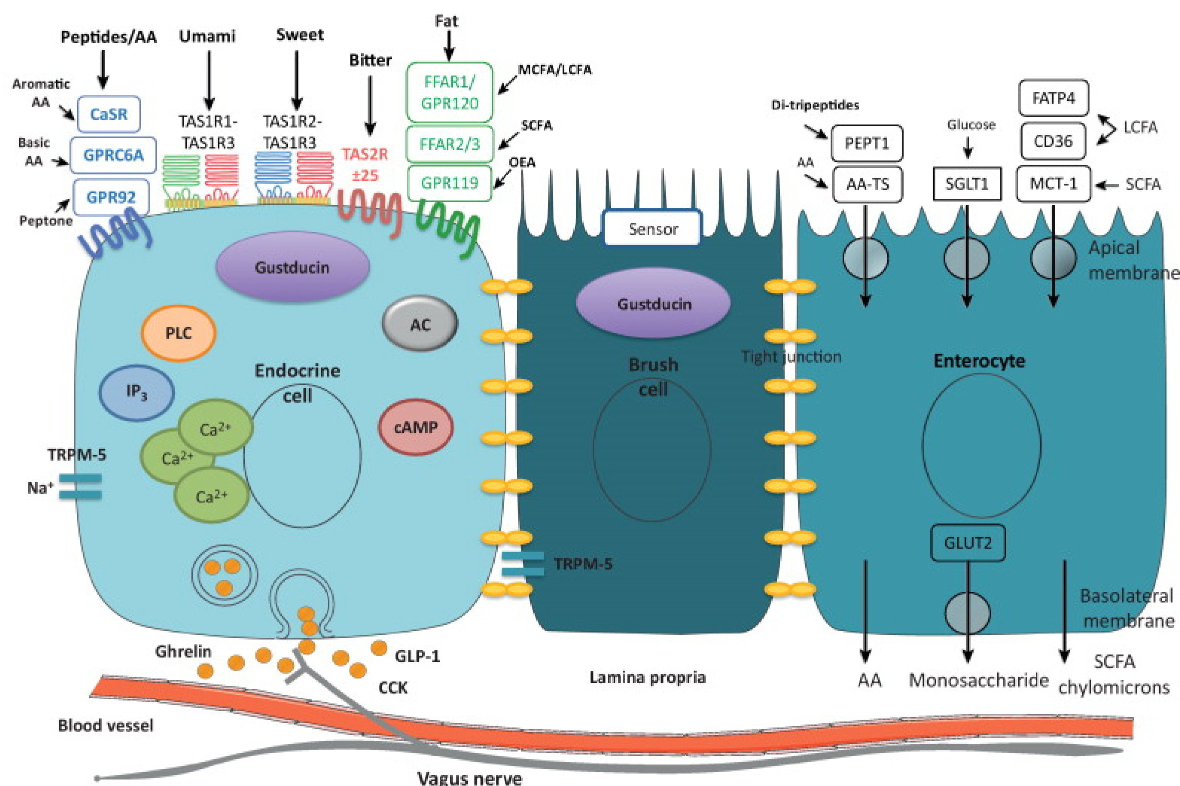


Figure 8. Simplified model of the pathways involved in chemosensory signalling in the gastrointestinal mucosa. Nutrients (sweet, bitter, fat, amino acids) are sensed by different G protein-coupled receptors (GPCRs) as well as transporters in several cell types (endocrine cell, brush cell, enterocyte) of the epithelial lining that cross regulate each other's expression. The GPCRs induce the release of second messengers that lead to the release of gut peptides which can communicate directly, via the bloodstream, or indirectly, via the vagal nerve, with the hypothalamus to control food intake (Janssen & Depoortere, 2013).

However, several enteroendocrine cell models have been successfully established and are available for *in vitro* screening bioassays (Verhoeckx et al., 2015).

SCT-1 is a cell line derived from a duodenal secretin tumour cells from transgenic mice, expressing many features of native intestinal hormone-secreting cells (Rindi et al., 1990). SCT-1 secrete CCK and GLP-1 in a manner that resembles that of humans and therefore they are routinely used in screening platforms to identify foods or compounds that modulate secretion of gastrointestinal hormones *in vitro* (Geraedts et al., 2011; Santos-Hernández et al., 2018).

While, STC-1 cells secrete these hormones in response to a range of physiological stimuli, levels may differ to native enteroendocrine cells (Reimann et al., 2008). Monosaccharides (Mangel et al., 1994), fatty acids (Hand et al., 2010), aromatic amino acids

(Cordier-Bussat et al., 1997; Wang et al., 2011), peptidomimetic compounds (Geraedts et al., 2011) and bitter tastants (Miyata et al., 2014) have all been demonstrated to dose dependently elicit CCK and GLP-1 secretion from STC-1 cells.

Hormonal secretion from SCT-1 cell line seems to be dependent on an increase in cAMP and cytoplasmic Ca^{2+}, which leads to changes in the transcriptional levels of hormonal genes or release of hormonal peptides. Increased levels of Ca^{2+} can be due to (a) an influx of Ca^{2+} across the plasma membrane through activation of voltage-gated calcium channels or (b) triggered inositol 1,4,5-triphosphate (IP3) by Ca^{2+} release of intracellular stores. In native enteroendocrine cells, activity of various G protein-coupled receptors (GPCRs) and nutrient transporters also alter intracellular levels of Ca^{2+} and cAMP (figure 8) (McCarthy et al., 2015).

In addition to stimulating the release of intestinal hormones, common luminal nutrients added to the growth medium are also able to modulate gene expression in STC-1 cells. As such, this cell line has been widely used to investigate the regulation of CCK gene expression. However, for the proglucagon gene, aberrant post-translational processing has been documented (Blache et al., 1994). When properly used, the STC-1 cell line has been proven a reliable and reproducible enteroendocrine cell model. Yet, considerable variations in hormone secretion levels have been reported by different laboratories for the same substance (Geraedts et al., 2009; Hand et al., 2013). Some studies have shown that protein hydrolysates induced greater release of CCK and GLP-1 when compared to undigested proteins (Caron et al., 2016; Cordier-Bussat et al., 1997). In contrast, other studies have demonstrated that intact protein is a much stronger stimulus for CCK and GLP-1 release in STC-1 cells than hydrolysates or specific peptides (Geraedts et al., 2011; Power-Grant et al., 2015). In addition to differences between different working groups, significant inter-experimental variability in the amount of CCK secreted has also been reported. Differences in culture protocols such as seeding density, cell feeding routine, washing steps, test buffer and passage number may contribute to this variability (McCarthy et al., 2015).

4. Dietary proteins

Protein is one of the three macronutrients we can obtain from our food and constitutes about half of the human body's dry weight. Proteins are the main supply of nitrogen in the diet, and despite the wide range of existing proteins, all of them are built with the same 20 amino acids linked by peptide bonds. Amino acids can be distinguished between essentials (must be supplied by the diet- His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val), nonessentials (can be synthesized by the body using other amino acids or simpler precursors- Asp, Asn, Glu, Ala, Ser, Cys, Tyr, Gly, Arg, Gln and Pro) and conditionally essential (under certain pathophysiological conditions become essential- Cys, Tyr, Gly, Arg, Gln, Pro and Tau) (Boye et al., 2012). Most of the proteins in our body have specific functions in the regulation of growth, repair, maintenance and replacement of the tissues and consequently, any loss in body proteins is a loss of cellular function.

Contrary to lipids and carbohydrates, the human body does not have true reserves of protein and, therefore, insufficiency of dietary protein is compensated by catabolizing some, but not all, proteins in our body's tissues. The protein pool that is irreversibly catabolized due to body metabolism is defined as the recommended daily protein intake, which varies with age, physiological state, and sex (Nadathur et al., 2017). In other words, protein nutritional requirement is "the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity, plus, in children or pregnant/lactating women, the needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health" (FAO/WHO/UNU, 2007).

A reference for protein intake in Europe was estimated at ~0.83 g/kg per day for the general adult population, with exception of young children and pregnant and lactating women who require higher protein intakes (Mariotti, 2016). However, in most of the industrialized countries, the average protein intake is 100 g/day, that is, 1.3 - 1.4 g/kg per day corresponding to ~16% of total energy intake (Elmadfa et al., 2009; Fulgoni, 2008).

Dietary proteins can be classified according to their nutritional value depending on the amino acid content and composition. Complete proteins contain all the essential amino acids in the right proportions required by the human body, whereas incomplete proteins are deficient in one or more essential amino acid. With the notable exception of collagen, proteins derived from animal sources are considered whole or complete proteins because they contain the entire spectrum of the 20 amino acids, including all essential amino acids, being best suited to human needs, as well as being highly digestible. However, plant proteins (with exception of soy protein) often lack or are low in one or more essential amino acids and are less digestible than animal proteins, and are therefore of lower quality and might even be considered as

incomplete protein sources (Apong, 2019; Hertzler et al., 2020). These differences between plant and animal proteins can be due to structural differences, as for example more β-sheet structures in plant proteins and less α-helixes than animal proteins (Carbonaro et al., 2012; Nguyen et al., 2015). The high amount of fibers and the presence of antinutritional factors in plant proteins, make plant proteins more resistant to proteolytic digestion (Duodu et al., 2003; Tulbek et al., 2017).

4.1 Selected protein sources

4.1.1 Plant origin sources

In global terms, around 80 % of food energy and 65 % of dietary protein are provided by plant foods (Sathe, 2002).

Plant proteins are complex and cereal, pulse and legume proteins differ in characteristics. Food grain legumes (e.g. beans, peas and lentils) play an important role in the human food supply and represent the main protein source in starchy foods-based diets consumed by a large number of people in developing countries (Los et al., 2018; Talari & Shakappa, 2018; Venkidasamy et al., 2019). Pulses, as they are also called, contain approximately 21 - 25 % crude protein. However, these percentages may vary slightly depending on genetic, maturity and environmental factors (Henchion et al., 2017). Pulses are rich in lysine, leucine, aspartic acid, glutamic acid, and arginine, but poor in sulphur containing amino acids (cysteine, methionine and tryptophan). When consumed together with cereals, which are rich in sulphur amino acids, they provide a well-balanced essential amino acid profile in order to meet human nutritional needs (Boye et al., 2010). Most of the protein found in pulse seeds is in the form of storage proteins called globulins (vicilin and legumin), which represent approximately 70 % of the total protein (Shevkani et al., 2019). Pulses contain many bioactive substances such as enzyme inhibitors, lectins, phytates and phenolic compounds, which are part of the defensive mechanism of the seed and may affect several biological functions when consumed. Some of these substances (e.g. enzyme inhibitors and lectins) are considered as antinutritional factors once they can reduce protein digestibility and nutrient absorption (Campos-Vega et al., 2010). Nevertheless, this effect can be reduced by cooking the foods before consumption (Lajolo & Genovese, 2002). On the other hand, health benefits were associated to the same bioactive compounds. Regular consumption of grain legumes is associated with reduction of cholesterol levels, and reduction of the risk of cardiovascular diseases, cancers and diabetes (Leterme, 2002; Martino et al., 2012; Singh & Basu, 2012; Talari & Shakappa, 2018). Considering these characteristics, there is a growing interest in the

use of these foods in the development of healthy ingredients, such as flour mixtures and extruded snacks, aiming to create functional and healthy foods.

Cultivated worldwide, peanuts are one of the largest agricultural crop, being mainly used for oil production and as a cheap protein source. Although often eaten as nuts, peanuts are actually legumes and have more protein than any other nut with levels comparable to or better than beans. Peanuts have a high digestibility with a true protein digestibility comparable with that of animal protein (Arya et al., 2016; Settaluri et al., 2012). Sometimes called a poor man's protein, as they are available at an affordable price, peanuts are an excellent source for vital nutrients such as proteins (24%), vitamins, minerals, fibres and bioactive compounds that prevent disease and promotes good health (Council, 2013; Sandefur et al., 2017; Settaluri et al., 2012). The main proteins are two globulins, arachine and conarchine, which represent approximately 95 % of the total proteins (Sebeia et al., 2013). Peanut proteins are rich in arginine, asparagine/aspartic acid and glutamine/glutamic acid, but poor in methionine, threonine and tryptophan (Davis & Dean, 2016; Settaluri et al., 2012).

Cereal grains (wheat, corn, rice, barley, sorghum, etc.) provide 62 % of the world food supplies being the main staples consumed globally. Especially in developing countries where average protein intake is below the recommended values, cereals play an extremely important role (Khan et al., 2014). When compared with legume seeds, cereal grains contain little protein, with an average of about 10 – 12 % in dry weight (Shewry & Halford, 2002). More than 50 % of the total protein content in the mature cereal seeds are storage proteins (prolamins and glutelins), which are rich in glutamine, proline, leucine and alanine. On the other hand, the essential amino acids lysine, tryptophan, methionine, histidine are limiting (Cunsolo et al., 2012; Koehler & Wieser, 2013).

Wheat is the largest plant protein source in the Western diet and is often used for daily food products (Krijne & Essink, 2011). Wheat is not utilized with the same efficiency as animal proteins and when consumed as an essentially single source of protein, high quantities are needed to meet human physiological needs for both total protein and specific indispensable amino acids. Especially lysine and also threonine and tryptophan are often limiting amino acids in these foods. Yet, when combined with other food proteins such as legumes, oil seeds or animal products, the proteins of wheat exhibit excellent nutritional complementarity (Young & Pellett, 1985). The true ileal digestibility of wheat protein is reported to be around 90 % (Bos et al., 2005).

Sorghum is a vital food crops for millions of people in Africa and Asia, where it is often the only viable food grain crop for many people who live in these regions (Bhagavatula et al., 2013; Taylor, 2004). Sorghum has similar protein amounts when compared with other cereals but lower protein quality. Lysine, which plays an essential role in growth in infants and maintenance in adults, being important for bone calcification and gastric secretions, and

playing a vital role in the immune system, is the first limiting indispensable amino acid in sorghum. Furthermore, sorghum proteins are poorly digestible, especially when cooked wet, leading to a decrease in amino acid bioavailability (Taylor & Taylor, 2011).

Corn is widely consumed in Mesoamerica and in Eastern and Southern Africa and it is very important for global food security (Henchion et al., 2017). Zein is the trivial name for the most abundant storage protein in corn. This prolamin accounts for 35-65 % of total protein and is found exclusively in the endosperm (Luo & Wang, 2012). Its poor water solubility and imbalanced amino acid profile (only traces of tryptophan and lysine) make zein not the ideal protein for human consumption. Zein is obtained from corn starch handling as a by-product and for many years was considered a waste protein without value. Recently, novel applications, taking advantage of its physicochemical and biological properties, sparked interest in this protein, especially in pharmaceuticals and food industries (Lorenzo et al., 2018; Luo & Wang, 2014; Sharif et al., 2019). Humans have used milk since the beginning time as a source of essential nutrients and energy, providing high-quality proteins, fats, minerals and vitamins.

4.1.2 Animal origin sources

Milk and milk products are nutrient-dense foods and their consumption plays an important role in the diets of children in populations with very low protein intakes and limited access to other animal source foods (FAO, 2022).

Whey is a by-product of cheese-making and casein production in the dairy industry, accounting for 20% (wt/wt) of total milk protein (Sindayikengera & Xia, 2006). It is a complex mixture of globular protein molecules such as β-LG (~50 wt/wt), α-LA (~20 % wt/wt), immunoglobulins (IgG; ~10 % wt/wt), BSA (~6 %, wt/wt) and other minor protein or peptide components like lactoferrin, lactoperoxidase, lysozyme, and growth factors (Muro et al., 2011; Walstra & Jenness, 1984). Whey protein is a good source of cysteine. This amino acid seems to enhance glutathione levels, which has demonstrated strong antioxidant properties, helping the body fighting various diseases such as cancer (Bounous, 2000). In addition, whey is a good source of branched-chain amino acid (BCAA) (leucine, isoleucine and valine), which have been associated with increased stimulus of skeletal muscle protein synthesis (Almeida et al., 2015), therefore whey is well known for its applicability in sports nutrition. Additionally, its functional potential to modulate adiposity, improve immune function and antioxidant properties has sparked interest in the food and drug industry (Ha & Zemel, 2003). Whey products have been used in baked goods, salad dressings, emulsifiers, infant formulas, and medical nutritional formulas. Depending on their composition, as a result of the extent and method of processing, three forms of whey can be distinguished: whey powder, whey protein

concentrate, and whey protein isolate. Isolates are the purest protein source available with protein concentrations of 90 % or higher (Bawa, 2007).

Meat played a vital role in human evolution and has always been consumed to satisfy the nutritional needs of human beings. Over the last century, global meat consumption has risen sharply with animal-derived protein accounting for nearly 40 % of humanity's total protein consumption, and FAO expects a substantial increase by 2050 if the trend goes on uninterrupted (Boland et al., 2013). Beef in particular has played a key role in food security by providing energy, protein, and essential micronutrients. Depending on the source and the fat content, raw meat can have protein contents between 20 to 25 %, which, due to the loss of water in cooking, can correspond to 28 to 36 % in the prepared food (Henchion et al., 2017). Meat protein is an excellent source of essential amino acids (arginine, valine, lysine and leucine) and has high net protein utilization and digestibility. In addition, meat is as well a good source of minerals (iron, zinc, and selenium) and vitamins (A, B9 & 12, D, and E) (Ahmad et al., 2018).

Collagen is the main structural protein of the different connective tissues in animals. It is mostly found in fibrous tissues, such as tendons and ligaments, but also abounds in cornea, cartilage, bones, blood vessels, the gut, and intervertebral discs, accounting for 25 to 35 % of the total protein mass in mammals (Sibilla et al., 2015). Collagen consists of three peptide chains rich in glycine and proline and the occurrence of 4-hydroxyproline and 5-hydroxylysine (Belitz et al., 2009). It can be extracted from various animal species and is generally derived from slaughter by-products. There is great demand for collagen in food and beverage industries because of its high protein content and functional properties, such as water absorption capacity, gel formation, and the ability to form and stabilize emulsions. Furthermore, collagen enhances the quality, nutritional and health value of the food products. It has been applied as protein dietary supplements, carriers, food additives, edible films and coatings. Due to collagen's excellent biological compatibility and degradability, with weak antigenicity, it has been widely applied in the cosmetic, biomedical, pharmaceutical, and tissue engineering fields. It has been used as a vehicle for drugs, proteins and genes, as well as substitute for human skin, blood vessels and ligaments (Hashim et al., 2015; Schmidt et al., 2016; Sionkowska et al., 2020).

4.2 New dietary proteins

The current livestock sector uses about 70 % of global agricultural land (FAO, 2009) and is responsible for approximately 14.5 % of global greenhouse gas emissions, representing the most important source of methane, polluting fresh and marine waters (Gerber et al., 2013). Half of the Earth's ice-free land area is used as farmland or grazing land, and the increasing global demand for food and increasing population are leading to deforestation of rainforests, savannahs and grasslands, which threatens species with extinction. However, despite intense global agriculture, nearly a billion people still have inadequate diets and insecure food supplies. On the other hand, the more frequent and intensive presence of processed foods, refined sugars, refined fats, oils and meats in our diet contributed to 2.1 billion people becoming overweight or obese (Tilman & Clark, 2014).

The rapid increase in meat consumption, especially in developing countries due to an increase in wages, is negatively associated with environmental sustainability (excessive resource consumption, water and land pollution, greenhouse gas emissions), and human health (increased risk of cardiovascular diseases, cancer and diabetes type 2) (Zhang et al., 2022). In contrast, vegetarian and meat-reduced diets have a positive impact on both factors (Dinu et al., 2017). By directly linking and negatively affecting human and environmental health, a shift towards reducing meat consumption is needed and the global food transition is one of the great challenges facing humanity. One way to achieve this goal is to replace meat with meat analogues.

In recent years, many types of alternative meat containing insects, egg whites, grains, legumes or fungi as a source of protein have been launched on the market (Michel et al., 2021; Zhang et al., 2022). However, there are some concerns that vegans, or diets containing plant-based protein as the main source of protein, are nutrient deficient due to unbalanced protein sources. This stems from the fact that unlike animal proteins, plant proteins may not contain all essential amino acids in the required proportions to meet human nutritional needs (Elorinne et al., 2016).

Biological value is often discussed in the context of matching essential amino acid intake with the body's demand pattern. This allows the identification of dietary protein mixtures enabling essential amino acid deficiencies in one protein to be supplemented with a relative excess in another protein, resulting in an adequate overall diet mix that can help to overcome this in strict vegan or vegetarian diets (FAO/WHO/UNU, 2007). Although the process of producing plant-based meat alternatives has been around for a long time in recent years, the market for plant-based meat alternatives is rapidly expanding to meet growing consumer demand. While soy and cereals such as wheat, rice, barley, and oats have been commonly used in meat alternatives, other protein sources such as pea, lentil, lupine, chickpea, mung bean and fungi are also gaining popularity among producers of plant-based meat alternatives.

Raw ingredients are processed to yield extracts and isolates, which are then subjected to processes to transform them into meat analogues (Bohrer, 2019; Choudhury et al., 2020).

Soybeans and peas are two widely used plant proteins due to their excellent functional properties, such as water retention, gelling, fat absorption, and emulsifying capacity in food products. Lentil, lupine, chickpea, pigeon pea, mung bean, and fava bean are other legume proteins studied for their physicochemical characteristics, including foam stabilization, emulsification, and gel formation (Ismail et al., 2020).

Soy protein is historically the most used raw ingredient in the preparation of meat analogues, making it the best-known alternative to animal protein (Zhang et al., 2021). Among the best well-known meat-like products is Tofu, Tempeh and Seitan. Tofu, perhaps the most widely recognized meat alternative, is derived from soybeans. Its production is comparable to cheese production, and it consists of three main stages: preparation of soybeans, coagulation of soy juice to form curds, and pressing curds to form tofu blocks (Liu, 1997). Tempeh is another popular soy-derived meat alternative. It is a fermented soybean block made from cooked soybeans and grains such as rice and millet, combined with the *Rhizopus oligoporus* culture.

Seitan, often known “wheat meat”, is another common vegetarian meat substitute. Seitan is made from wheat flour and is produced by washing wheat flour dough until the starch dissolves, resulting in a chewy mass of proteinaceous gluten. However fairly common among the vegetarian community, seitan has the disadvantage that it cannot be consumed by people who are intolerant or allergic to gluten (Malav et al., 2015).

Traditional plant-based meat alternatives are produced using simple techniques, such as fermentation, chemical-based protein coagulation, pressing, heating, steaming, cooling, and washing (Malav et al., 2015; Sá et al., 2020). Extrusion, shear cell technology, and 3D printing are developed modern processing techniques (Ismail et al., 2020).

Extrusion is a common and widely used practice that converts 50 - 70 % protein-containing plant-based materials into fibrous products. It is a thermomechanical process that combines pressure, heat, and mechanical shear. During extrusion, protein undergoes approximately four main stages of conformational changes: unfolding of the molecular chains, association, aggregation, and cross-linking with potential degradation or oxidation (Kyriakopoulou et al., 2019). In the extruder, the proteins are exposed to high temperatures and pressures that cause the protein to denature and the loss of its tertiary or even secondary structure. The denatured proteins realign in the direction of flow as they move through the screw, exposing binding sites that allow the proteins to cross-link in a new way. This cross-linking is what texturizes the proteins and transforms globular plant proteins into structures that more closely resemble the fibrous and laminar construction of meat. At the end of the extruder, the water in the mixture evaporates quickly due to the high temperatures and pressure release,

causing the material to expand and creating the final puffed appearance (Ismail et al., 2020). There are two types of extrusion processes considering the amount of water added during the process: low-moisture extrusion (20 - 40 % moisture added) and high-moisture extrusion (40 - 80 % moisture added). Low-moisture extrusion technology was developed earlier than high-moisture extrusion technology and therefore low-moisture products are the most available in today's market. Low-moisture textured proteins have a sponge-like appearance and typically must be rehydrated before use. The high-moisture extruded products have a texture similar to animal meat with a rich, dense fibrous structure, strong elasticity and high moisture content, requiring no further processing before use (Zhang et al., 2019; Zhang et al., 2021). The protein denaturation that occurs during extrusion, which leads to the exposure of enzyme access sites, and the partial or total destruction of thermo-labile compounds can result in improved protein digestibility *in vitro*, positively influencing protein quality. The mechanical shear pressure applied during the extrusion process also plays an important role in the disruption of the protein bodies, resulting in the changes of the physical, chemical and nutritional quality of the extruded food products, improving their protein digestibility (Arribas et al., 2017; Zhang et al., 2017; Zhang et al., 2019).

While some plant-based foods, such as soy and quinoa, provide all nine essential amino acids, one or more indispensable amino acids are lacking in many legumes and cereals. Thus, the combination of soy, legumes and cereal grains for the development of alternative plant-based meat products can ensure more balanced amino acid profiles, similar to those of natural meat. Another concern is the digestibility of plant protein. Soy beans and fungi present digestibilities similar to animal-derived protein, but cereals and legume proteins have much lower digestibilities values (Bohrer, 2019). This can constitute a disadvantage for the replacement of animal proteins by plant proteins.

Food producers are trying to understand how plant proteins can partially or fully replace traditional animal protein ingredients by alternative plant-based foods to provide optimal nutrition, taste and functionality. Plant-based meat analogues production is not simply the reproduction of a meat-like texture to mimic that of real meat products, but also the appearance, sensory properties, and even nutrition and safety of such products, which requires extensive and profound research in order to overcome the various challenges associated with these factors. In addition, there is a need to explore efficient protein extraction processes to ensure high yields and preserve protein quality and functionality, understand the structure/function relationship, develop cost-effective protein functionalization strategies, identify high-value applications, investigate the diversity of cultures and ensure abundant supply. Science and technology must keep up with the exponential increase in demand for new plant proteins. The development of plant-based meat alternatives will not only open up new opportunities in reducing environmental impact, and overusing natural resources, but also

improving animal welfare, and developing food products with potential health benefits (Ismail et al., 2020; Zhang et al., 2021).

5. Determination of Protein Quality

Protein quality can be defined as the ability of a food protein to meet the body's metabolic demand for nitrogen and it can be determined by its essential amino acid profile, digestibility, and bioavailability of the individual amino acids Score (FAO, 2013).

Digestibility and bioavailability are not fixed attributes of the food, but rather affected by internal and external factors. Factors such as food matrix (e.g., levels and types of fat and carbohydrates, and antinutritional compounds), protein folding and crosslinking, pH, temperature, and ionic strength affect protein digestibility (Joye, 2019). Therefore, food processing, which has a substantial impact on these factors has a huge impact on protein digestibility and nutrient bioavailability.

Bioavailability takes into account three main parameters: digestibility and solubility of the element in the gastrointestinal tract, absorption of the element by the intestinal cells and transport into the circulation, as well as incorporation from the circulation to the functional entity or target. Additionally, bioavailability includes two other terms: bioaccessibility and bioactivity. Bioaccessibility is defined as the fraction of a compound that is released from its food matrix within the gastrointestinal tract and thus becomes available for intestinal absorption. It includes the sequential events that occur during digestion of food for transformation into potentially bioaccessible material, but excludes absorption/assimilation through epithelial tissue and pre-systemic metabolism (both intestinal and hepatic). Bioactivity, in turn, includes events linked to how the nutrient or bioactive compound is transported and reaches the target tissue, how it interacts with biomolecules, the metabolism or biotransformation it may undergo, and the generation of biomarkers and the induced physiological responses (Etcheverry et al., 2012).

Digestibility is generally defined in terms of the amino acid balance along the small intestine (mouth to terminal ileum: ileal digestibility) or throughout the entire intestine (mouth to anus: fecal digestibility). This concept is based on the principle that the difference between intake and loss provides a measure of the extent of digestion and absorption of food protein by the gastrointestinal tract. Amino acid bioavailability can be defined as the proportion of ingested amino acids that is absorbed and reaches systemic circulation being incorporated into body protein synthesis.

5.1 *In vivo* models for protein quality assessment

Protein quality methods assess animal growth or, in humans, nitrogen balance, where both digestibility and the suitability of the amino acid pattern of absorbed amino acids (biological value) determine net protein utilization.

The net protein utilization, or NPU, is the ratio of amino acid mass converted to proteins to the mass of amino acids supplied (Marinangeli & House, 2017). Biological value is a measure of the proportion of absorbed protein from a food, which becomes incorporated and can be used in protein synthesis in the cells of the body. Biological value measures protein quality by measuring the amount of nitrogen consumed and excreted. Endogenous losses of urinary and fecal nitrogen must be determined, which requires the feeding of nitrogen-free diets. Then, the amounts of urinary and fecal nitrogen after consumption of the test protein are determined. The differences in excreted nitrogen between the two dietary conditions are then calculated and multiplied by 100. The biological value provides a measurement of how efficient the body utilizes protein consumed in the diet. However, the biological value ignores the part of the dietary protein that is not absorbed and appears in the feces (Brody, 1999). Various methods for evaluating protein quality have been developed over the years. Protein efficiency ratio (PER), protein digestibility corrected amino acid score (PDCAAS) and digestible indispensable amino acid score (DIAAS) are among the most common *in vivo* methods and each of them has advantages and disadvantages (table 2).

Table 2. Advantages and disadvantages of protein quality measurements (Nosworthy & House, 2017).

Measurement	Advantages	Disadvantages
PER	Easy for calculation. Measurement of growth. PER values can be greater for the test food than for the reference (casein).	Assumption that all diet is used for growth. Rodent sulphur amino acids requirements are greater than human requirements, so growth may be limited.
PDCAAS	Measurement of protein digestibility. Amino acid content is quantified.	Fecal digestibility is used. Single crude protein measurement. Colonic alteration of fecal nitrogen. PDCAAS values are truncated to 100 %. Bioavailability of amino acids not determined. Multiple amino acids analyses required.
DIAAS	Measurement of protein digestibility. Ileal digestibility is used. Amino acid content is quantified. Individual amino acid digestibility.	Invasive. Limited data currently available. Ileal digestibility must be calculated. Multiple amino acid analyses required.

5.1.1 Protein Efficiency Ratio (PER)

The Protein Efficiency Ratio uses a rodent feeding trial to determine the quality of a test protein compared with a reference protein (casein) (figure 9). Weanling rats are fed an experimental diet or a casein control diet, each containing 10 % protein, for 28 days. At the end of the experiment, the total weight gain of the rats is divided by the amount of protein consumed. To compare PER values across different laboratories, the raw PER value is adjusted to the average PER of casein (2.5). The protein rating of a food is then calculated by multiplying the adjusted PER by the grams of protein in a reasonable daily intake. However, the method neglects contributions to maintenance and other metabolic processes by assuming that all of the protein the rat consumes is used for growth. Furthermore, rats require higher levels of the sulfur-containing amino acids than humans do, leading to an underestimation of the quality of food targeted (Marinangeli & House, 2017). This, together with the practical difficulties and poor sensitivity of the nitrogen balance method led to the adoption of the protein digestibility-corrected amino acid score approach.

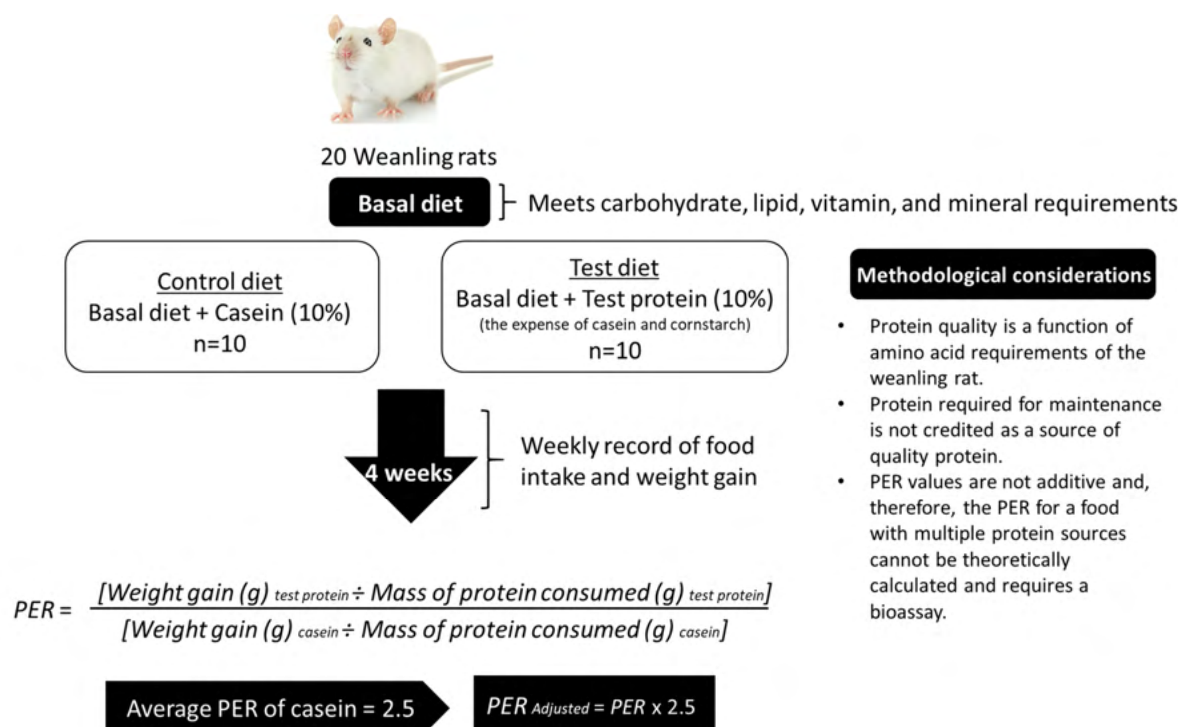


Figure 9. Scheme of the determination of the protein efficiency ratio (Marinangeli & House, 2017).

5.1.2 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

The PDCAAS method was developed in 1989 by a Joint FAO/WHO Expert Consultation on Protein Quality Evaluation for routine evaluation of protein quality for humans (FAO/WHO/UNU, 1991).

PDCAAS measures the amount of each indispensable amino acid in a protein sample, as well as the protein sample's digestibility. The amino acid score reflects the amount of each indispensable amino acid in a test protein compared with the amount of the same amino acid in a reference provided by the FAO (usually, the essential amino acid requirements of a child aged 6 months to 3 years). The limiting amino acid score is the ratio of the first limiting amino acid in 1 g of protein from the test food to that found in a reference protein or reference requirement. The PDCAAS of a food is determined by multiplying the limiting amino-acid score of a test protein and its true fecal nitrogen digestibility. True fecal nitrogen digestibility is determined by a rodent assay where rats are fed a known amount of nitrogen from the test protein and then fecal nitrogen excretion is measured (apparent protein digestibility) (figure 10). The fecal nitrogen excretion from the rats on a protein-free diet is then subtracted from fecal nitrogen excretion of the test protein, which accounts for endogenous protein nitrogen excretion derived from bacterial cells and digestive secretions. The result is referred to as true fecal protein digestibility.

An amino acid score of 1.0 or greater indicates that there is no deficiency in that amino acid in the test protein. Foods with low contents of essential amino acids and/or low digestibility coefficients of true fecal nitrogen will result in low PDCAAS values and, thus, being characterized as a low quality protein.

Because available protein in food will be first limited by digestibility, which cannot exceed 100 %, PDCAAS values cannot exceed 100 %. Thus, in calculating PDCAAS values, amino acid score values higher than 100 % are truncated not allowing indication of the potential of a high-quality protein to optimize the amino acid composition of food mixtures with low protein quality (Marinangeli & House, 2017; Nosworthy & House, 2017).

Although the PDCAAS method has been used for over 30 years as the preferred method to assess protein quality for its ability to meet the amino acid requirements of the human body, it has been criticised for a number of reasons. These include the fact that the PDCAAS relies on true fecal nitrogen digestibility, and does not account for the bioavailability of individual indispensable amino acids; PDCAAS overestimated values due to limited bioavailability of specific forms of amino acids resulting from the presence of anti-nutritional factors or Maillard compounds; true protein digestibility values can be falsely enhanced due to bacterial assimilation of amino acids; PDCAAS values truncate at 1, which doesn't allow the recognition of high quality proteins; true fecal protein digestibility values determined in rats, which have different amino acids requirement patterns for both growth and maintenance than

humans. Facing constant criticisms the to the PDCAAS method, the FAO introduced in 2011 an updated amino acid scoring system, the Digestible Indispensable Amino Acid Score (FAO, 2013).

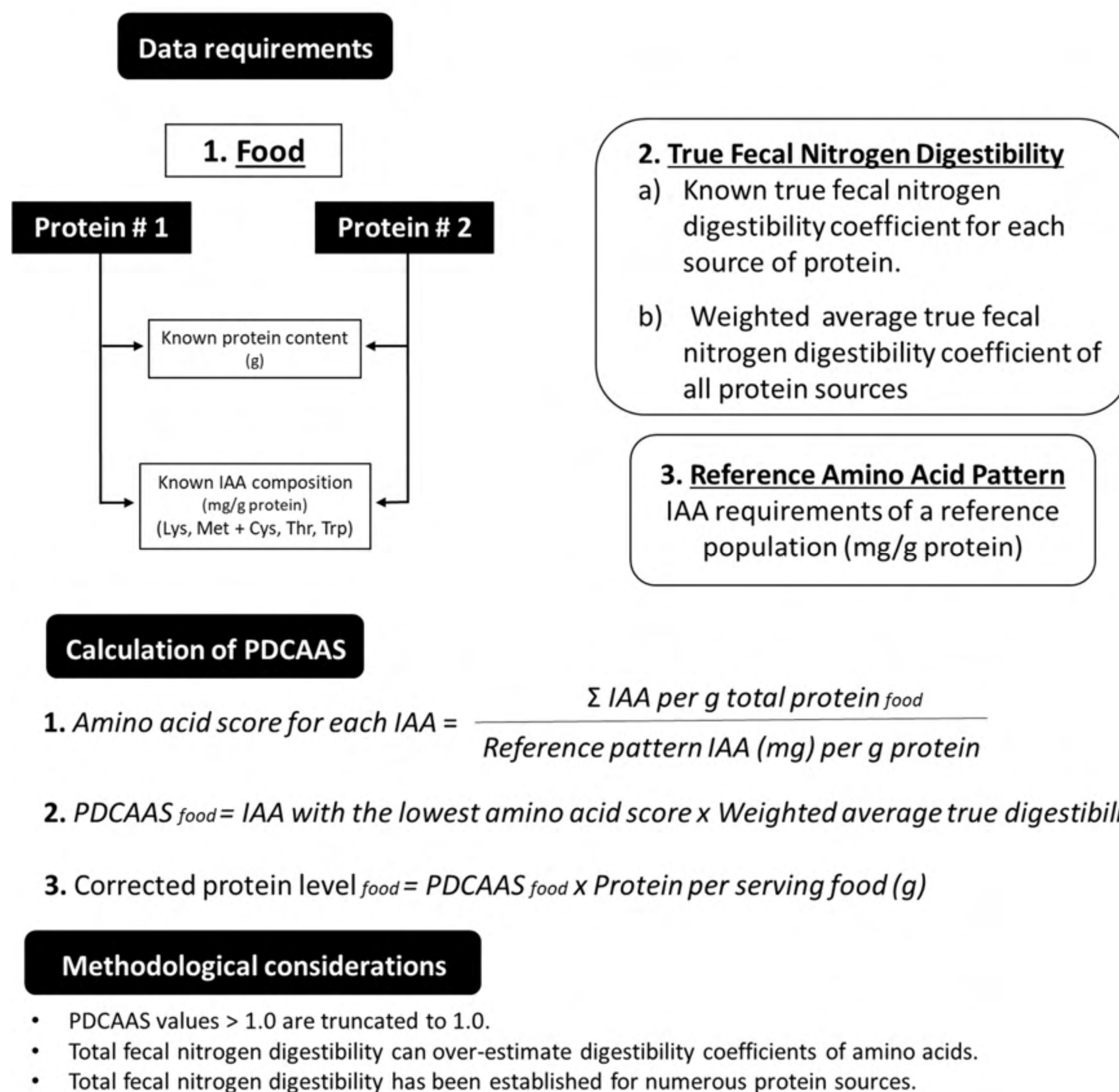


Figure 10. Scheme of PDCAAS determination (Marinangeli & House, 2017).

5.1.3 Digestible Indispensable Amino Acid Score (DIAAS)

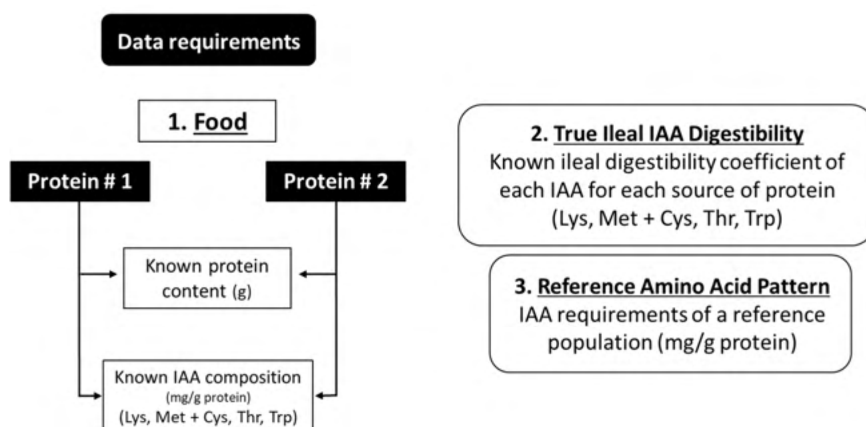
Unlike to the PDCAAS, which uses the true (corrected for gut non-dietary amino acids) fecal digestibility of the entire protein based on nitrogen digestibility, DIAAS uses the ileal digestibility of each individual amino acid as a constituent of food to determine the true ileal digestibility of the indispensable amino acids present within the food mixture. In case multiple protein-containing ingredients are present, the sum of digestible amino acids for each ingredient is calculated. This distinction is very important since true ileal amino acid digestibility can vary significantly across amino acids, even within the same protein source (Wolfe et al., 2016).

Determination of digestibility at the ileal level is more accurate than determination at the fecal level because in the hindgut there is little absorption of amino acids, yet the abundant microflora there will digest and utilize undigested proteins, peptides, or amino acids coming out of the small intestine. Furthermore, the microflora in the hindgut can also synthesize amino acids. As a result, the catabolism and synthesis of amino acids by the hindgut microflora will inevitably modify the undigested profile of dietary amino acids, which will confound fecal measurements of protein or amino acid digestibility, leading to over- or underestimation of digestibility. Additionally, fecal protein is largely bacterial protein, the amino acid whose composition has no resemblance to the undigested dietary protein leaving the ileum. Ileal digestibility overcomes all these problems and therefore, DIAAS is considered to be more accurate than PDCAAS (Rowan et al., 1994; Wolfe et al., 2016).

Another important aspect of DIAAS is that it recognizes that, for some processed dietary protein sources, amino acids may have undergone structural changes and, although the altered amino acids may be absorbed, they are not available for protein synthesis (Fontaine et al., 2007; Moughan & Rutherfurd, 2019). In addition, reactive digestible lysine rather than total digestible lysine should be considered to determine the DIAAS of processed and cooked foods (Herreman et al., 2020). In these cases, conventionally determined true ileal amino acid digestibility values are not accurate indicators of availability. This is an important point because most food consumed by humans undergoes some form of processing, either at the manufacturing level or through home cooking (Wolfe et al., 2016).

DIAAS determination requires the absolute protein content and levels of indispensable amino acids for a given food. The proportion of each digestible amino acid is compared with that of a reference amino acid pattern and multiplied by 100 (figure 11). Updated age-related AA reference scoring patterns were implemented: 0–6 months (infant), 0.5–3 years (children), and > 3 years (rest of the population). There are three categories based on the DIAAS value to classify proteins: <75 (no quality claim), 75–99 (high-quality protein), and ≥100 (excellent quality protein). The most limiting digestible indispensable amino acid content (DIAA) defines the DIAAS value of a protein and unlike the PDCAAS, the DIAAS values are not truncated

allowing scores >1 to acknowledge the potential of a high-quality protein to complement low-quality protein in mixed diets.



Calculation of DIAAS

1. *Determine the level of each digestible IAA in the food* = $\sum_{r=1}^{n} (IAA_{protein\,(r)}\,(mg) \times \text{Ileal IAA digestibility coefficient}_{protein\,(r)})$

2. *Amino acid score for each IAA* = $\frac{\sum \text{Digestible IAA (mg) per g total protein}_{food}}{\text{Reference pattern IAA (mg) per g protein}} \times 100$

3. $DIAAS_{food}$ = *IAA with the lowest amino acid score*

4. *Corrected Protein Level* $_{food}$ = $DIAAS_{food}$ x *Protein per serving food (g)*

Methodological considerations

- DIAAS values remain untruncated and can exceed 100.
- Only food with a DIAAS > 75 are eligible for protein content claims.
- Limited availability of ileal digestibility coefficients for IAA across foods.

Figure 11. Scheme of DIAAS determination (Marinangeli & House, 2017).

5.1.3.1 Human DIAAS assessment

As mentioned above, to calculate the DIAAS of a food human measurements of the true ileal digestibility of individual indispensable amino acids (IAAs) at the end of the small intestine is required. Digestibility is assessed using standard methods of oro-ileal balance, which can only be achieved through invasive naso-ileal intubation (figure 12A), in healthy participants or ileostomized patients (figure 12B).

The classic and standard method for measurement of true ileal protein and amino acid digestibility involves collecting the digesta in the terminal ileum during the postprandial period to determine the amount of undigested nitrogen or/and amino acids (Moughan et al., 2005). A triple-lumen radio-opaque tube is introduced through the nose of healthy volunteers with intact bowel. One of the lumens inflates a balloon at the end of the tube that together with the

peristaltic movements helps the migration of the tube along the intestinal tract. When the tube reaches the terminal ileum, a perfusion of polyethylene glycol (PEG, non-absorbable marker) is started through the second lumen, in order to evaluate the total ileal effluent flow. The test meal, containing intrinsically ^{15}N-labeled test protein undergoes digestion and absorption and ileal effluents containing non-absorbed dietary amino acids are continuously collected from the third lumen during the 8 h postprandial period (figure 12A) (Bandyopadhyay et al., 2021).

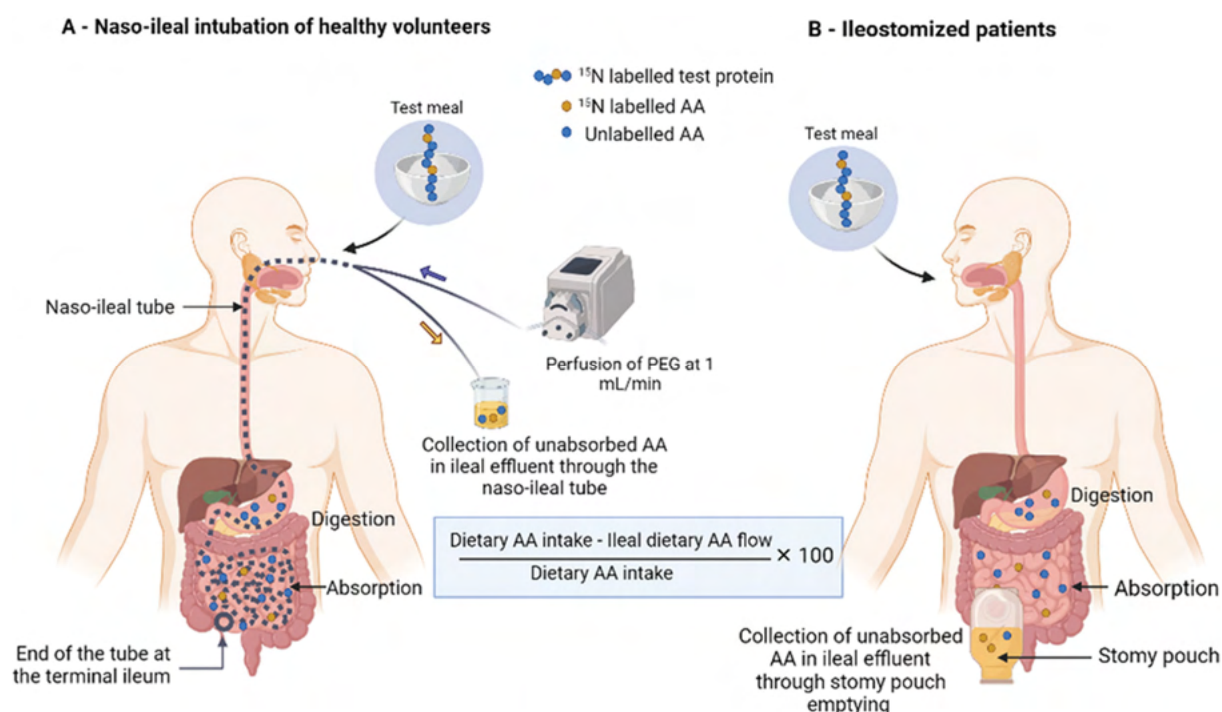


Figure 12. Schematic representation of the oro-ileal balance method to measure ileal amino acids digestibility with (A) intubated healthy volunteers or with (B) ileostomized patients using labelled protein (Bandyopadhyay et al., 2021).

Although used for more than 25 years, the naso-ileal intubation method is limited by the invasiveness of the procedure and the need for hospitalization of the volunteers for several days, the variable tolerance of the tube among subjects and the small amounts of digesta that can pass through the tube, the need of multiple markers, the inter-individual variability for the tube migration, the extent to which the digesta sample represents the total digesta, and the need for fine material to feed the subjects due to the risk of blocking the tube with food particles (Wolfe et al., 2016). In ileostomized patients, where the colon and rectum have been partially or completely removed and the terminal ileum exteriorized, ileal effluents are collected directly into a pouch (figure 12B), making this procedure relatively non-invasive and with the possibility of total digesta collection, the use of non-absorbable markers can be avoided. These patients, however, may suffer from different gut disorders leading to morphological and microbiological changes in their ileum, so the digestibility values measured may differ from healthy volunteers.

In addition, the ability to recruit patients with a permanent ileostomy is limited, which leads to a limited use of this method. For both methods the digestibility of each amino acid is determined by the ratio of the absorbed amino acid to the intake (Bandyopadhyay et al., 2021).

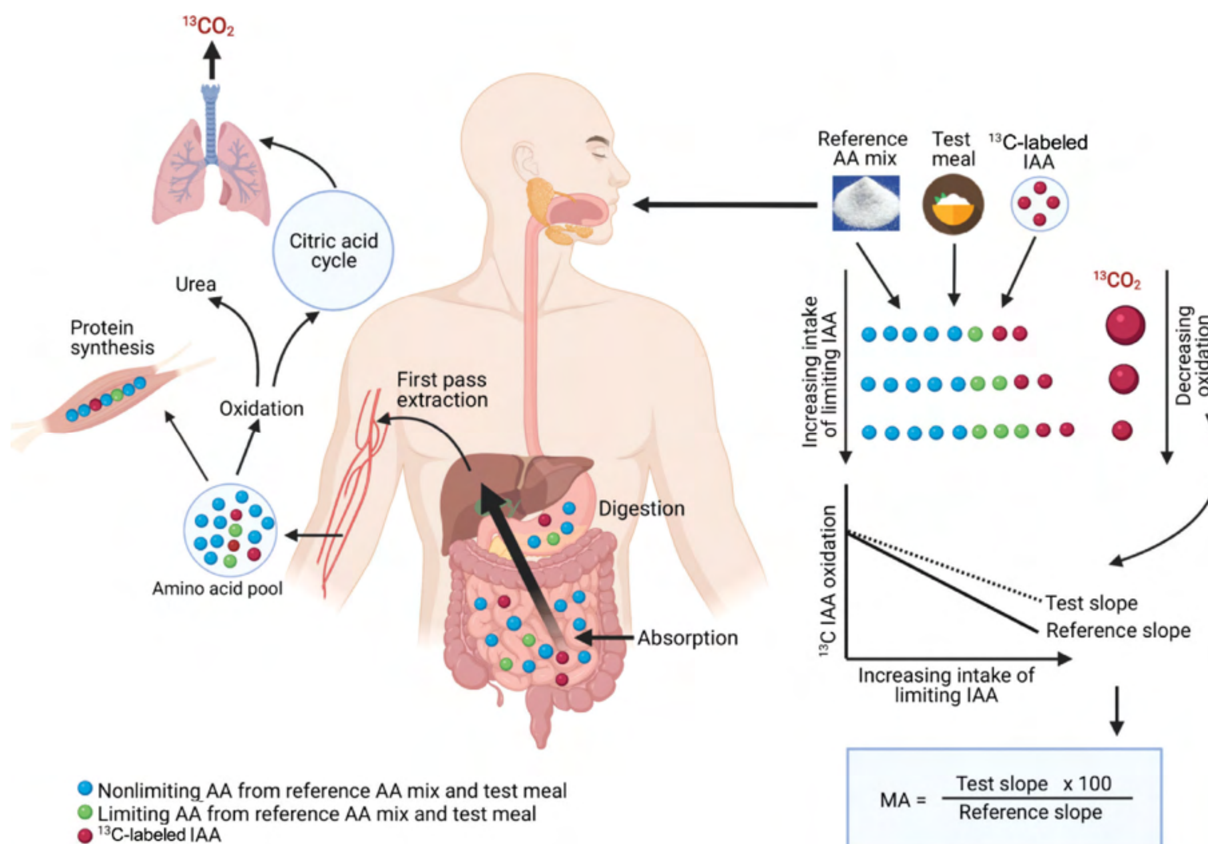


Figure 13. Schematic representation of the principle and application of the IAAO method to estimate metabolic availability of limiting amino acids in test proteins (Bandyopadhyay et al., 2021).

A significant effort has been made over the past 2 decades to develop non-invasive or less invasive methods to measure indispensable amino acids digestibility in humans. In 2013, Prolla and colleagues measured the metabolic availability of lysine in humans using the non-invasive indicator amino acid oxidation technique (figure 13) (Prolla et al., 2013), which had been validated against animal models (Levesque et al., 2011; Moehn et al., 2005).

The indicator amino acid oxidation (IAAO) slope ratio method is a non-invasive adaptation of the growth assay, which uses the oxidation of an orally administered ^{13}C labelled "indicator" indispensable amino acid as a proxy to estimate the contribution of an unlabelled test dietary indispensable amino acid to protein synthesis. The subjects are provided with increasing intakes of limiting indispensable amino acids (IAA) at the subrequirement concentration from a reference amino acid mixture or a combination of reference amino acid mixture and test protein with constant intake of ^{13}C-labeled indicator indispensable amino acid

over the study days, in a repeated measures design. With increasing intake of limiting/test IAA, the incorporation of ^{13}C-labeled indicator IAA into tissue protein synthesis increases with the subsequent reduction in its oxidation, which is measured as $^{13}CO_2$ in breath (the higher the oxidation of indicator indispensable amino acid, the lower the protein synthesis). The IAAO response slopes are obtained by measuring both reference amino acid mixture and test protein at the same concentrations of limiting/test IAA intakes. The metabolic availability of limiting/test IAA in a test protein is computed by comparing the estimated IAAO response slope of test protein to the estimated slope of reference amino acid mixture, which is assumed to have 100 % metabolic availability (Bandyopadhyay et al., 2021; Elango et al., 2012).

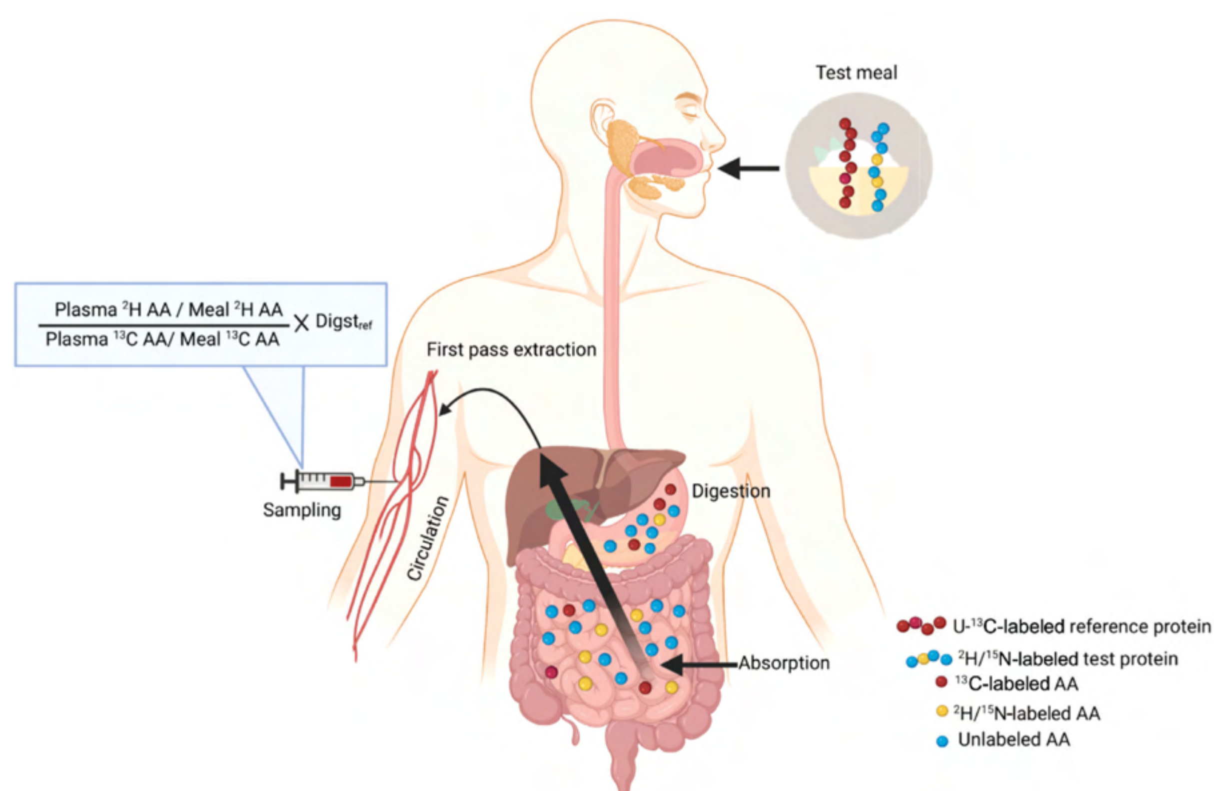


Figure 14. Schematic representation of the principle of the dual isotope tracer method to measure small intestinal AA digestibility (Bandyopadhyay et al., 2021).

However non-invasive, this method is limited due to the fact that only one amino acid can be evaluated for its metabolic availability within any experiment (Elango et al., 2012; Humayun et al., 2007).

The use of intrinsically labelled food proteins (with stable isotopes such as ^{13}C, ^{15}N, and ^{2}H) has helped circumvent invasive oro-ileal balancing techniques while allowing differentiation between endogenous and exogenous proteins.

The recently developed minimally invasive dual isotope tracer in which an intrinsically isotope-labelled test protein is fed simultaneously with a different isotope-labelled “standard” protein of known digestibility (figure 14). Two different intrinsically labelled proteins, a test protein (^{2}H or ^{15}N) and a reference protein (^{13}C) of predetermined digestibility are simultaneously fed in a plateau feeding protocol. After absorption and first-pass splanchnic extraction (assuming that amino acids from both test and reference proteins undergo similar first pass and splanchnic metabolisms), amino acids from both proteins enter the systemic circulation. The ratio between the plasma appearance of the individual amino acids from $^{2}H/^{15}N$-labeled the test protein and the ^{13}C-labeled reference protein of known digestibility allows the determination of the true ileal amino acid digestibility of the test protein (Bandyopadhyay et al., 2021; Devi et al., 2018).

In addition to overcome the invasiveness and complexities around collecting ileal digesta in direct methods, this method allows the measurement of small intestinal digestibility of multiple amino acids in a single trial and is applicable to vulnerable groups and pathophysiological conditions (Devi et al., 2018). However, this methods needs to be validated against conventional assays that determine digestibility at the terminal ileum (Bandyopadhyay et al., 2021). A resume of the amino acid digestibility and metabolic availability assays used in humans is presented in table 3.

5.1.3.2 Animal DIAAS assessment

Ideally, true ileal amino acid digestibility coefficients for human foods should be determined directly in humans. However, it is technically, economically and ethically difficult to collect digesta from the terminal ileum in humans. Therefore, animal models have been widely used for this purpose, with rats and pigs being the most commonly employed.

The lab rat is easy to raise and relatively inexpensive to house. However, they are selective eaters and foods must be ground and mixed with other components in the diet to ensure that the rat does not select only one part of the diet. In addition, rats may be able to digest better poorly digestible proteins when compared with humans. Thus, caution is needed when making estimations for poorly digestible proteins (Deglaire & Moughan, 2012).

Although more technically demanding, from a purely biological perspective, the pig is the preferred model for human nutrition studies due to the high anatomical, physiological and behavioural similarities. Another advantage of testing the foods in the pig is that the pig readily consumes foods in a range of formats (Deglaire & Moughan, 2012; Hodgkinson et al., 2020). In pigs, the ileal digesta is collected using two different techniques: slaughter and ileal cannulation. With the slaughter technique, the terminal ileum is dissected from the pig immediately post-mortem or from an anesthetized pig, which is then immediately euthanized.

Within the ileal cannulation approach, the T-cannulation, post-valve T-cecum cannulation are the most commonly used. All of these techniques rely on the use of an indigestible dietary marker. In the T-cannulation technique a flanged wide-bore cannula (in the shape of a T) is surgically implanted approximately 10 cm anterior to the ileocecal junction. The cannula protrudes through the abdominal wall and is consequently exteriorized. Normally capped, the cannula can be uncapped to attach a plastic bag and collect the digesta (Wolfe et al., 2016). The digestibility is then calculated as the difference between the ingested dietary amino acids and the remaining amino acids at the terminal ileum, after correction for the gut endogenous protein (Moughan et al., 1998; Skilton et al., 1988).

Table 3. Principle, strengths, and limitations of the amino acid digestibility and metabolic availability assays used in humans (Bandyopadhyay et al., 2021).

Methods	Principle of measurement and equation	Strengths	Limitations
Direct balance	- Measures disappearance of ingested protein-derived AAs from intestinal lumen - Calculated as the difference between the amount of ingested dietary AAs and that recovered in the terminal ileum For intrinsically labelled test proteins: $TID = \frac{\text{Dietary AA intake} - \text{Ileal dietary AA flow}}{\text{Dietary AA intake}} \times 100$ For unlabelled test proteins: $TID = \frac{\text{Dietary AA intake} - (\text{Total ileal AA flow} - \text{Endogenous AA flow})}{\text{Dietary AA intake}} \times 100$ - Two models are adopted in humans: naso-ileal intubation and ileostomy model	- Standard and direct method for measuring true ileal digestibility with repeatable results; - Provides digestibility estimates of all AAs in a single trial; - Ileostomy model is non-invasive and does not require a non-absorbable marker to estimate flow rates of ileal effluents.	- Naso-ileal intubation model is invasive, requires sampling of ileal digesta and not suitable for routine application in humans; - Uncertainties around the recovery of the non-absorbable markers in intubation model; - Expensive assay; - Ileostomy model cannot be employed for healthy humans; - Errors associated with the measurement of endogenous AA losses if test proteins are not intrinsically labelled; - Overestimates AA availability of heat-treated and chemically processed foods; - Microbial colonization in terminal ileum of ileostomates could confound the digestibility estimates; - Discounts colonic absorption of AAs, if any;

			- Not applicable for routine measurements, vulnerable age groups, and pathological conditions with altered digestion and absorption.
AAO slope ratio	- Oxidation of a ^{13}C-labeled IAA (indicator) is used as a surrogate for protein synthesis; - Compares oxidation response slope of an indicator IAA to graded intakes of test IAA from test protein to that of reference crystalline AAs $MA = \frac{\text{slope of } ^{13}C \text{ recovery for the test protein}}{\text{slope of } ^{13}C \text{ recovery for reference AA}} \times 100$ - Bioavailability of crystalline IAA assumed to be 100%; - Test IAA must be the first limiting IAA in the diet; - Oxidation rates of indicator must be linear to the graded intakes of test IAA; - IAAO slopes for test protein and reference AAs must have a common origin at a fixed base intake of test IAA in crystalline form	- Non-invasive, requires breath collection and rate of CO_2 production; - Analytically simple and relatively less expensive; - Does not require test proteins to be intrinsically labeled, less expensive compared to other methods.	- Repeated measures design with higher subject burden, difficult for routine application in vulnerable groups; - Provides MA estimate of one only IAA at a time; - The interindividual variability of the MA estimates is not reported.
Dual isotope tracer	- Measures appearance of ingested protein-derived AAs in systemic circulation; - Compares appearance of labelled AAs in plasma from intrinsically labelled food proteins to that of a simultaneously ingested but differently labelled reference protein of known digestibility in relation to the meal administered; - An example equation for ^{2}H-labeled test protein and ^{13}C-labeled reference protein: $TAAD = \frac{\text{plasma } ^{2}H_{AA} / \text{meal } ^{2}H_{AA}}{\text{plasma } ^{13}C_{AA} / \text{meal } ^{13}C_{AA}} \times 100 \times Dig_{Std} \times F_{TCF}$	- Minimally invasive method requires blood collection; - Provides digestibility estimates of almost all AAs in a single trial; - Measures digestibility of proteins in habitually consumed meal preparations; - Suitable for application in humans and vulnerable groups.	- Expensive, as requires test and reference proteins to be intrinsically labelled; - Indirect method. Digestibility of reference protein needs to be established in target population; - Uncertainty introduced by the transamination/ deamination reactions; - Analytical complexity.

AA- amino acid; Dig_{Std}- digestibility of the reference protein; F_{TCF}- transamination correction factor; IAA- indispensable amino acid; IAAO- indicator amino acid oxidation; MA- metabolic availability; TAAD- true amino acid digestibility; TID- true ileal digestibility

Despite concerns such as how well the digesta samples reflect the total digesta, the adequacy of the indigestible marker compound, the correction of the total amino acid flux to the non-dietary amino acid flux, and any effects that small intestinal bacteria may have on digestibility, a considerable amount of work has been done to develop and test such animal-

based assays, and these have showed similar true ileal amino acid digestibility values between pigs and humans (Deglaire et al., 2009; Deglaire & Moughan, 2012; Rowan et al., 1994; Sousa et al., 2022).

Currently, DIAAS cannot be fully implemented by the industry because there are limited data for the true ileal digestibility of amino acids in foods (FAO, 2013). Notwithstanding the promising non-invasive isotope-based methods recently developed and the good correlation between animal and human assays, the heavy ethical, technical and economic problems associated to the *in vivo* assays lead to the need to develop *in vitro* methods that can predict the true digestibility of ileal amino acids in humans. Furthermore, the increasing market of plant-based (vegan) protein sources desperately needs an alternative to the *in vivo* methods to assess the digestibility of their products.

5.2 In vitro models for protein digestion

During the last years, there is a growing interest in understanding the mechanisms of digestion. This knowledge is not only important to design new food formulations with health benefits, but also to predict allergenicity risks and to evaluate new drugs behaviour. However, studying digestive processes is a hard and challenging task due to multiple factors such as the high complexity of the digestive tract and its mechanisms, the complex nature of food and meals, a high variability between individuals and the limitations of the available techniques. Currently, knowledge of digestive processes comes from three different methodologies: *in vivo, in vitro*, and *in silico*. Although *in vivo* assays provide more relevant data than *in vitro* and *in silico* assays, the latter are preferred since they are performed under simple and well-stablished conditions, and can easily overcome the ethical, economic and duration problems associated to the *in vivo* methodologies.

There are a multitude of different *in vitro* digestion methods, varying in complexity and purpose and, ideally, they are cheap, high throughput, and produce accurate and physiologically relevant results. *In vitro* studies have been widely used for screening different food formulations (Hur et al., 2011), study digestive mechanisms (Gidley, 2013), pharmacokinetic and allergenicity studies (Dupont & Mackie, 2015), as well estimation of glycaemic index of a food (Englyst et al., 1992).

In the 1990s, the use of *in vitro* methods to study food digestion became very popular, where different models try to simulate the human gastrointestinal tract. They can go from a simple pepsin enzymatic reaction in a beaker (static models), to the most complex and sophisticated dynamic models, where parameters like temperature, pH gradients, enzyme types and concentrations, quantities and composition of digestive secretions, residence times, flow and mixing, motility, diffusion and mass transfer, and absorption mechanisms can be

replicated (Gouseti et al., 2019). In practice, any *in vitro* method will inevitably fail to match the precision that can be achieved by actually studying a food *in vivo* due to the inherent complexity of the process (Coles et al., 2005). Consequently, some compromise between accuracy and ease of utilisation of any *in vitro* digestion model is required.

5.2.1 Static *in vitro* digestion models

Static models are the simplest and fastest techniques based on a single reactor normally including three digestion steps (oral, gastric, and intestinal), with a fourth stage replicating large intestinal digestion being occasionally included. These methods try to simulate the physicochemical and enzymatic environment of each digestive compartment. Nevertheless, the products of digestion remain inside the reaction vessel during the whole digestion process, i. e. there is no gastric emptying and no absorption. In addition, physical processes that occur *in vivo* such as shear, mixing, hydration, changing conditions over time, are not mimicked (Gouseti et al., 2019). The experiments are normally performed using homogenized foods or isolated compounds in a closed system, at 37 °C under mixing conditions. The pH is fixed at physiologically relevant values at the beginning of each step (pH 1 - 3 for the stomach, pH 6 - 7 for the small intestine and about 7 for the colon) and the digestive fluids consist of water with electrolytes, enzymes, and possibly other compounds (mucins, bile salts, etc.), depending on the experimental protocol. Sample volume may vary from µL to tens of mL.

In vitro models start with an oral phase, which lasts for few seconds to minutes, with addition of amylase and neutral pH. During this phase, due to the short duration and pH close to neutral, no significant compound dissolution and protein hydrolysis is expected (Wickham et al., 2009). The gastric phase is performed under acidic conditions (pH 1 - 3) with addition of pepsin for 1 to 3 h at 37 °C (Gouseti et al., 2019; Orlien et al., 2021). Concerning gastric enzymes, a minimum amount of 4,000–5,000 IU/L of pepsin seems to be required for optimal protein digestion. The intestinal phase follows with previous neutralization, normally using NaOH, and addition of pancreatic enzymes and bile salts. As most of the nutrients are absorbed in the jejunum (pH 6.7 - 8.8) and ileum (pH 6.8 - 7.7), the intestinal phase is normally adjusted to a pH between 6.5 and 7.5, for 1 to 5 h at 37 °C (Ekmekcioglu, 2002).

After digestion, the samples can be centrifuged or filtrated in order to get the supernatant where the soluble components are expected to be found. The amount of solubilized component can be used as a measure of the bioaccessibility of a nutrient or bioactive component. An alternative methodology is the introduction of a dialysis bag containing sodium bicarbonate, after gastric digestion of the food sample, and dialysis of soluble components across a semi-permeable membrane without removal of the dialyzed

compounds. The use of a dialysis bag with a specific pore size even allows the discrimination between high and low molecular weight components (Ekmekcioglu, 2002; Etcheverry et al., 2012).

Static models incorporating cell cultures have been developed to study absorption of the digested material. These models can use a monolayer of cells such as Caco-2, MDCK, and HepG2, or membrane models such as PAMPA (parallel artificial membrane permeation assay) and Ussing chambers. Many absorption models have been reviewed in relation to drug absorption studies, however, the same principles pertain to nutrient absorption (Deferme et al., 2008; Fedi et al., 2021; Motilva et al., 2015).

Often, in studies using static methods, the selection of the enzymes and incubation conditions is determined by the aim of the study. Thus, the application of such methods to a single nutrient led to the use of a single enzyme, for example, lipases for lipid digestion, proteases for protein digestion, and amylases for starch digestion.

Over the years, different static *in vitro* protocols have been published, but often their outcomes were difficult to compare, due to differences in type and concentration of enzymes, pH, and time sets. The use of a single purified enzyme is advantageous as it helps in the standardization of the *in vitro* model by allowing for more reproducible results between different laboratories. Although, the digestion of a nutrient is influenced by other food components and, consequently, the use of complex enzyme mixtures provides results that more closely reflect the real situation *in vivo* than the use of simple purified enzymes (Alegría et al., 2015).

In an attempt to harmonise static *in vitro* digestion methods, a network of scientists collectively working in the European (COST) action INFOGEST has published a consensus standardised protocol. The experimental conditions, such as the presence of digestive enzymes and their concentrations/activity, pH, digestion time, and salt concentrations, were based on physiologically data of the fed state for a typical meal (Minekus et al., 2014). This INFOGEST protocol led to a better standardization of the experiments and greater consistency of the results obtained through the static *in vitro* experiments, which allows a better comparability of *in vitro* digestion studies directed to the upper gastrointestinal tract (Egger et al., 2016).

In 2019 an improved and amended version of the digestion method was released (INFOGEST 2.0) (Brodkorb et al., 2019) in order to solve some challenges associated with the original method. In this improved version of the method, the use of gastric lipase is recommended. The determination of digestive enzymes activities is a critical step and one of the major sources of variability in the results between different laboratories (Wang et al., 2017). Therefore, is recommended to determine the enzyme activity for each new batch of enzyme or after prolonged (Brodkorb et al., 2019), using defined assays previously described in the supplementary materials of Minekus et al., 2014.

Briefly describing the method (figure 15), in the oral phase simulated salivary fluid (pH 7) with or without α-amylase (enzyme activity of 75 U/mL) is mixed with the food for 2 min. In the gastric phase, simulated gastric fluid (pH 3) and pepsin (enzyme activity of 2000 U/mL) is added to the reaction vessel and kept in a rotating wheel or shaking incubator for 2 h at 37 °C. In the final intestinal phase, simulated intestinal fluid (pH 7) and either pancreatin (from porcine pancreas with a trypsin activity of 100 U/mL of digesta) or a mixture of individual enzymes, trypsin, chymotrypsin, pancreatic amylase, pancreatic lipase and pancreatic colipase together with bile salts is added to the reaction and mixed using a rotating wheel or shaking incubator for 2h at 37 °C (Minekus et al., 2014).

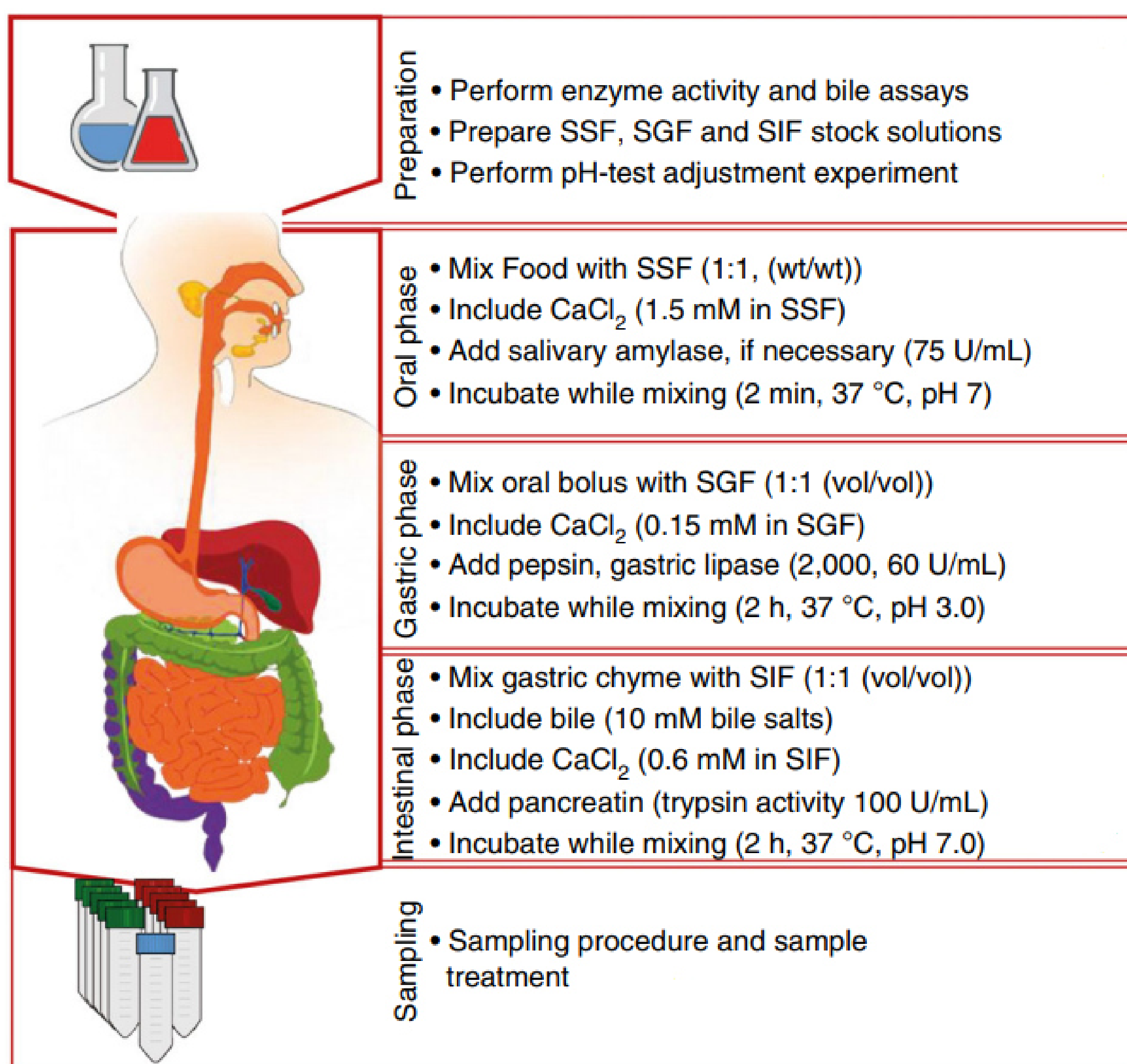


Figure 15. Flow diagram of the INFOGEST 2.0 digestion method (Brodkorb et al., 2019).

Static models are predominately used for digestion studies on simple foods and isolated or purified nutrients, and are therefore ideal for assessments of the digestibility. Since they are generally easy to up-scale, these static methods can be applied for screening large amounts of samples (Wickham et al., 2012).

Although static models can be practical, inexpensive, and feasible choices to assess many experimental conditions and a large number of samples, digestion is a dynamic process, and in food digestion, substrate-enzyme ratio, pH profiles, and transport of digested products are important parameters. Therefore, these models exhibit strong limitations once they cannot reproduce the mechanical forces and dynamic conditions that foods experience in the digestive tract. Consequently, several dynamic models simulating parts of the human gastrointestinal tract have been developed and are currently in use.

5.2.2 Semi-dynamic model

A semi-dynamic method (Mulet-Cabero et al., 2020) has been developed in order to fulfil the gap between the static and dynamic IVD methods. One of the main limitations of static models is the use of a fixed pH value for each stage of digestion, more specifically, pH 7.0 for the oral phase, pH 2.0-4.5 for the gastric phase and pH 7.0 for the intestinal phase. In the human body, the pH in the stomach can reach values of up to 7.0 after eating a meal due to the buffering capacity of the ingested food. Then, gastric pH is dynamically reduced to 2.0 by the release of HCl stimulated by the consumption of the meal, in parallel with gastric emptying. Designed, based on the harmonised INFOGEST static model, the semi-dynamic *in vitro* digestion method includes crucial kinetic aspects associated with the gastric phase, including gradual acidification, fluid and enzyme secretion and emptying.

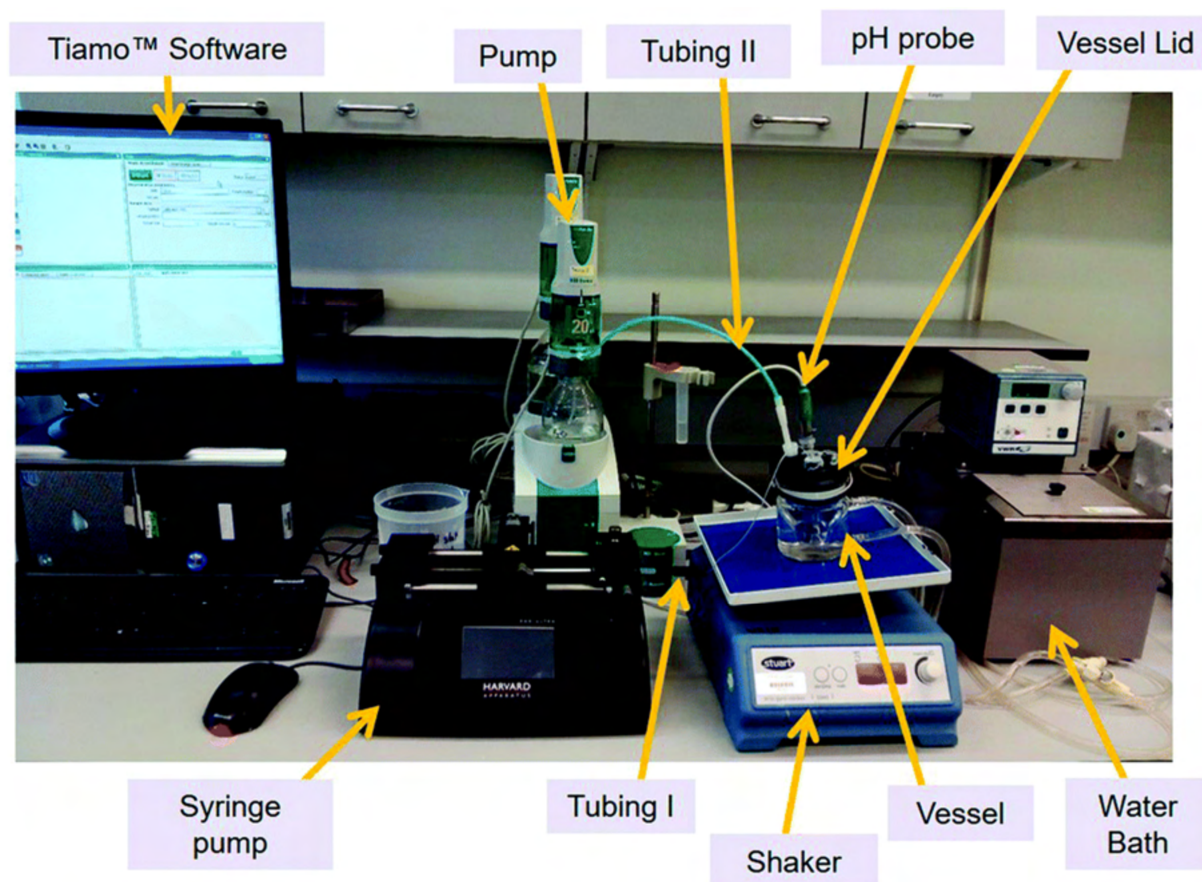


Figure 16. Apparatus used for the gastric digestion phase (Mulet-Cabero et al., 2020).

These characteristics make this method suitable for the study of structural changes in the food during gastrointestinal digestion, such as the effects of the food matrix on the disintegration and nutrient delivery. While the oral and intestinal phases remain practically the same as in the static method, the gastric phase is dynamic, requiring a specific apparatus (figure 16) to simulate the gastric phase pH decrease and emptying. The caloric value of the meal has to be known to estimate the gastric emptying parameters (energy emptying rate, 2 Kcal/min). The emptying is performed in a step-wise manner by manually taking the selected aliquots at calculated times from the bottom of the vessel using a selected laboratory tool with an end diameter of ~3 mm. Every aliquot taken from the gastric phase proceeds the digestion separately (figure 17).

There are still some limitations considering the shape of the vessel used to simulate the gastric phase and gastric motility. The J-shape of the stomach and the mechanical forces of the antrum are particularly difficult to simulate. Therefore, the standard semi-dynamic IVD protocol recommends poor mixing of gastric contents, especially in the top part of the reaction vessel, avoiding the use of magnetic agitation, to better simulate the low mixing that occurs in

the body, where only the antrum experiences significant shear (Mulet-Cabero et al., 2020; Xavier & Mariutti, 2021).

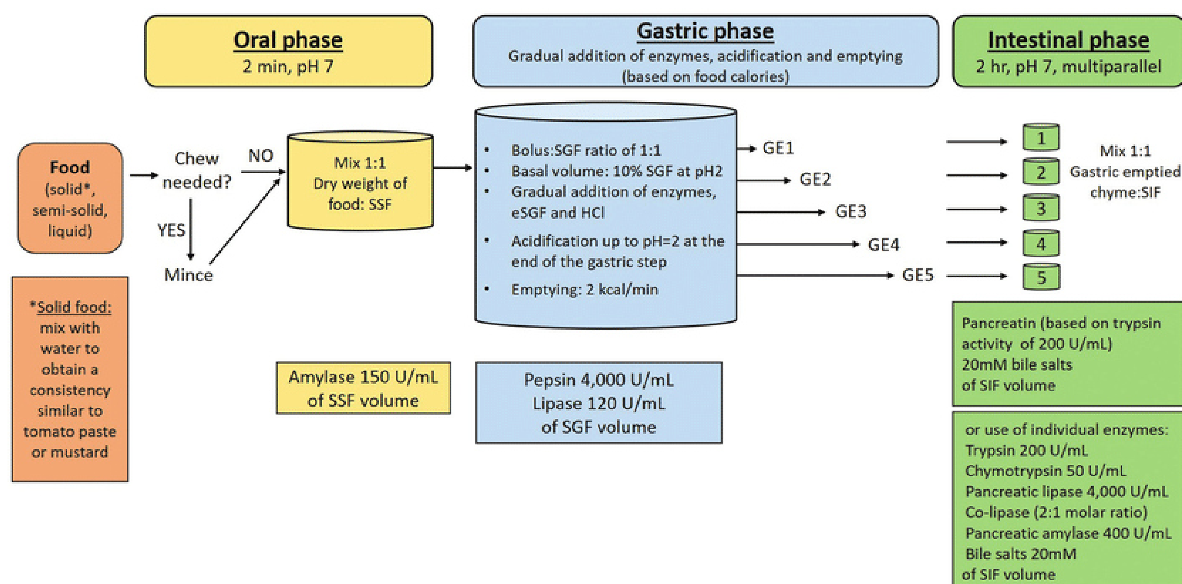


Figure 17. Overview and flow diagram of the simulated semi-dynamic *in vitro* digestion method. Simulated salivary fluid (SSF) simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and gastric emptying (GE). Enzyme activities are in units per mL of the simulated digestive solution added at each digestion phase (Mulet-Cabero et al., 2020).

5.2.3 Dynamic *in vitro* digestion models

Static models are quite basic and hardly recreate the complexity of the digestive tract. In contrast, dynamic models include physicochemical and mechanical processes and temporal changes in luminal conditions as occur *in vivo*, such as pH regulation, flow of the food and injection in real time of digestive enzymes in the different compartments of the gastrointestinal tract. Therefore, it is more likely that these new and more complex models are able to simulate the digestive process more accurately.

Dynamic systems can be either monocompartmental, simulating one compartment of the gastrointestinal tract, or multicompartmental, simulating several compartments. As example of monocompartmental systems, there is the Dynamic Gastric Model – DGM, the Human Gastric Simulator – HGS, and the Artificial Colon – ARCOL. DIDGI®, TIM, the Simulator or the Human Intestinal Microbial Ecosystem - SHIME®, Engineered Stomach and small Intestinal - ESIN, Simulator of the Gastro-Intestinal tract - simgi® are examples of multicompartmental systems (Dupont et al., 2019; Orlien et al., 2021).

Dynamic systems have the challenge of maintaining the balance between technological complexity and biological significance. Not all the existing dynamic *in vitro* models can mimic

mechanic, kinetic, and chemical physiological conditions of the human digestive system altogether. Some only simulate chemical conditions, some primarily focus on mechanical conditions, and very few include all mechanical, dynamic, and chemical conditions (table 4).

5.2.3.1 Dynamic Gastric Model (DGM)

The Dynamic Gastric Model, simulates elegantly the fundus and the antrum of the stomach (figure 18) (Wickham et al., 2012). Digestions using the DGM are performed in real-time, and the duration of each experiment is designed around the estimated gastric residence time of the meal used. Depending on composition, calorific content, and meal size, experiments can last between 25 min (glass of water) and 4.5 h (high-fat FDA breakfast). Masticated food is introduced in real-time or as a bulk from the top into the fundus, where it will find a previously added 20 mL volume of gastric priming acid. Acid and enzyme solutions are added through a perforated hoop situated at the top of the fundus, allowing a flow of secretion through the reaction vessel. The flow rates of these secretions are controlled dynamically. Gastric acid addition slows gradually in response to the acidification of the meal as detected by the pH electrode inserted within the fundus, while gastric enzyme addition slows in response to the gradual decrease in food bolus volume as recorded in response to ejection of samples from the antrum. Inside the fundus, the bolus is subjected to a rhythmic compression caused by the cyclic pressurization of the water jacket at 37°C around it. The DGM antrum consists of a barrel and a piston, which moves inside a water jacket pulling portions of food bolus through an inlet valve from the fundus into the antrum, it is the up and down movement of the barrel during processing that exerts shear stresses on the antral contents. At pre-set intervals, the inlet valve closes and the outlet valve opens, allowing the ejection of the already processed chyme. Gastric sieving is simulated within the DGM using a “dead volume”. It is a defined space between the barrel and the piston whose volume is kept fixed during ejection, allowing large and dense particles to remain in the antrum and pass through repeated processing cycles. At the end of the simulated digestion, any material remaining in this dead volume is ejected to simulate the phase III contraction (housekeeper wave) that completely empties the human stomach at the end of gastric digestion. After ejection, samples can be subjected to further digestion using a static duodenal model. For that, the pH of the samples is increased and a physiological mix of bile salts and pancreatic enzymes, is added to simulate conditions found within the duodenum (Dupont et al., 2019).

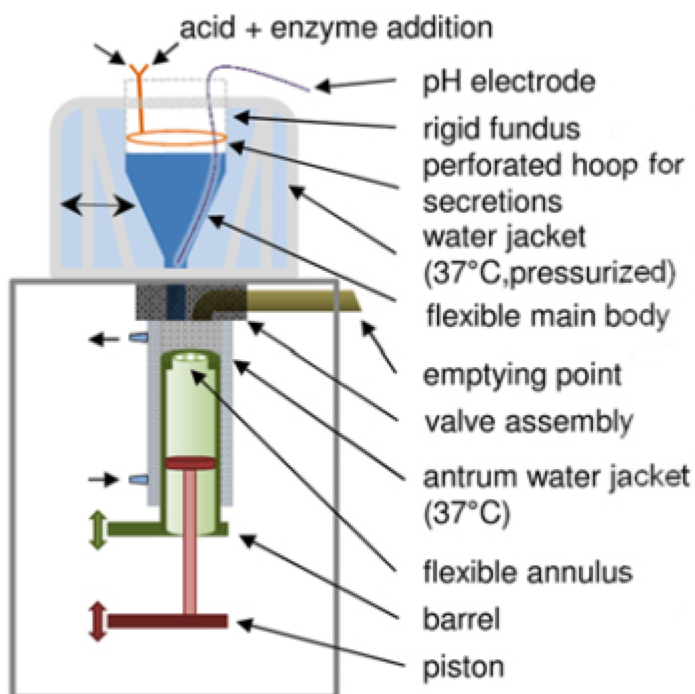


Figure 18. Schematic representation of the main components of the dynamic gastric model (DGM) (Thuenemann et al., 2015).

5.2.3.2 Human Gastric Simulator (HGS)

Human Gastric Simulator has been designed to reproduce the fluid mechanical conditions responsible for the disintegration and mixing of gastric contents during digestion. The HGS consists of a cylindrical latex vessel that simulates the stomach compartment, and it mimics the antral contraction wave activity of the stomach by a series of rollers that continuously impinge and compress the compartment wall with increasing amplitude (figure 19). The entire system operates at 37 °C and the gastric secretions are added through tubes entering the top of the vessel. The secretion rate and secretion composition (pH, enzymes, salts, mucins) can be adjusted, depending on the goal of the study. A mesh with 1 mm openings is used to simulate the sieving effect of the pylorus and control the gastric emptying. Samples are emptied through a small tube (0.01 m inner diameter) in the distal portion of the vessel. Oral and/or small intestinal stages can be incorporated either before or after testing using the HGS, respectively (Kong & Singh, 2010).

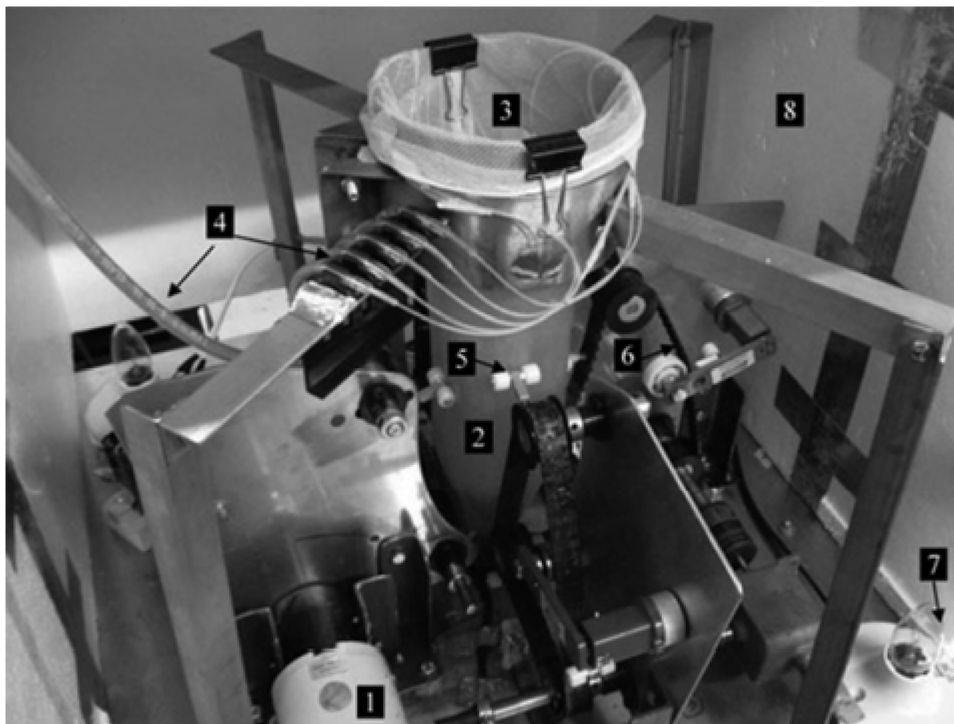


Figure 19. Human gastric simulator. 1- Motor; 2- Gastric compartment; 3- Mesh bag; 4- Simulating secretion tubes; 5- Teflon rollers; 6- Conveying belt; 7- Insulated chamber (Kong & Singh, 2010).

5.2.3.3 *The artificial colon (ARCOL)*

The artificial colon is a one-stage fermentation model that integrates the main parameters of the colonic fermentation environment of humans or animals. Parameters such as pH, temperature, anaerobiosis, colonic residence time, supply of simulated ileal effluents, presence of a complex, high-density, metabolically active microbiota, and passive absorption of water and microbial metabolites. It is the first model in which the maintenance of anaerobiosis within the fermenter is ensured solely by the metabolic activity of the microbiota and not by washing with N_2 or CO_2, as usually done in other colonic *in vitro* models.

The bioreactor is inoculated with fresh feces from healthy volunteers or animals, after suspension into phosphate buffer and filtration through a double layer of gauze. A culture medium, reproducing the composition of ileal effluents and containing various sources of carbohydrates, proteins, lipids, minerals and vitamins, is continuously introduced into the bioreactor to feed the microbiota, while the fermentation medium is continuously withdrawn from the bioreactor. The pH and temperature are kept constant by adding NaOH and heating with a water double-jacket. A dialysis system using hollow fiber membranes (cut-off 30 kDa) maintains the appropriate electrolyte and metabolite concentrations and the operating volume (Dupont et al., 2019).

5.2.3.4 DIDGI®

DIDGI® is a model focused on the upper parts of the digestive tract and is a multicompartmental system consisting of two consecutive compartments simulating the stomach and the small intestine (figure 20). A glass jacket filled with water pumped using a temperature-controlled water bath surrounds each compartment keeping the temperature constant. The computer program can be configured with parameters and data obtained from *in vivo* studies in animals or human volunteers reproducing the quantity and duration of a meal (gastric and intestinal transit times), the pH curve for the stomach, the secretion rates into the different compartments and the gastric and small intestine emptying rates. Anaerobic conditions can be simulated by injecting air with N_2 inside the system. A Teflon membrane with 2 mm holes is placed between the gastric and the intestinal compartment to mimic the sieving effect of the pylorus in human (Dupont et al., 2019).

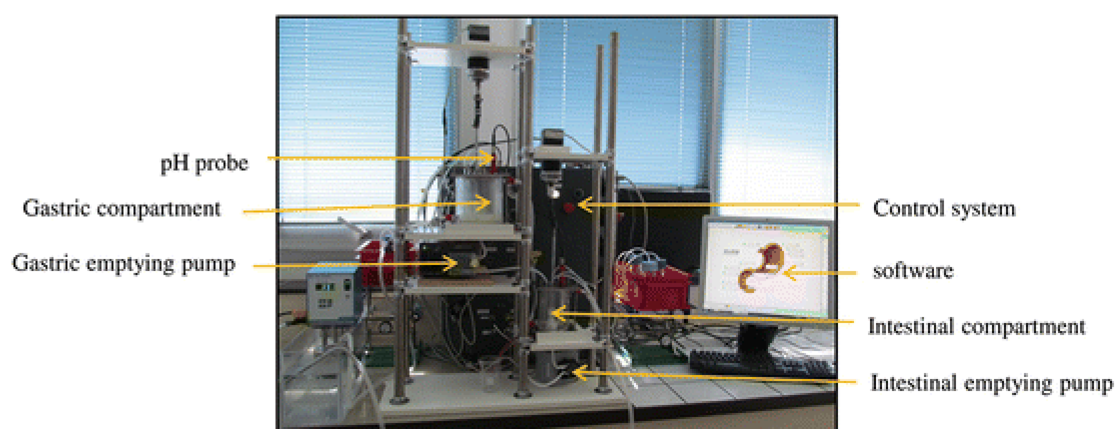


Figure 20. The DIDGI® system and its components (Ménard et al., 2015).

5.2.3.5 TIM

The gastric and small-intestinal model (TIM-1) was described in details in 1995 (Minekus et al., 1995) and in 1999 the large-intestinal model (TIM-2) was developed (Minekus et al., 1999). A continuous process of optimization, such as simulation of infant GI conditions (Roussel et al., 2016) or elderly (Denis et al., 2016) and development of the advanced gastric model 'TIMagc', which simulates the specific conditions in the corpus and antrum part of the stomach, including peristaltic motility and pressure forces (figure 21) (Bellmann et al., 2016) is still ongoing.

TIM-1 comprises four compartments, representing the stomach, duodenum, jejunum and ileum (figure 22). Peristaltic valve pumps connect the different compartments that allow the transfer of controlled amounts of chyme. Before starting the gastric step, the meal is

masticated with a food processor and mixed with artificial saliva containing electrolytes and α-amylase. Once in the gastric compartment, gastric secretion containing electrolytes, pepsin and lipase is added. The pH follows a predetermined curve or at a variable rate in time and is adjusted with the addition of hydrochloric acid. Once the intestinal phase begins, duodenal secretion composed by electrolytes, bile and pancreatin is added into the system and the pH is controlled with sodium bicarbonate at pre-set values for each compartment. According with their solubility, digestion products are removed by two different systems. Water soluble products are removed by dialysis through membranes connected to the jejunal and ileal compartments with a molecular weight cut-off of ~10 kDa. Lipophilic products, which cannot be removed efficiently by these membranes, since they are incorporated in micelles that are too big to pass the membrane, are removed through a 50 nm filter that passes micelles but retains fat droplets (Minekus, 2015).

TIM-2 is a continuous single-stage fermentation model designed to simulate the dynamic conditions in the proximal colon and to reproduce its metabolic activity. This model reproduces the peristaltic mixing of proximal colonic luminal content as well as the absorption of water and fermentation products (Minekus et al., 1999).

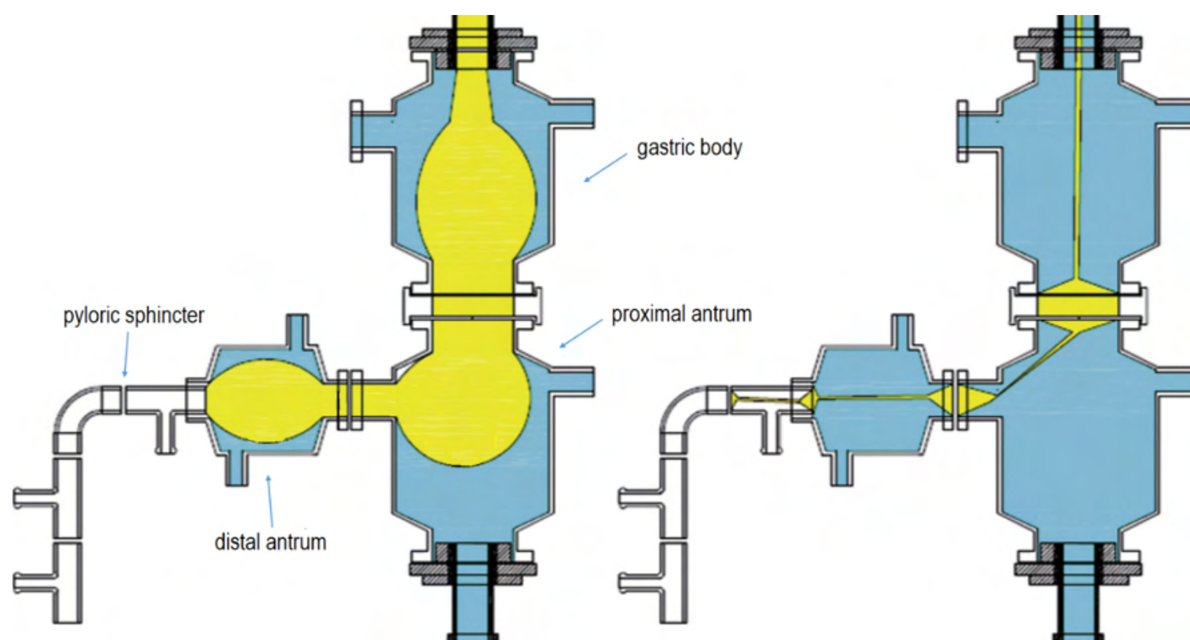


Figure 21. Schematic presentation of the TIM advanced gastric compartment (TIMagc). The left and right pictures show a filled and completely empty gastric compartment, respectively. A- body; B- proximal antrum; C- distal antrum; D- pyloric sphincter (Minekus, 2015).

The system can be inoculated with microbiota originating from healthy volunteers (single donor or pooled microbiota), or from people with a disease (e.g., inflammatory bowel disease). The model consists of four interconnected glass compartments, with a flexible

membrane inside (figure 23). The water present in between the glass jacket and the membrane regulates the temperature of the system. Peristaltic movements are achieved by applying pressure on the water at regular intervals and in a certain sequence. The flexible membrane contracts are mixing the luminal contents and moves them through the system. The system is continuously measuring the pH, which is kept at the physiological value of 5.8, by secretion of 1 mol/L NaOH to neutralize the acids that are produced by the microbiota.

The system is equipped with a dialysis system that has been configured to keep physiological concentrations of microbial metabolites in the lumen preventing accumulation of microbial metabolites, which would lead to the inhibition or death of the microbes in the model. When the volume in the system rises due to addition of the food or through dialysis, a sensor activates a dial-out pump and maintains the volume at a constant level of ~120 mL.

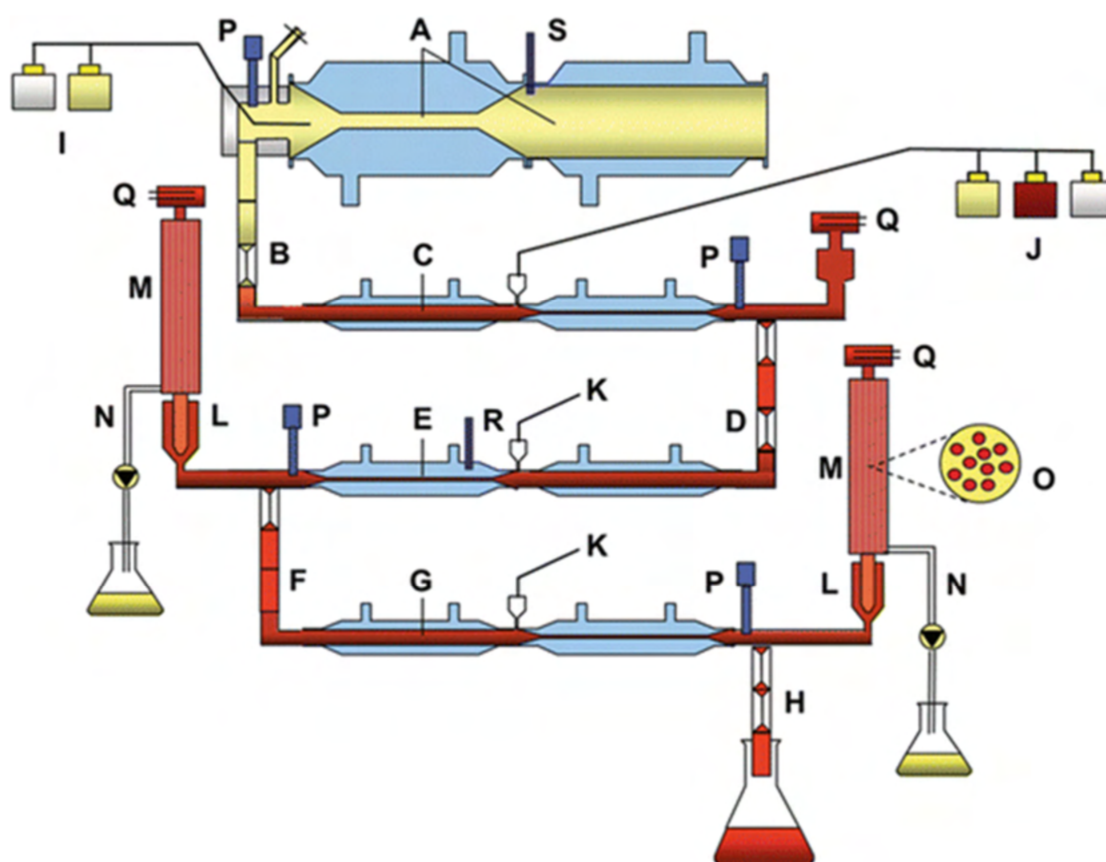


Figure 22. Schematic presentation of TIM-1 (Minekus, 2015).

A- gastric compartment; B- pyloric sphincter; C- duodenal compartment; D- peristaltic valve; E- jejunal compartment; F- peristaltic valve; G- ileal compartment; H- ileal-cecal valve; I- gastric secretion; J- duodenal secretion; K- bicarbonate secretion; L- pre-filter; M- filtration system; N- filtrate with bio-accessible fraction; O- hollow fiber system (cross section); P- pH electrodes; Q- level sensors; R- temperature sensors; S- pressure sensor.

The anaerobic conditions inside the system are ensured by flushing it with gaseous nitrogen. The microbiota in the system is fed with a simulated ileal efflux medium, which mimics the composition of the components that reach the colon from the terminal ileum, consisting of some complex carbohydrates, some protein (since not all proteins are digested in the small intestine), some residual bile (not all bile is resorbed in the small intestine), and some minerals and vitamins (Venema, 2015).

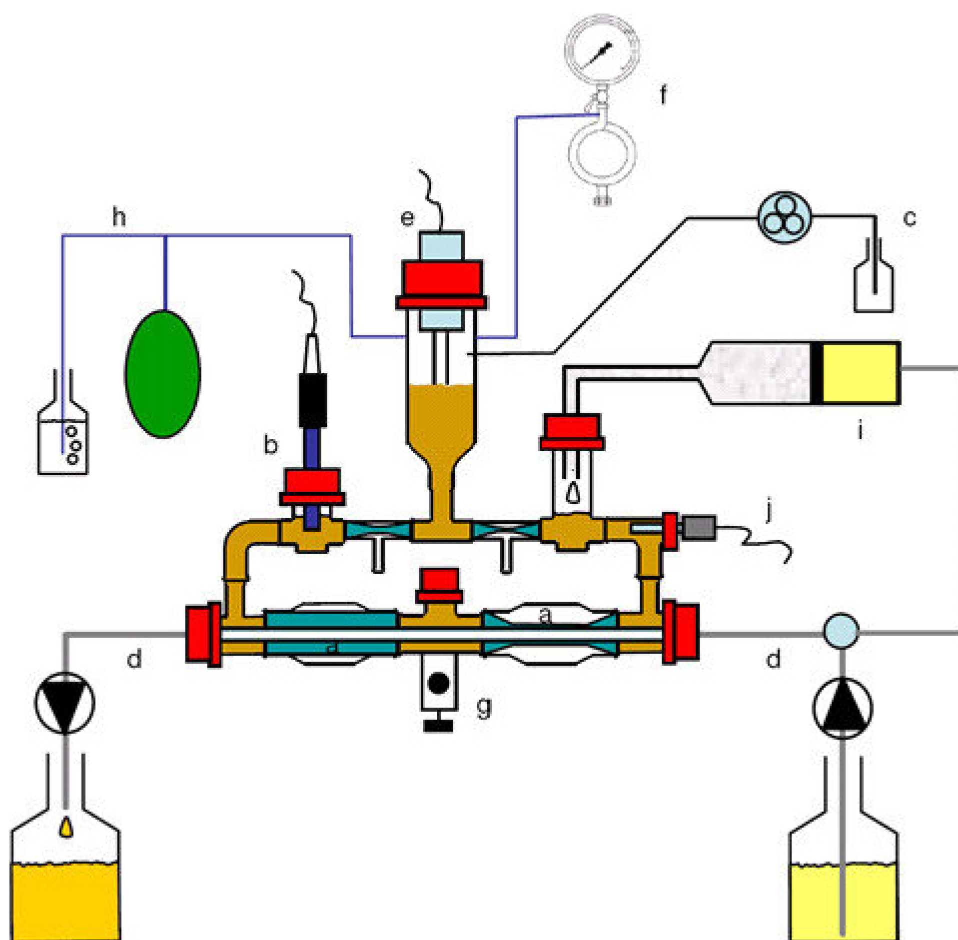


Figure 23. Schematic figure of the TIM-2 (Rehman et al., 2012).
a- peristaltic compartments containing faecal matter; b- pH electrode; c- alkali pump; d- dialysis liquid circuit with hollow fibre membrane; e- level sensor; f- N 2 gas inlet; g- sampling port; h- gas outlet; i- 'ileal efflux' container; j- temperature sensor

5.2.3.6 Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

The Simulator of the Human Intestinal Microbial Ecosystem is one of the few gut models that mimics the entire gastrointestinal tract incorporating stomach, small intestine and different colon regions (figure 24) (Molly et al., 1993). This model operates at 37 °C and is composed by double-jacketed glass vessels connected through peristaltic pumps. The first two reactors

use the fill-and-draw principle to simulate different steps in food uptake and digestion, with the peristaltic pumps adding a defined amount of SHIME® nutritional medium (3x/day) together with pepsin to the stomach and pancreatic enzymes with bile liquid in the small intestine. After digestion in the gastric and intestinal compartments, the digesta is pumped in the ascending colon vessel where colon digestion is initiated. The colon compartments are continuously stirred with constant volume and pH control (pH between 5.6 and 5.9 in the ascending, 6.1–6.4 in the transverse and 6.6–6.9 in the descending colon). By changing the flow rates from the gastric and intestinal compartments it is possible to modulate retention times in the upper digestive tract, while in the colonic compartments, retention times are mainly modulated through a change in compartment volume. Retention times may vary between 24 to 72 h, depending on the target group of interest.

Fecal microbiota is used to inoculate the colonic compartments of the SHIME® reactor due to the inaccessibility of the human colon region to collect a representative microbial inoculum. In contrast to other gut models, microbiota isolated from fecal material of one single individual is used to the detriment of microbiota from pooled fecal samples from different human volunteers. The system is kept anaerobic by daily flushing the compartments with N_2 gas or a 90/10 % N_2 /CO_2 gas mixture (Van de Wiele et al., 2015).

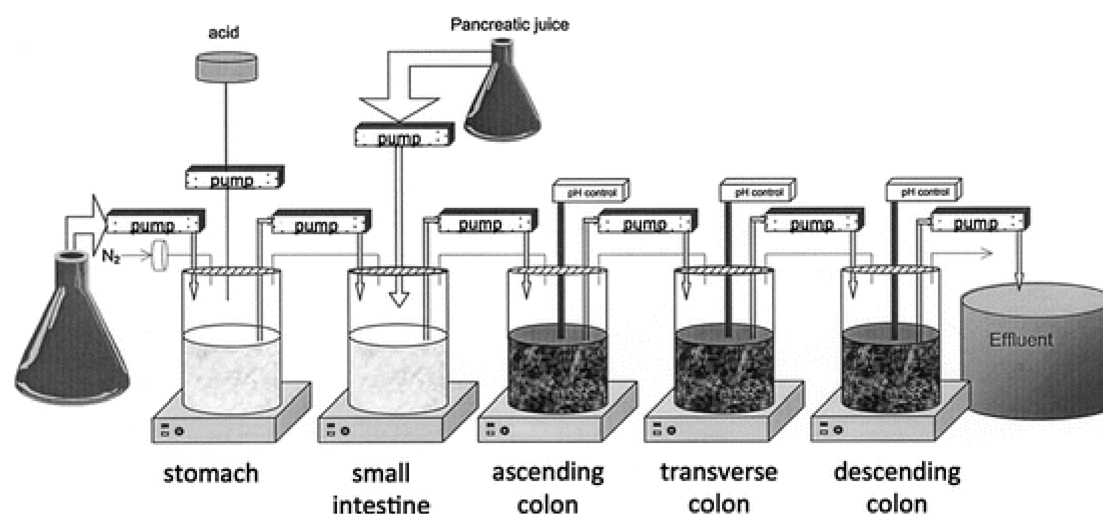


Figure 24. Schematic representation of the SHIME® (Van de Wiele et al., 2015).

5.2.3.7 Engineered Stomach and small intestine (ESIN)

Engineered Stomach and small intestine is a multi-compartmental dynamic *in vitro* model of the human stomach and small intestine (Guerra et al., 2016). This model is composed by six successive compartments: a meal reservoir, a salivary ampoule, the stomach, and the three sections of the small intestine (duodenum, jejunum and ileum) (figure 25).

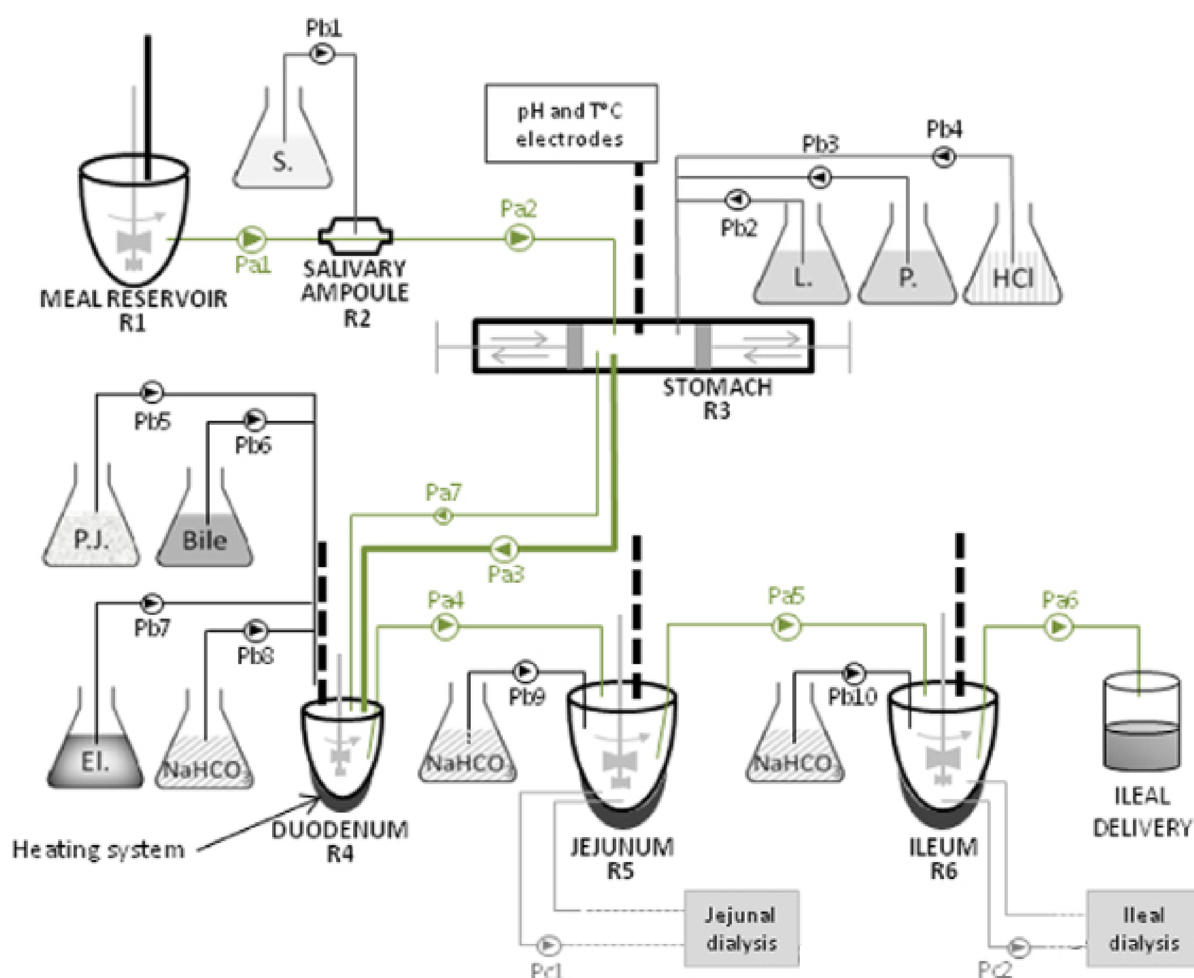


Figure 25. Schematic overview of the new Engineered Stomach and small Intestine (ESIN) model. Transit pumps (''Pa'') regulate chyme transit from one compartment to the next one. Secretions pumps (''Pb'') dispense digestive secretions, whereas dialysis pumps (''Pc'') are responsible for the absorption of digestion products and water and regulation of jejunal and ileal levels. S- saliva, L- lipase, P- pepsin, PJ- pancreatic juice, El- Electrolytes, HCl- hydrochloric acid, NaHCO3- sodium bicarbonate (Guerra et al., 2016).

The meal reservoir allows a progressive introduction of food particles with realistic size into the gastric compartment, while the salivary ampoule secretes "saliva", which is progressively mixed with the food. In the stomach vessel, when solid particles reach a size between 1–2 mm, they can pass the pylorus into the second vessel, while solid particles bigger than 2 mm

stay in the gastric vessel for further digestion. *In vivo* data from parameters of human digestion such as body temperature, temporal and longitudinal changes in pH, salivary-, gastric-, pancreatic- and biliary- secretions, transit times, chyme mixing, and passive absorption of digestion products were used to calibrate the system (Dupont et al., 2019).

5.2.3.8 Simulator of the gastrointestinal tract (Sigmi®)

The simgi® is a dynamic simulator developed to reproduce gastrointestinal digestion and colonic fermentation. The model consists of five interconnected compartments that simulate the stomach, small intestine, and the ascending, transverse, and descending colon that can operate jointly or independently (figure 26).

Figure 26. The simulator of the gastrointestinal tract (Simgi®) (CIAL-CSIC).

The stomach section has two transparent and rigid plastic vessels that cover a flexible silicone container and the peristaltic movements are achieved by pumping water at 37 °C through the jacket between the plastic modules and the flexible container. The stomach compartment has different ports for the entry of experimental food components, gastric juice and acid. The small intestine and the colonic vessels are continuously stirred reactors operating under anaerobic conditions and controlled pH (by addition of NaOH and HCl). The small intestine compartment receives the gastric content and mixes it with pancreatic juice and bile. The intestinal and

colonic vessels contain ports for the transit of intestinal content, sampling, continuous flushing of nitrogen allowing a permanent anaerobic atmosphere and control of pH and temperature. Flow rates of the digestive secretions, temperature, and pressure values are controlled through a computer program (Dupont et al., 2019).

A wide range of gastrointestinal models has been designed to reproduce the complexity of the human digestive system (table 4). They have provided valuable scientific information on assessing the bioaccessibility of nutrients and food pollutants, and developing and testing drug formulations under digestive conditions. While TIM, ESIN, and DIDGI are highly sophisticated systems and focus on reproducing the mechanical, dynamic, and chemical physiological conditions in the stomach and small intestine, SHIME and SIMGI are systems that fully focus on the large intestine fermentation and cannot mimic accurately the contractions of the stomach and small intestine. HGS and DGM aim to simulate the mechanical, dynamic, and chemical conditions of the stomach only, while ARCOL is a reactor that only simulates fermentation in the large intestine. However, it is still not possible to fully mimic the complex human digestive system, it is possible to simulate the digestive system's critical mechanical, dynamic, and biochemical processes in a robust, repeatable manner.

As we deepen our understanding of the digestive process and all the interactions involved, it will be possible to better fine-tune the simulations, expand their potential and more accurately address the human situation *in vivo* (Sensoy, 2021). Increased interest in modifying the matrix and structural characteristics of foods to optimize their digestion and absorption behaviour for health benefits requires further efforts and technological innovations to improve *in vitro* models for digestion studies.

Table 4. Characteristics of the selected *in vitro* dynamic models used in food digestion studies (Sensoy, 2021).

System	**Mouth**	**Stomach**			**Small intestine**			**Colon**	**Temperature control**
	Mixing pH Saliva	**Secretions**	**Mixing**	**Emptying**	**Secretions**	**Mixing**	**Absorption**	**Microbiota Mixing Absorption**	
TIM	Prepared as a bolus	Syringe pumps	Peristaltic pumps	Peristaltic pumps	Syringe pumps	Peristaltic pumps	Dialysis (Hallow fibers)	TIM-2 Feces Anaerobic conditions Peristaltic pumps Dialysate system	Heating elements connected with temperature sensors for each compartment
SHIME	Prepared as suspension	Fill and draw	Magnetic stirrer	Fill and draw	Fill and draw	Magnetic stirrer	-	Feces Anaerobic conditions Magnetic stirrer	Heater and Thermostat
ESIN	Meal reservoir, progressive	Peristaltic pumps	Two inox pistons	Peristaltic pumps	Peristaltic pumps	Shaft stirrers with adjustable	Dialysis (Hallow fibers)	-	Water bath and heating films

	introduction of food for 20 min (1–8 mm)					rotors			
DIGDI	Prepared as a bolus	Peristaltic pumps	Agitation with a rotating blade actuated by a motor	Peristaltic pumps	Peristaltic pumps	Agitation with a rotating blade actuated by a motor	-	-	Water bath
SIGMI	Prepared as a bolus	Peristaltic pumps	Peristaltic pumps	Peristaltic pumps	Peristaltic pumps	Magnetic stirrer	-	Feces Anaerobic conditions Magnetic stirrer	Water bath
DGM	Prepared as a bolus Can be loaded in real time	Through perforated hoop Peristaltic pumps	Piston and barrel up and down movement	Piston and cyclical movement	-	-	-	-	Water bath
HGS	Prepared as a bolus	Peristaltic pumps	Rollers, belts, driving shafts, and pulley system	Peristaltic pump	-	-	-	-	60 W light bulbs and thermostat
ARCOL	-	-	-	-	-	-	-	Feces Anaerobic conditions Magnetic stirrer Dialysate system	Heater

6. *In vitro* digestibility

Protein remains the only nutrient requiring animal-based bioassays for routine regulation and labelling purpose. These bioassays are not consistent with socially responsible research directives to replace animal studies and therefore FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition (FAO, 2013) recommended to "developing and validating *in vitro* methods for predicting amino acid digestibility and bioavailability in humans". Furthermore, the growing vegan industry urges for alternative methods to conventional animal methods to assess the nutritional quality of their products.

During the last years, several studies using *in vitro* approaches to calculate PDCAAS in different protein sources have been published (De Bhowmick & Hayes, 2022; Le Roux et al., 2020; Nosworthy et al., 2018; Schaafsma, 2005; Tavano et al., 2016).

Schaafsma (2005) stated the predictive quality of tiny-TIM for estimating true ileal digestibility of proteins and amino acids. According to the author, the PDCCAS can be calculated as follows: "The test product is analyzed for amino acid and/or protein nitrogen. Products are tested by digesting a quantity of a meal that contains 5 g protein. After 5 h of

digestion, the dialyzed fraction is sampled and analyzed for amino acids and/or protein nitrogen. A blank run is performed to determine the contribution of secreted proteins. The method allows the correction of the amino acid score for true ileal digestibility of the first limiting essential amino, which is much more relevant than correction for true fecal digestibility of the whole protein, as proposed by FAO/WHO." (Schaafsma, 2005).

Tavano et al. (2016) tested seven different *in vitro* methods to determine the protein digestibility and calculate the PDCAAS of chickpea fractions. In this study, *in vitro* digestibility was determined using a pepsin-pancreatin incubation, considering soluble nitrogen via Kjeldahl, and hydrolysed peptide linkages by TNBS, and OPA methods. *In vitro* digestibility was also determined using trypsin, chymotrypsin and peptidase or trypsin, chymotrypsin, peptidase and pronase solution. PDCAAS was calculated based on the essential amino acid scoring pattern for 1- to 2-year-old children (FAO/WHO/UNU, 2007), using the protein digestibility values obtained via according with the following formula:

$$\text{PDCAAS} = \frac{\text{limiting amino acid content in sample protein}}{\text{same amino acid content of reference protein}} \times \text{protein digestibility}$$

Nosworthy et al. (2018) assayed the *in vitro* digestibility of beans using a multi-enzyme cocktail containing trypsin, chymotrypsin and protease. The equivalent of 62.5 mg of protein was heated to 37 °C, and adjusted and stabilized to pH 8 for 10 min. After adding the digestive cocktail to the sample, the subsequent pH drop was recorded for 10 min. The *in vitro* protein digestibility was calculated as follows, where the ∆pH10 min is the change in pH in 10 min from the initial pH of about 8. *In Vitro* Protein Digestibility (IVDP) % = 65.66 + 18.10 x ∆pH (10 min), and the *in vitro* PDCAAS was calculated multiplying the Amino Acid Score by the *In Vitro* Protein Digestibility (%).

Le Roux et al. (2020) digested infant formulas using the *in vitro* dynamic system DIDGI® adapted to the gastric and intestinal parameters of a newborn of four weeks. Digestion experiments were performed over three hours and samples were collected before digestion (time 0 min) and in both compartments (gastric and intestinal) at 30, 60, 90, 120, and 180 min after the beginning of the digestion. The total AA contents were determined after acid hydrolysis and the free AA contents were determined after deproteinization of the samples. The quantity of AA released during digestion was expressed as the percentage of free AA (expressed in g/100 g infant formula) related to the total AA (g/100 g infant formula). Undigested samples and intestinal digesta in the intestinal compartment at 3 h of digestion (or emptied from the intestinal compartment over 3 h) were analyzed for total N and soluble N (micro-Kjeldahl method) after the removal of insoluble particles by centrifugation. Molecular weight distributions of the resulting soluble fractions were determined by size exclusion chromatography (SEC). The SEC system was calibrated by injecting eight molecular weight

markers, which allowed to determine the retention times defining the limits of each molecular weight range: >10 kDa, 10 - 5 kDa, 5 - 2 kDa, 2 - 1 kDa, and 1 - 0.2 kDa. The proportion of soluble proteins and peptides in a given molecular weight range was determined as the percentage of area under the curve between the respective limits (% Area SEC). The soluble N fraction corresponds to the nitrogen contained in the proteins, peptides, and free AA of the soluble fraction. For each molecular size range, and because free amino acids are supposed to be undetectable at 214 nm, the proportion of soluble N in this range (% N SEC) was calculated as follows:

$$\%\text{N SEC (x kDa)} = \frac{\text{Total soluble N}_{\text{(digesta)}} - \text{Soluble N}_{\text{(free AA)}}}{\text{Total N}_{\text{(infant formulas)}}} \times \%\ \text{Area SEC (x kDa)}$$

Total soluble $N_{(digesta)}$ corresponds to the quantity of *soluble N* (mg) in the digesta at the end of digestion in the intestinal part (both intestinal compartment and intestinal emptied fraction). Total $N_{(infant\ formula)}$ corresponds to the total *N* in the infant formula (mg). Soluble $N_{(free\ AA)}$ corresponds to the quantity of soluble *N* corresponding to free amino acids (free AA) (mg). *In vitro* apparent protein digestibility was calculated based on the soluble *N* lower than 10 kDa, i.e., as measured in the peptides by SEC and cumulated to the free AA nitrogen. It was determined in the intestinal compartment at 180 min and in the intestinal fraction emptied over 180 min. In both cases, it was calculated as follows:

$$\textit{In vitro}\ \text{apparent protein digestibility (\%)} = \frac{(SH\ [\text{N SEC}_{(<10\ \text{kDa})}] + \text{Soluble N}_{\text{(free AA)}})}{\text{N}_{\text{(infant formulas)}} + \text{N}_{\text{(secretions)}} \times \%\ \text{substrate}} \times 100$$

NSEC and *Soluble N (free AA)* (expressed as mg/kg digesta) corresponds the *soluble N* content in the intestinal compartment or in the intestinal emptied fraction. % substrate was the percentage of the infant formula initially introduced in the system that was present in the intestinal compartment or in the intestinal emptied fraction (g infant formula/100 g digesta), estimated using the emptying equation; a known flows in the system. $N_{(infant\ formulas)}$ (expressed as mg/kg infant formula) was the total *N* content of the meal introduced in the system. $N_{(secretions)}$ (expressed as mg/kg infant formula) was the total nitrogen content of the simulated bile secretion and pancreatin solution. Both digestibility values (intestinal compartment and emptied fraction) were averaged after weighting each value according to the substrate repartition in these two fractions. The PDCAAS-like score (protein digestibility-corrected amino acid score) was calculated by adapting the FAO/WHO methodology in which the true fecal protein digestibility (normally determined *in vivo* in growing rats) was replaced by the *in vitro* apparent protein digestibility calculated as described above. The calculations were done according the following equations:

$$\text{PDCAAS-like} = \text{Amino acid score (of limiting AA)} \times \text{in vitro apparent digestibility}$$

$$\text{Amino acid score} = \frac{\text{Amino acid content of the infant formula}}{\text{Amino acid content of the reference}}$$

De Bhowmick & Hayes (2022) determined the *in vitro* k-Protein Digestibility-Corrected Amino Acid Score (k-PDCAAS) values of six different, Irish seaweeds using the rapid k-PDCAAS method by Megazyme. Protein samples were sequentially digested using pepsin, trypsin, and chymotrypsin at neutral pH and undigested proteins were removed by precipitation with trichloroacetic acid (TCA). Based on the amino acid profile and protein content of each seaweed, the *in vitro* protein digestibility and k-PDCAAS scores were calculated. The α-amino acid concentration present in the sample was quantified using 2% ninhydrin solution with respect to L-glycine standards, and the absorbance was recorded at 570 nm. PDCAAS values were calculated using the Megazyme Mega-CalcTM programme (k-PDCAAS Mega-Calc) available from the Megazyme website (https://support.megazyme. com/support/solutions/articles/8000062829-protein-digestibility-k-pdcaas-mega-calc).

PDCAAS is a useful routine method but, as mention before, there are several disadvantages associated, such as the fact that protein digestibility may not correctly reflect essential amino acid digestibility, and by truncating PDCAAS to 100%, information is lost about the power of a protein to balance the amino acid composition of other proteins or protein mixtures. In addition, PDCAAS values can be overestimated because of limited bioavailability of specific forms of amino acids, that falsely enhance values of true protein digestibility (Marinangeli & House, 2017) On the other hand, DIAAS can be used for mixed diets, and because they are not truncated, these scores allow differentiation among excellent or very good sources of dietary protein (FAO, 2013).

Conceptually, the digestible indispensable amino acid score could be assessed by *in vitro* gastrointestinal digestion experiments that mimic relevant physiological conditions, as a substitute for invasive human and animal studies. To date, only two studies determining DIAAS by means of *in vitro* gastrointestinal models (Ariëns et al., 2021; Havenaar et al., 2016).

Havenaar et al. (2016) measured true ileal digestibility of protein and indispensable amino acids under human conditions simulated in a gastrointestinal model (tiny-TIM). Dialysate samples were collected in 2-hour aliquots (membrane cut-off 5-7kDa) and hydrolysed to measure nitrogen and amino acids. The true ileal digestibility of the protein was measured as amount of nitrogen in the dialysate aliquots. The true ileal digestibility (corrected for endogenous fraction using data of the blank experiments) was calculated as a percentage of intake (to compensate for difference of protein intake) according with the following formula:

$$\text{True ileal digestibility (\%)} = \frac{(\Sigma)\text{ sample [mg]} - (\Sigma)\text{ sample [mg]}}{\text{Intake}_{\text{(nitrogen or amino acid) [mg]}}} \times 100$$

The true ileal protein digestibility was then corrected for the total nitrogen recovery, which corresponded to the amount of nitrogen in dialysate plus gastric and intestinal residue. Based on the concentrations of amino acids (mg/100 g product) in the tested products and on the amount of protein added in tiny-TIM, the intake of the individual amino acids was calculated. Together with the data from the amino acid analysis of the dialysate samples, true ileal digestibility of all individual amino acids was determined as percentage of amino acid intake (equation above). The true ileal amino acid digestibility could also be calculated as an absolute amount (mg) per gram protein and as a ratio of the amino acid requirement pattern for children (>3 years of age), adolescents and adults (DIAA). As described by FAO (FAO, 2013), the DIAAS of the tested products was calculated with using the following equation:

$$\text{DIAAS} = \frac{\text{Digestible indispensable amino acid [mg] in 1g test protein}}{\text{Digestible indispensable amino acid [mg] in 1g reference protein}} \times 100$$

Ariëns et al. (2021) used the INFOGEST static protocol to determine the degree of hydrolysis, true ileal digestibility, *in vitro* digestible indispensable amino acid score (IVDIAAS) and total absorbable amino acids and total essential amino acids of different protein concentrates. Samples were collected before digestion, at the beginning of the gastric phase, after 2h of the gastric phase, at the beginning of the intestinal phase and at the end of the intestinal phase. The digest aliquots were separated in a filtrate and retentate by ultrafiltration using a 5 000 MW filter. Amino acids contents from the source material, digests, retentate and filtrate were quantified. The true ileal digestibility was calculated, either basing calculation on a start protein or digestive mixture including digestive enzymes (for which a correction is needed using an “empty” digest) and based on retentate or filtrate using the following formulas:

$$\text{TID}_{\text{AA}}\text{ (\%)} = \frac{(\text{AA filtrate}_{\text{prot}} - \text{AA filtrate}_{\text{control}})}{\text{AA start}_{\text{prot}}} \times 100$$

$$\text{TID}_{\text{AA}}\text{ (\%)} = \frac{(\text{AA filtrate}_{\text{prot}} - \text{AA filtrate}_{\text{control}})}{(\text{AA digestion}_{\text{prot}} - \text{AA digestion control})} \times 100$$

$$\text{TID}_{\text{AA}}\text{ (\%)} = \frac{\text{AA filtrate}_{\text{prot}}}{\text{AA digestion}_{\text{prot}}} \times 100$$

As control was used an “empty” digest containing the same components as in the “real” digests, only without the addition of a tested protein. Based on the TID, the *in vitro* DIAAS (IVDIAAS) was calculated as suggested in the FAO report (FAO, 2013) using the following formula:

$$\text{IVDIAAS (\%)} = \frac{\text{TID}_{\text{filtrate}} \text{ x mg of digestible indispensable amino acid in 1g dietary protein}}{\text{mg of the same digestible indispensable amino acid per 1g of reference protein}} \text{ x 100}$$

The use of *in vitro* methods to measure digestibility coefficients can provide a rapid and cost-effective approach to bioassays and potentially provide sufficient sensitivity for regulatory requirements. However, the two *in vitro* methods mentioned above have their limitations and downsides.

Virtually simultaneously with the completion of our *in vitro* digestibility protocol, the publication by Ariens et al (2021) has appeared. Like us, their method is based on the Infogest model. Ariens et al struggled with the homogeneity of pancreatin affecting the reproducibility of the method. They tried to solve this by reducing the required physiological enzyme activity by tenfold. Unfortunately, this solution results in a reduced digestion of the food proteins and thus in a protocol that no longer corresponds to human physiology. At the same time, an empty digest without food input was used to subtract the contribution of the autodigested digestive enzymes. It is known that such empty digestions lead to an increased degradation of the digestive enzymes, which leads to an overestimation of the digestibility of the food protein. In the method of Ariens at al, the bioavailable fraction was prepared by filtration with a cutoff of 5 kDa. It is questionable what size of peptides should be accepted, respectively how large peptides would be degraded by brush border membrane enzymes to bioavailable small peptides (Hooton et al., 2015). Most likely 5 kDa is too big and should rather be around 1 kDa (peptides with a length of 8 to 10 amino acids). In addition, filtrations often do not result in an optimal accurate separation, lead to clogging on the membrane and to a greatly reduced yield of the two fractions.

On the other hand, the tiny-TIM method (Havenaar et al., 2016) is a very expensive and complex equipment and it is not accessible for every lab. At the same time, this system unfortunately made very similar decisions regarding the use of the enzyme blank and the cutoff of the bioavailable fraction as Ariens et al.

In addition, and despite the extreme scientific relevance of these two works, none of the two *in vitro* methods were validated towards *in vivo* data. Therefore, further research is needed, where the same products are digested in parallel in both *in vitro* and *in vivo* models (using controlled human bioavailability assays).

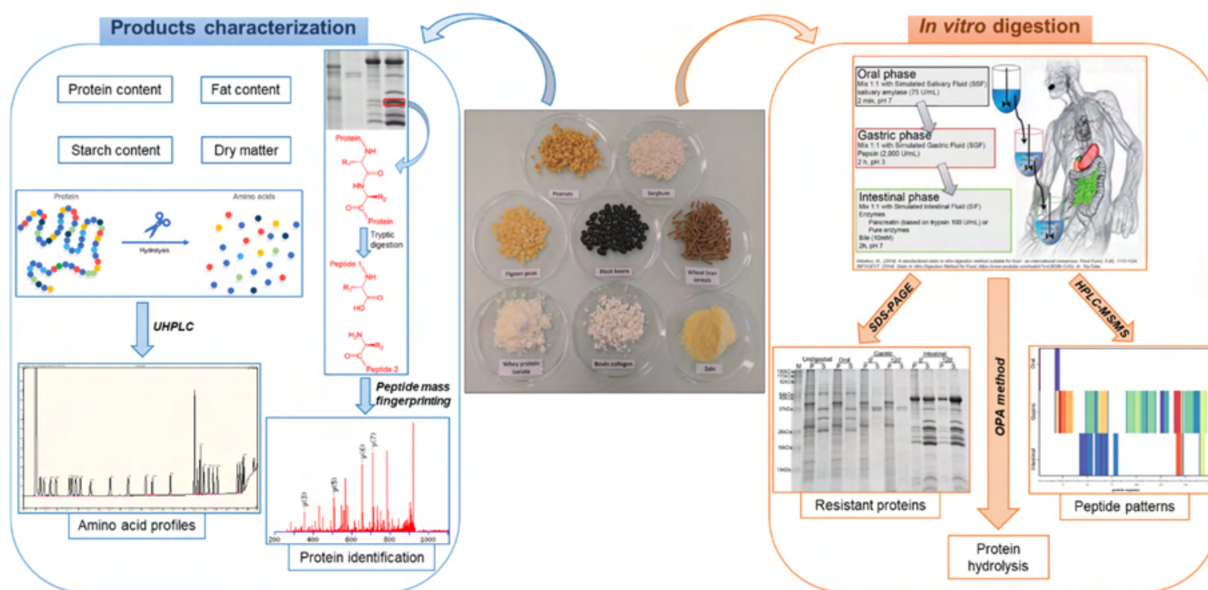
Aims and working plan

Determination of the protein quality and digestibility is important to estimate their influence on human (and animal) protein supply, as well as their ecological and economical values. Quality of proteins is so far (Ref FAO 1991) assessed with the "protein digestibility-corrected amino acid score" (PDCAAS) method. This method is based on the ratio of the limiting essential amino acid (established for a child at preschool age) in the test protein compared to the same amino acid in a reference protein. This ratio is corrected with a factor for the fecal nitrogen digestibility (%) established in a rat model. Unfortunately, this correction and the use of rat is not optimal, leading to imprecise results. Due to several shortfalls of this method, the FAO recommends (FAO 2011) the replacement of this method by the "Digestible Indispensable Amino Acid Score" (DIAAS) method. DIAAS considers the ratio of digestible essential amino acid in mg per g of the test protein versus the amount of the same amino acid in a reference protein. This so called ileal digestibility has to be determined for each food in human or pig trials, which is a huge challenge fraught with ethical problems. In agreement with the principle of 3-R (*Replace, Reduce, Refine*) for animal trials, *in vitro* methods need to be developed and validated, allowing the analysis of the bioavailability of nutrients and metabolites from different food for human and animal nutrition without the need for *in vivo* assays.

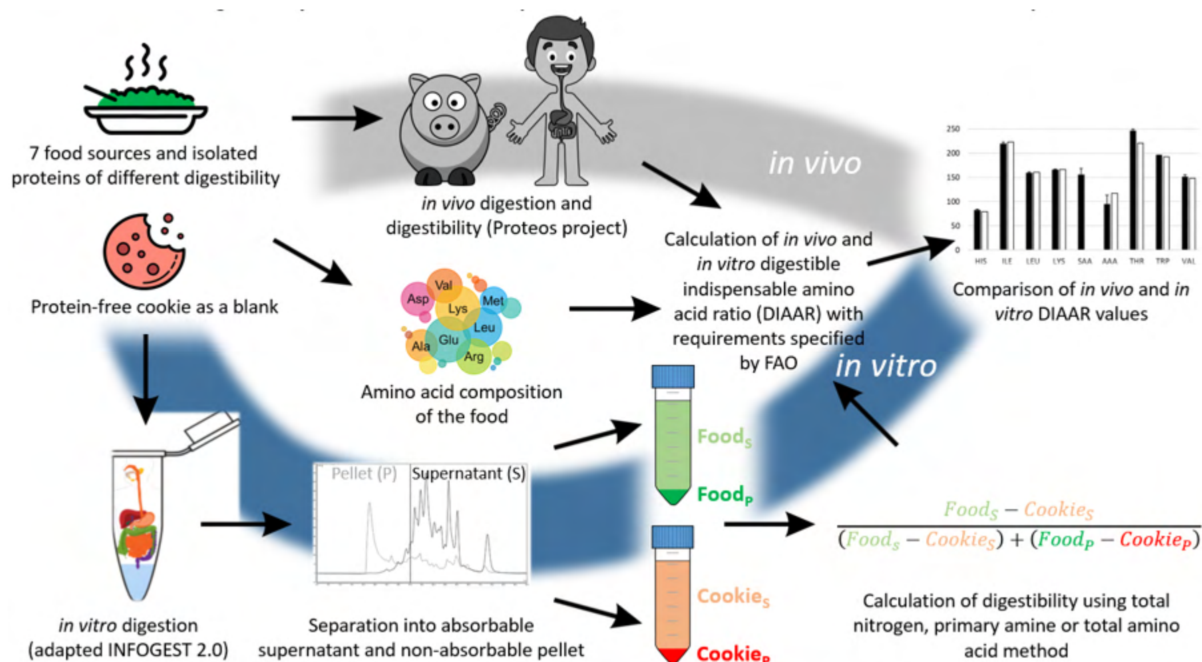
The main goal of this doctoral thesis was to develop and validate with *in vivo* data, an *in vitro* digestion method able to assess the protein quality, in order to reduce the need of *in vivo* assays for this purpose. As starting point of this work, the harmonized INFOGEST *in vitro* digestion method was taken. In our lab, eight protein sources were subjected to *in vitro* digestion and in parallel, the exact same samples were *in vivo* digested in pig and human within the international PROTEOS project coordinated by researchers at the Riddet Institute (NZ).

In order to reach this final goal, the following research was performed:

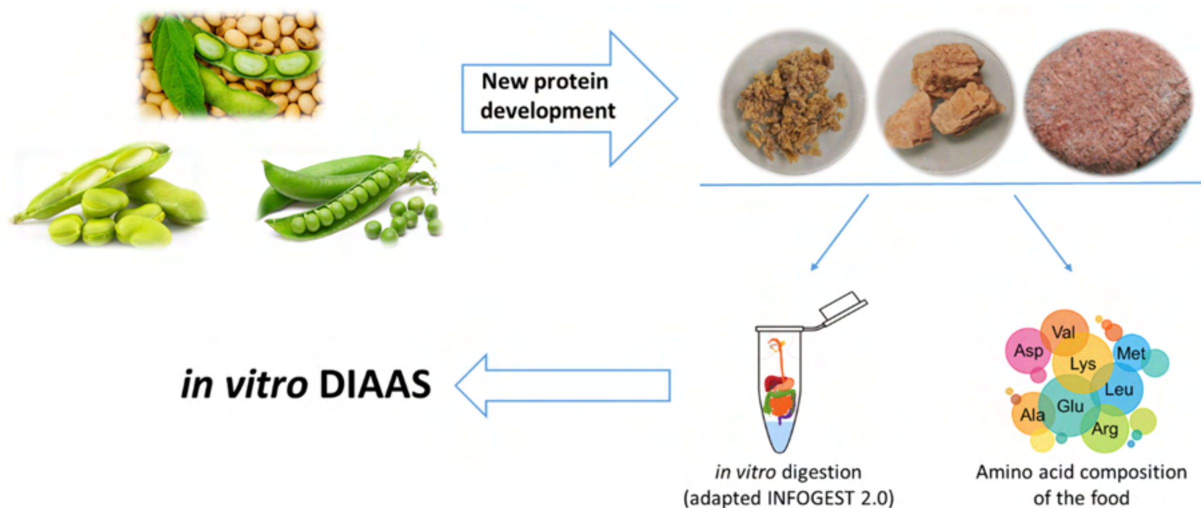
1) Characterizing and testing the digestibility of the eight selected proteins with the INFOGEST harmonized *in vitro* digestion method.



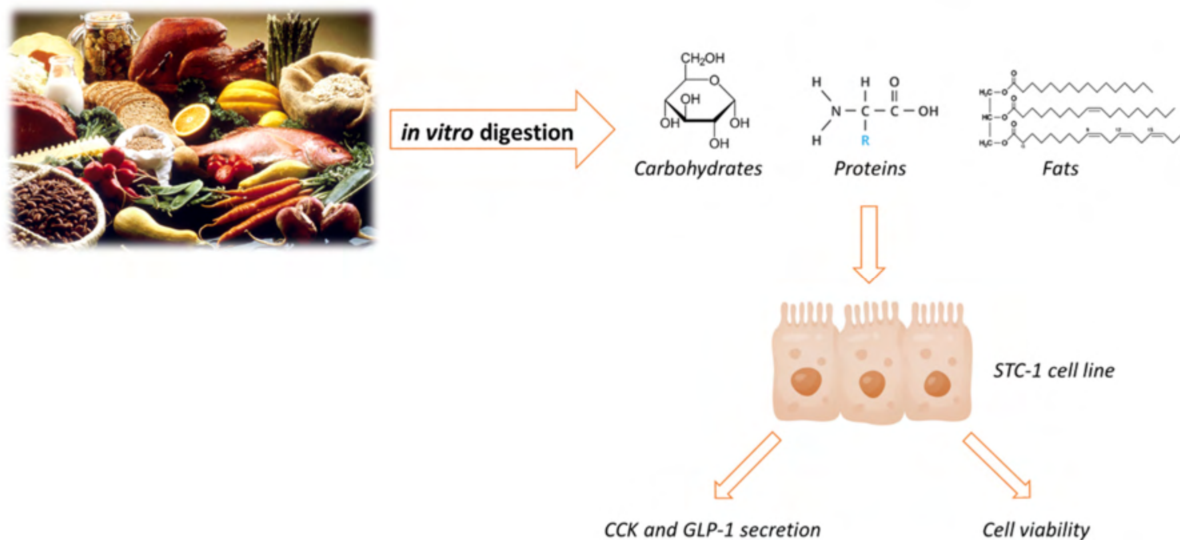
2) Amendment of the INFOGEST *in vitro digestion method and d*evelopment of techniques to measure and calculate protein digestibility for the evaluation of protein quality and comparison with *in vivo* data.



3) Application of the *in vitro* digestibility protocol to evaluate the protein quality of alternative sources of highly transformed plant proteins.



4) Assessment of the physiological relevance of the *in vitro* digestion protocol by evaluating the influence of digestion products on satiety using STC-1 cell lines.



Results

Chapter 1

Characterizing and testing the digestibility of the eight selected proteins with the INFOGEST harmonized *in vitro* digestion method

Manuscript 1

Protein digestion of different food sources using the INFOGEST static digestion model

Raquel Sousa[1], Reto Portmann[1], Sébastien Dubois[3], Isidra Recio[2], Lotti Egger[1]

[1]Agroscope, Schwarzenburgstrasse. 161, 3003 Bern, Switzerland
[2]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain
[3]Agroscope, Tioleyre 4, 1725 Posieux, Switzerland

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Abstract

In vitro digestion systems are valuable tools for understanding and monitoring the complex behavior of food degradation during digestion thus, proving to be good candidates to replace the *in vivo* assays. The aim of the present work was to evaluate the applicability of the harmonized INFOGEST static protocol to different protein powders and complete foods. Three isolated proteins (collagen, whey protein and zein) and five foods (peanuts, sorghum, black beans, pigeon peas and wheat bran) were separated by SDS-PAGE, and 138 major proteins were identified by in-gel digestion of electrophoretic bands followed by HPLC-MS/MS. Individual amino acid composition was analyzed by HPLC, showing the EA/NEAA ratios in the substrates (from low to high): wheat bran cereals, peanuts, collagen, zein, whey protein, sorghum, pigeon peas and black beans. Results also revealed that sorghum, whey protein and zein are good sources of BCAA. No intact protein originating from the food was visually detected after the intestinal step of *in vitro* digestion with the INFOGEST protocol for all the substrates. However, digestion resistant peptides were detected in all substrates after the intestinal digestion phase. Protein hydrolysis was high in whey protein isolate and pigeon pea and low for wheat bran cereals and bovine collagen.

Keywords: Amino acid; *In vitro* digestion; Peptides; Proteins; Protein hydrolysis

Abbreviations:

SDS, Sodium dodecyl sulfate; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, High performance liquid chromatography; MS, Mass spectrometry; LC-MS, Liquid chromatography-mass spectrometry; UHPLC, Ultra-high-performance liquid chromatography; FAO, Food and Agriculture Organization of the United Nations; DIAAS, Digestible indispensable amino acid score; EAA, Essential amino acids; NEAA, Nonessential amino acids; BCAA, Branched-chain amino acids; FAA, Free amino acids; RT, Room temperature; DTT, Dithiothreitol; TBP, Tributylphosphine; OPA, *o*-phthalaldehyde; UV/VIS, Ultraviolet–visible; IVD, *In vitro* digestion; WPI, Whey protein isolate; GLU, Glutamic acid; PDCAAS, protein digestibility-corrected amino acid scores; BCA, Bicinchoninic acid

1. Introduction

Food proteins can be classified according to their nutritional value depending on their amino acid content and composition. Complete proteins contain the essential amino acids (EAA) in the right proportions required by the human body, whereas incomplete proteins are deficient in one or more EAA (Moughan, et al., 2016; Nadathur et al., 2017).

The nutritional quality of the protein fraction of foods should be studied *in vivo* (in humans or animals), however these experiments are expensive, technically difficult, time consuming and most often entail serious ethical problems (Dupont et al., 2019; Minekus et al., 2014). Thus, the need for *in vitro* models that closely mimic the physiological processes occurring during human digestion led to the development of *in vitro* digestion models as alternatives to *in vivo* experiments. These models were designed taking into account the physiological conditions regarding the occurrence and concentration of digestive enzymes, the pH values in gastric and intestinal phases, digestion time and salt concentrations, among other factors. One of the main goals of the former COST action INFOGEST, involving more than 200 scientists from 32 countries working in the field of digestion, was the elaboration of a harmonized *in vitro* digestion protocol. This consensus protocol was first published in 2014 (Minekus et al., 2014) and updated recently (Brodkorb et al., 2019). The performance of this digestion protocol on milk proteins has been validated using pigs as animal models (Egger et al., 2017) and human digests (Sanchón et al., 2018).

Although no *in vitro* method can reflect the full complexity of *in vivo* digestion (Coles et al., 2005; Hur et al., 2013), *in vitro* models have proven to be useful alternatives to animal and human models as screening tools for addressing diet-related questions such as digestibility, bioavailability, release of bioactive compounds and structural changes in food (Egger et al., 2017; Espert et al., 2019; Hur et al., 2013; Kopf-Bolanz et al., 2012; Marcano et al., 2015). In addition, there is a growing interest in how proteins are digested and the health implications of protein digestion products, for example to predict allergenicity of novel protein sources (Pali-Schöll et al., 2019; Verhoeckx et al., 2019).

The main purposes of this work were to characterize eight different protein substrates -wheat bran cereals, sorghum, pigeon peas, black beans, peanuts, zein, whey protein isolate and collagen- and to assess gradual protein hydrolysis using the INFOGEST *in vitro* gastrointestinal digestion protocol. These very same products were previously selected for *in vivo* digestibility experiments (Institute Riddet, 2019; PROTEOS, 2010), and the future goal will be the validation of *in vitro* digestibility toward these *in vivo* digestible indispensable amino acid score (DIAAS) values.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and enzymes used were purchased from Sigma Aldrich.

2.2 Food sources composition (fat, carbohydrates, protein and dry matter)

The fat content in the different food samples was determined according to the ISO standard 1735:2004. Protein content was quantified with the Kjeldahl method according to ISO 8968-3:2007/IDF 20-3: 2007 (ISO 8968-3, 2007). Drying loss was analyzed with ISO standard 5534:2004/IDF 4:2004 (ISO 5534, 2004), calculating dry matter by subtracting the weight loss from the original weight. Starch was analyzed using the Total Starch Assay Kit (AA/AMG) from Megazyme (Megazyme u.c., Wicklow, Ireland).

2.3 Determination of total AA

Total amino acids of each substrate were determined as described in the ISO 13903:2005 (ISO 13903, 2005). Briefly, after oxidation, 24 h of acid hydrolysis (HCl, 6M) and derivatization (Waters, 2007), and the amino acid profile was determined by ultra-high-performance liquid chromatography (UHPLC) (AccQ-Tag Ultra 2.1 x 100 mm, 1.7 µm, Waters) coupled with a UV detector (Vanquish, Thermo Fisher Scientific).

2.4 Protein extraction

Wheat bran protein extraction was adapted from the method described by Chatzifragkou et al. (2016) (Chatzifragkou et al., 2016). Briefly, EtOH (70%, 1:2 w/v) was added to the minced sample. After a 30-minute incubation time at 50 °C, the sample was centrifuged (10 000 × g, 4 °C for 10 min) and the pellet was resuspended with EtOH (70%, DTT 1%). The incubation-centrifugation-resuspending cycle was repeated twice. Thereafter, the EtOH concentration was reduced from 70% to 20% with 2.5 ml of H_2O. The sample was kept at -20 °C overnight. After a centrifugation (15 000 × g, 2 °C for 20 min), the pellets were collected and resuspended with Tris buffer (100 mmol/L, SDS 1%, DTT 1%, pH 7.4).

Collagen proteins were precipitated with EtOH (SDS 1%, TBP 1 %) at -20°C for 24 h. After centrifugation (17 900 × g, 4°C for 15 min), the supernatant was discarded and the pellet was resuspended in Tris buffer (100 mmol/L, SDS 1 %, pH 7.4). The sample was sonicated (5 pulses, power 60% for 2 sec) and centrifuged again. The supernatant was discarded and the pellet was resuspended in Tris buffer (100mmol/L, SDS 1%, DTT 1%, pH 7.4).

Sorghum proteins were extracted with Tris buffer (100mmol/L, SDS 1%, DTT 1%, pH 7.4), sonicated (5 pulses, power 60% for 2 sec) and incubated at 95 °C for 5 min. Thereafter, 1 ml of MeOH was added and the sample was kept at -20 °C for 24 h. The sample was centrifuged (17 900 × g, 4 °C for 15 min), the supernatant was discarded and the pellet was

resuspended in Tris buffer (100 mmol/L, SDS 1%, pH 7.4). After another sonication (5 pulses, power 60% for 2 sec) and centrifugation (17 900 × g, 4 °C for 15 min), the supernatant was transferred into a new tube.

Using a mortar and pestle, the samples from peanuts, pigeon peas and black beans were ground and then mixed with Tris buffer (100 mmol/L, SDS 1%, DTT 1%, pH 7.4). The samples were incubated at 95 °C for 5 min, then left overnight at room temperature (RT) under constant gentle mixing on a rotating wheel. After sonication (5 pulses, power 60% for 2 sec), MeOH was added and the samples were centrifuged (17 900 g, 4 °C for 10 min). The supernatant was discarded and Tris buffer (100 mmol/L, SDS 1%, DTT 1%, pH 7.4) was added. The samples were incubated at RT for 1 h, sonicated (5 pulses, power 60% for 2 sec) and centrifuged (17 900 × g, 4°C for 10 min), and the supernatants were transferred into new tubes.

Whey protein isolate and zein samples were dissolved with Tris buffer (100 mmol/L, SDS 1%, pH 7.4), then sonicated (5 pulses, power 60% for 2 sec). MeOH was added, samples were centrifuged (17 900 × g, 4 °C for 10 min), the supernatant was discarded and the pellet was resuspended with Tris buffer (100 mmol/L, SDS 1%, pH 7.4). The samples were incubated at RT for 1 h and sonicated (7 pulses, power 60% for 2 sec). After centrifugation (17 900 × g, 4°C for 10 min), the supernatants were transferred into new tubes.

2.5 Gel electrophoresis

After extraction, proteins from undigested samples were quantified with the bicinchoninic acid (BCA) protein determination kit (Pierce®, Thermo Scientific). Equal amounts of protein were diluted with 6x sample buffer (Tris–HCl, 350 mM, pH 6.8, SDS 10%, DTT 100 mM, glycerol 50%) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide). Samples obtained from *in vitro* digestion (IVD) were centrifuged, and the pellet and the supernatant were mixed with 6x sample buffer and denatured at 95 °C for 5 min. Then, all the samples were diluted 1:80 with 1x sample buffer and additional dilutions for the oral phase (1:8), gastric phase (1:4), and intestinal phase (1:2), respectively. A molecular weight marker (Benchmark™, Invitrogen, Basel, Switzerland) was included on each gel. Gels were stained with colloidal Coomassie (Donghoon Kang et al., 2002).

2.6 Peptide mass fingerprinting

Polyacrylamide gel pieces were manually excised from the protein bands. Gel pieces were washed three times, alternating between 100 µL destain buffer (ammonium bicarbonate 25 mmol/L, acetonitrile 50% v/v) and 100 µL digestion buffer (ammonium bicarbonate 25 mmol/L). Then, they were digested with 2 µL trypsin (4 mg/L) in 20 µL of digestion buffer at 37

°C overnight. After tryptic in-gel digestion, the peptides were separated using high-performance liquid chromatography (HPLC) (Rheos 2200, Flux Instruments) equipped with an XTerra MS C18 column (3.5 mm, 1.0 mm 3 150 mm, Waters). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland) using an electron spray ionization interface. Protein identifications were performed by submitting the fragmentation data to the Mascot search engine (Matrix Science, London, UK), using UniProt (2018). Identifications were manually validated according to the following criteria: protein score above 40, peptide score above 25, identification of at least two different peptides and identification of at least three consecutive fragmentation ions per peptide.

2.7 In vitro digestion: INFOGEST static model

All substrates were subjected to the *in vitro* gastrointestinal INFOGEST protocol (Brodkorb et al., 2019). In addition to the eight protein sources, a blank digestion of a protein-free cookie, containing only fat and carbohydrates, was performed in order to avoid the auto-digestion of the digestive enzymes. Briefly, 40.8 g of purified corn flour, 15.7 g of sucrose, 0.7 g of baking powder, 0.5 g of ground ginger and 36.9 g of margarine were mixed and divided into portions of ~35 g, then baked for 30 min at 175 °C. Black beans and pigeon peas were previously cooked; 40 g of each were soaked in water for 18 h. They were cooked in 200 ml of water with 288 mg of salt for 10 min (pigeon peas) or 20 min (black beans). Thereafter, they were ground to simulate the effect of mastication. The enzyme activities and bile concentration were measured prior to the digestion experiment according to the assays described in the harmonized protocol (Minekus et al., 2014).

In brief, the amount of each food corresponding to 0.04 g of protein was dissolved in 1 mL of water and mixed with 1 mL of simulated salivary fluid (pH 7, 37 °C) containing amylase (300 U/mL of digesta) for 2 min. Then, 2 mL of simulated gastric juice (pH 3, 37 °C) containing pepsin (2000 U/mL of digesta) were added and incubated for 120 min. Subsequently, 4 mL of simulated intestinal juice (pH 7) containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L of total digesta) were added and incubated for 120 min. The whole digestion protocol was performed at 37 °C under constant gentle mixing on a rotating wheel. The digestion was stopped after 120 min of gastric digestion by increasing the pH to 7 with NaOH (1 mol/L), and the intestinal phase by using the protease inhibitor 4-(2 aminoethyl) benzensulfonylfluorid (AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland). Immediately after stopping the digestion, all samples were snap frozen in liquid nitrogen. After freezing, all samples were separated in a soluble (S) and insoluble (P) fraction by centrifugation.

2.8 Amino acid counting

Identification of peptides in the simulated digests was performed similar to protein identification with the previously described modifications (Kopf-Bolanz et al., 2012). Briefly, digested samples were filtered through Amicon columns (Ultracel YM-30, Millipore, Zug, Switzerland), the peptides were identified via HPLC (Rheos 2200, Flux Instruments) equipped with an XTerra MS C18 column (3.5 mm, 1.0 mm 3 150 mm, Waters), coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland). For amino acid counting, the samples were measured in multiple overlapping narrow-mass windows spanning m/z−1 between 100 and 1300, and all raw files were merged for an identification search with Mascot (Matrix Science, London, UK), using a database containing the major proteins previously identified in the studied food samples. The amino acids identified within the peptides present in the protein of interest were summed up and numbers were displayed along the protein sequence. The color code was chosen, with red representing the maximal number of identified amino acids within the corresponding protein and digestion phase (Egger et al., 2018), green representing medium numbers of identified amino acids within a peptide, and blue representing a minimal number of identified amino acids. White regions represent unidentified amino acids.

Table 1: Composition of the eight protein sources in fat, total nitrogen (TN), starch and dry matter. The protein content was calculated based on a 6.25 conversion factor and Kjeldahl results. n.d. = not determined.

g/100 g of food			**Dry matter**	**Starch**	**Total fat**	**Protein**
Complete foods	1.	Sorghum	91.58	71.63	4.43	9.63
	2.	Wheat bran cereals	95.27	27.62	5.49	14.15
	3.	Black beans	92.06	33.60	2.29	22.97
	4.	Pigeon peas	92.17	44.11	2.45	26.11
	5.	Peanuts	98.28	n.d	48.91	31.98
Isolated proteins	6.	Collagen	93.3	n.d	n.d	103.83
	7.	Whey protein	93.13	n.d	n.d	84.16
	8.	Zein	96.23	n.d	n.d	92.86

2.9 Quantification of free amino groups (OPA method)

Free amino groups in the supernatant from the digests were measured by the *o*-phthaldialdehyde (OPA) method (Kopf-Bolanz et al., 2012). In brief, the samples were diluted 10 times with perchloric acid (0.5 mol/L) in order to precipitate proteins and longer peptides. Once derivatized with OPA and in the presence of 2-mercapto-ethansulfonic acid, the produced 1-alkylthio-2-alcylisonindol compound was measured with an UV/VIS photometer at 340 nm. Results were calculated based on a glutamic acid standard curve. A blank digestion (protein-free cookie) was defined as background and the obtained OPA values were subtracted from the OPA values of the eight protein sources.

3. Results

3.1. Substrates composition

In vitro digestion with the harmonized INFOGEST protocol was performed with the exact same eight protein sources that were previously selected by the PROTEOS project for the establishment of the DIAAS values in humans and pigs (Mathai, 2018). The ingredients used in this experiment were chosen to reflect protein sources used in diets around the globe. All samples were analyzed for their composition in fat, protein, starch and dry matter (Table 1), as well as for their total amino acid content in grams per gram of food (Figure. 1) and as a relative distribution in the protein fraction (supplemental Figure 1). To allow the comparability of protein hydrolysis between the samples, they were normalized according to a protein content of 0.04 g, based on a conversion factor of 6.25 for all sources, prior to *in vitro* digestion.

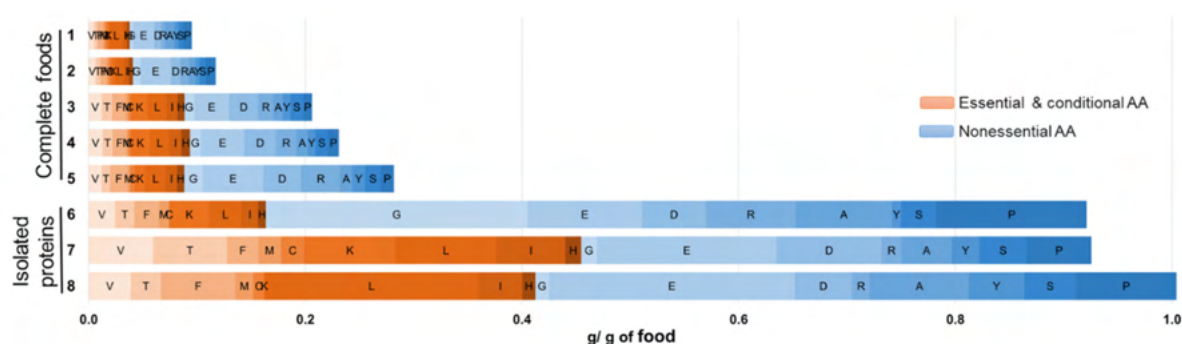

Figure 1. Amino acid profile (%) for the eight different protein sources.
1: Sorghum; 2: wheat bran cereals; 3: black bean; 4: pigeon pea; 5: peanut; 6: collagen; 7: whey protein; 8: zein. EAA are shown in orange and NEAA in blue (Rutherfurd et al., 2015).

As expected, the eight substrates were different in composition, with contents of protein between 8.25 g and 89.21 g; starch and fat values reached 49.91 g and 71.63 g per 100 g of product, respectively. The content of individual amino acids for each substrate was analyzed

by UHPLC, not considering tryptophan due to its destruction during acid hydrolysis. As expected, the three isolated powders had a protein content higher than 80%. Despite their relatively low protein contents, black beans, pigeon peas and sorghum, together with whey protein and zein powders, had the highest ratio of essential/non-essential amino acids (EAA/NEAA, ≥ 0.60). Moreover, sorghum, whey protein and zein are good sources of branched-chain amino acids (BCAA), which represent more than 20% of the total amino acid content of these substrates (supplemental Figures 1, 2). In contrast, wheat bran cereals, peanuts and collagen have lower EAA/NEAA ratios (≤ 0.48).

3.2. Protein identification

The substrates were further characterized in their composition of individual proteins. Therefore, proteins were extracted and separated by SDS-PAGE for further identification with liquid chromatography-mass spectrometry (LC-MS) after tryptic in-gel digestion. The individual proteins present in the substrates are shown in Figure 2, and 59 main proteins out of 138 are listed in Table 2.

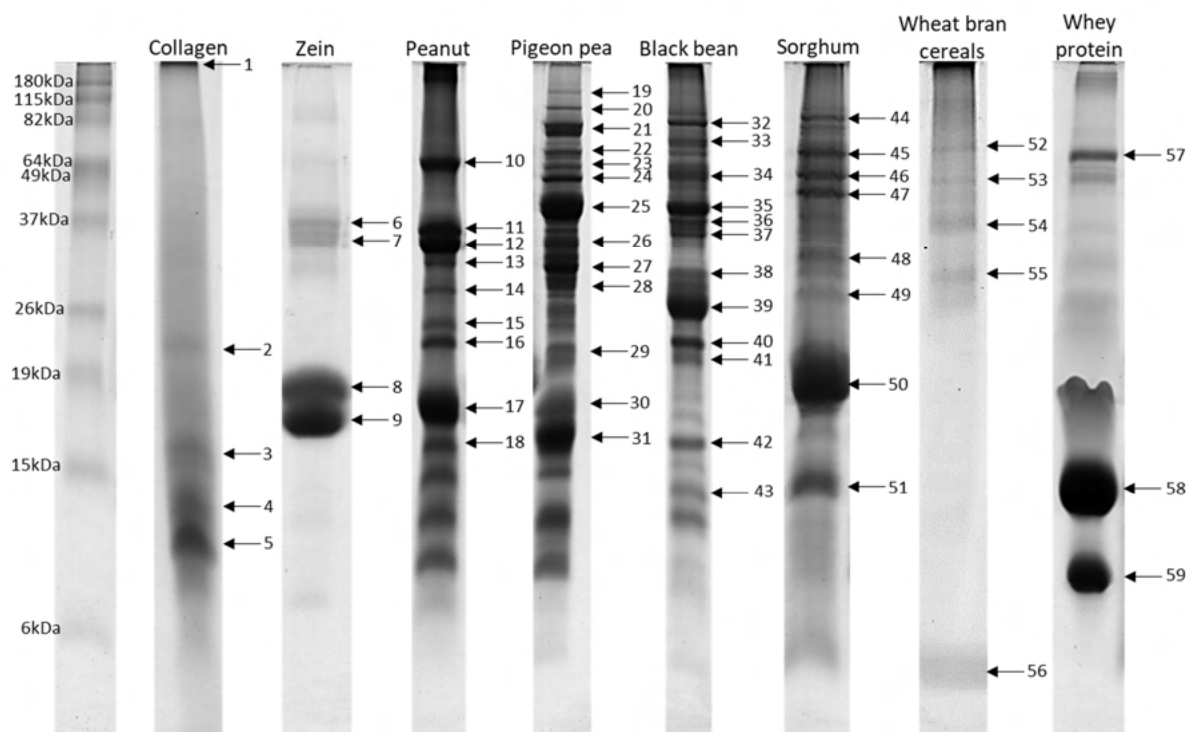

Figure 2. Protein separation in the eight different foods and protein powders using SDS-PAGE. Each number corresponds to an excised band in which one or more proteins have been identified. The identified proteins are listed in table 2.

With the exception of sorghum, the proteins identified for all substrates had been previously described. In the collagen powder, the proteins from the five excised bands corresponded to different collagen chains: VI alpha 3 chain (1), alpha-1 III chain (3), alpha-1 I chain (4), alpha-

2 I chain (5) and decorin (2). Decorin is a component of connective tissues, binding to type I collagen fibrils and playing a role in matrix assembly was also identified in this sample.

Alpha zein is the major fraction of corn prolamin (about 80 %) (Luo & Wang, 2016; Shukla & Cheryan, 2001). In the analyzed sample, zein alpha 19D1 (8) and 22 kDa alpha-zein 14 (8) were identified, as well as fragments of Z1D alpha zein (6, 7) and Z1A alpha zein (9) proteins. In peanuts, the two major proteins arachin (10-12, 17-18) and conarachin (14) as well the allergens Arah1 (10, 13), Arah2 (16) and Arah3 (15) were identified in different bands. In pigeon peas, different storage proteins were identified, namely, vicilin (23), a fragment of covicilin (22), legumin A (24) and different phaseolin types (26, 27). Fragments of two allergens were also present, allergen Lenc 1.0101 (31) and 1.0102 (30). In black bean samples, two different types of the storage protein phaseolin (35-38) (α and β-type), as well as two anti-nutritional factors, phytohemagglutinin (39) and alpha amylase inhibitor-1 (42, 43), were identified. In sorghum, most of the identified proteins were not yet described (44-51) in the UniProt database. Nevertheless, it was possible to identify the dominant protein fraction in grain sorghum, kafirin (50). The proteins chitinase B1 (49) and LEA3 (50) were also identified. These proteins are involved in responses against pathogen infection and environmental stresses, respectively (Magwanga et al., 2018; Ratnavathi et al., 2016). In the wheat bran cereals, mainly storage proteins were identified. Gobulin-3 (56) and different types of glutenin (52, 53) and gliadin were detected. In whey protein isolate, as expected, the major proteins were identified as serum albumin (57), β-lactoglobulin (58) and α-lactalbumin (59).

Table 2. Identified proteins after tryptic digestion of the bands excised from SDS-PAGE using peptide mass fingerprinting. The numbers refer to band numbering in figure 2.

Collagen		20	Seed lipoxygenase-3	42	Alpha amylase inhibitor-1
1	Collagen type VI alpha 3 chain	21	Seed biotin-containing protein SBP65	43	Alpha amylase inhibitor-1
2	Decorin	22	Convicilin (fragment)	***Sorghum***	
3	Collagen alpha-1 (III) chain	23	Vicilin	44	Pyruvate phosphatase dikinase
4	Collagen alpha-1 (I) chain	24	Legumin A	45	Putative uncharact. protein Sb01g012640 Putative uncharact. protein Sb03g03960
5	Collagen alpha-2 (I) chain	25	P54 protein	46	Putative uncharact. protein Sb01g005440

					Putative granule bound starch synthase
Zein		26	Phaseolin α- type Phaseolin β- type	47	Globulin-2 Elongation factor 1-alpha
6	Z1D alpha zein protein (fragment)	27	Phaseolin	48	Putative uncharact. protein Sb03g046810
7	Z1D alpha zein protein (fragment)	28	Provicilin (fragment)	49	Chitinase-B1 Putative uncharact. protein Sb04g007585
8	Zein alpha 19D1 22 kDa alpha-zein 14	29	Albumin-2	50	19kD-like alpha kafirin B3 LEA3 protein
9	Zein 1A alpha zein protein (fragment)	30	Allergen Lenc 1.0102 (fragment)	51	Putative uncharact. protein Sb03g006870 Oleosin
Peanut		31	Allergen Lenc 1.0101 (fragment)	***Wheat bran cereals***	
10	Allergen Arah1, clone P41B	***Black bean***		52	High molecular weight glutenin subunit
11	Arachin 3	32	Phaseolin α-type	53	Glutenin subunit
12	Arachin 6	33	Heat shock protein hsp70	54	Gamma Gliadin
13	Main Allergen Arah1	34	Group 3 late embryogenesis abundant protein	55	Gamma-gliadin B
14	Conarachin	35	Phaseolin	56	Globulin 3 Non-specific lipid-transfer protein (fragment)
15	Arachin Arah3 isoform	36	Phaseolin α- type Phaseolin β- type	***Whey protein***	
16	Arachin Arah2	37	Phaseolin α- type Phaseolin β- type	57	Serum albumin
17	Arachin 6	38	Phaseolin α- type Phaseolin β- type	58	Beta-lactoglobulin
18	Arachin 3	39	Phytohemagglutinin	59	Alpha-lactalbumin
Pigeon pea		40	Lectin		
19	Alpha-1, 4 Glucan phosphorylase L isozyme, chloroplastic/amyloplastic	41	Triosephosphate isomerase		

3.3. Protein hydrolysis after gastric and intestinal IVD

The evolution of protein hydrolysis was monitored by SDS-PAGE for all eight substrates (Figure 3 and supplemental Figure 3). As examples, protein hydrolysis of whey protein isolate (3a) and black beans (3b) after the oral, gastric and intestinal endpoints are shown (Figure 3). A blank experiment with water was performed and separated on gel to show the protein bands originating from the digestive enzymes (supplemental Figure 3g).

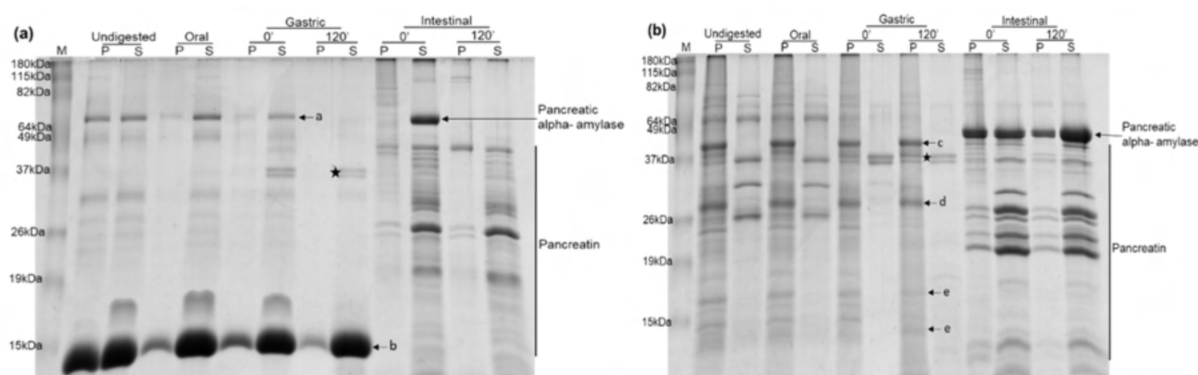

Figure 3. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phases from IVD of whey protein (3a) and black bean (3b); a: serum albumin; b: β-lactoglobulin; c: phaseolin; d: phytohemagglutinin; e: alpha amylase inhibitor-1; asterisk: pepsin.

In the case of whey protein isolate (Figure 3a), as has been previously well documented (Dupont et al., 2010; Kopf-Bolanz et al., 2012), β-lactoglobulin (b) was resistant to pepsin in the gastric phase and immediately hydrolyzed at the beginning of the intestinal phase. In contrast, serum albumin hydrolysis occurred at the beginning of the gastric phase and was no longer present at the end of this phase. No intact proteins from the whey protein isolate (WPI) were present neither in the supernatant nor in the pellet after completion of the intestinal digestion phase (Figure 3a, intestinal, 120 min). The samples from black bean digestion showed that phaseolin and phytohemagglutinin were resistant throughout the gastric phase, as has been previously reported (Liener & Thompson, 1980; Romero & Ryan, 1978; Vaintraub et al., 1979). Alpha amylase inhibitor-1 also presented a high resistance to gastric digestion, although a slight degradation at the end of this phase was visible. Despite their persistence during the gastric phase, no intact proteins from black beans were visible on gel after completion of the intestinal phase.

3.4. Peptide patterns

The most abundant and/or main proteins from each substrate were selected for analysis for gradual peptide generation during IVD. As examples, the peptide patterns after

oral, gastric, and intestinal digestion are shown for β-lactoglobulin (Figure 4a, WPI) and phaseolin (Figure 4b, black beans). The peptide patterns for the other substrates are shown in supplementary Figs. 4a-f. The patterns are color coded along the protein sequence, representing the abundance of amino acids, which were identified within the peptides present in the protein of interest. β-lactoglobulin was mainly intact after the oral phase. At the end of the gastric phase, the number of counted peptides increased significantly, especially at the N-terminus. After the intestinal phase, a higher number of peptides were still visible in two regions of the C-terminus, but most of the protein was digested and therefore no longer visible (Figure 4a). The peptide pattern from phaseolin, resulting from black bean digestion, shows some protein degradation at the oral phase, probably due to the cooking process performed before digestion. The number of counted peptides over the whole protein sequence increased during the gastric phase. Only a small amount of peptides were detected at the end of the intestinal digestion, indicating a high degradation of this protein.

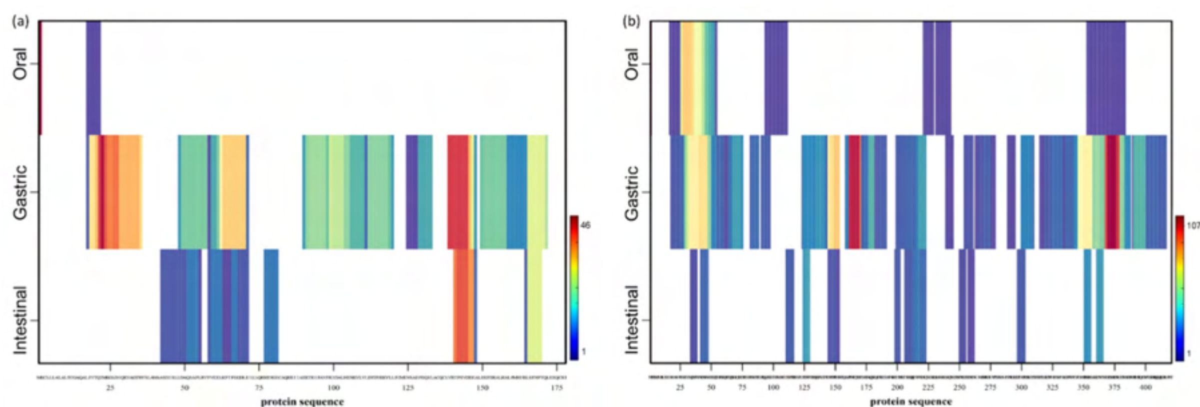

Figure 4. β-lactoglobulin (a) and phaseolin peptide patterns (b) at the end of oral, gastric and intestinal phases of *in vitro* digestion of whey protein isolate and black beans, respectively. The color coding ranges from red (representing the maximal number of identified amino acids) to green and blue (representing medium to low numbers of identified amino acids). White regions represent amino acids without identification within any peptide.

3.5. Formation of free amino acids and short peptides

The formation of free amino groups was determined using the OPA method, detecting primary amine groups in the supernatant of the digested samples after precipitation with perchloric acid. The substrates were all normalized to the amount of protein input of 0.04 g analyzed by Kjeldahl; therefore, the analyzed glutamic acid (GLU) equivalents can be used to compare the amount of protein hydrolysis in the different protein sources with higher values corresponding to a greater release of free amino acids and di- or tripeptides (Figure 5).

WPI clearly had the highest release of GLU equivalents comparing the three protein powders. In contrast, far less GLU equivalents were released from the two other protein powders, zein and collagen (Figure 5). From the five food samples, the highest amounts of GLU equivalents

were released from pigeon peas, which were as high as WPI. Sorghum, black beans and peanuts released less amino acids, dipeptides or tripeptides during digestion, and the lowest values were found in the wheat bran cereal sample.

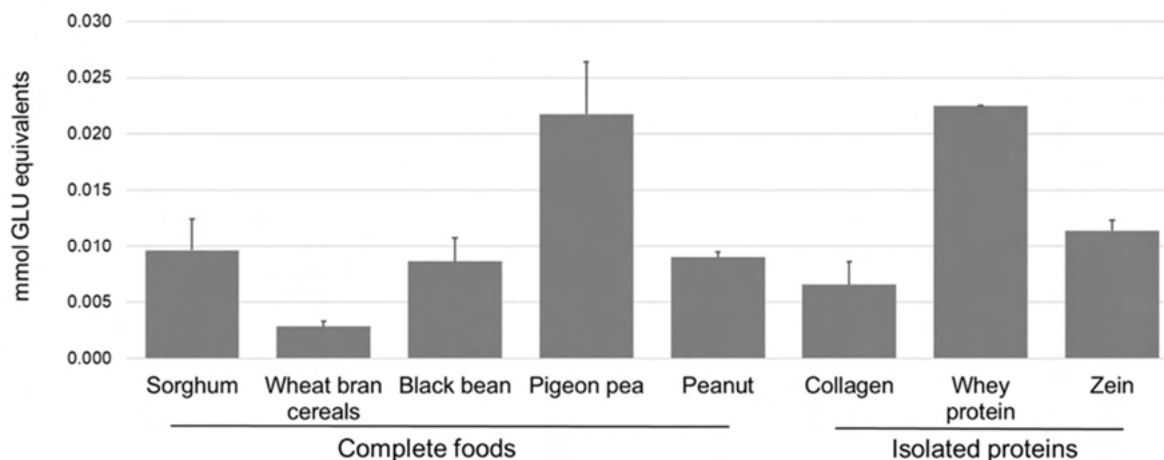

Figure 5. Absolute values of mmol glutamic acid equivalents corresponding to free amino groups quantified in the supernatant after IVD. Error bars indicate standard deviations of two measurements (each measurement was performed in duplicate).

4. Discussion

The eight protein sources were different in protein and EAA content as well as content of starch, fat and EAA/NEAA ratios (Table 1, Figure 1, supplemental Figure 2). Therefore, differences in protein hydrolysis and digestibility were expected.

4.1. Product characterization

Analysis of individual amino acids in the eight substrates showed that some of the products could be considered as incomplete proteins due to the low abundance or complete lack of one or more EAA. Protein sources of plant origin with the exception of some legumes are many times lacking one or more EAA, reducing the protein quality for human consumption (Young & Pellett, 1994). Sorghum, for example, was described to be rich in leucine and glutamine but poor in lysine and threonine (Awadalkareem et al., 2008; Salunkhe et al., 1977). Also, wheat protein was shown to be rich in glutamine and proline but poor in lysine, threonine, methionine, histidine and isoleucine (Khan et al., 2014; Xiao-ling et al., 2008). Proteins from legumes are described as having all the EAA (rich in lysine and leucine), as well as being rich in glutamine and asparagine, but they are often limited in sulfur amino acids (cysteine and methionine) (Duranti, 2006; Roy et al., 2010). The corn protein zein is due to the absence of lysine and due to the high leucine/isoleucine ratio, considered as a low nutritional quality

protein (Larkins et al., 1984). Our results showed high values for glutamine, leucine and proline in this protein, as other authors have stated earlier (Keith & Bell, 1988; Nehete et al., 2013). Peanuts were shown to be low in threonine and in sulfur amino acids, but high in arginine, asparagine and glutamine (Davis & Dean, 2016; Settaluri et al., 2012). Proteins from animal sources are often considered proteins of high nutritional value; however, as seen in Figure 1, collagen is rich in NEAA but poor in cysteine, tyrosine, methionine, histidine and completely lacking tryptophan. It is therefore considered a poor-quality and incomplete protein (Paul et al., 2019). In contrast, whey proteins have a high nutritional value due to their favorable amino acid composition. Our results were in agreement with the literature describing whey protein as rich in glutamine, asparagine and BCAA (Lollo et al., 2012) (supplemental Figure 2).

4.2. Protein hydrolysis during IVD

Protein hydrolysis was monitored during and after IVD at the levels of intact proteins, peptides and release of free amino acids and small peptides. To do so, it was first necessary to identify the individual proteins present in the eight samples by peptide mass fingerprint (Figure 2). For the detection of undigested proteins in the different protein sources, soluble and insoluble fractions were analyzed by gel electrophoresis after oral, gastric and intestinal IVD (Figure 3, supplemental Figure 3). As expected, some proteins were resistant to gastric digestion, like β-lactoglobulin (Figure 3a, band b) in WPI, and phaseolin, phytohemagglutinin and alpha amylase inhibitor-1 in black beans (Figure 3b; bands c, d, and e, respectively). In the other substrates, resistant proteins were also identified after the gastric phase (supplemental Figure 3). When evaluating all eight sources after completion of the intestinal phase, no intact protein could be visually detected after the intestinal digestion in the soluble or the insoluble fraction. Additionally, the protein bands visible after 120 min of intestinal digestion were identified by mass spectrometry as proteins of the digestive enzymes present in the pancreatin.

4.3. Generation of peptides during IVD

As a next step in the hydrolysis process, the peptide patterns generated during IVD were analyzed (Figure 4, supplemental Figure 4). Interestingly, although the intact β-lactoglobulin band remained constant throughout the whole gastric phase, a high number of peptides from this protein were identified at the end of the gastric phase, indicating that it was not completely resistant to pepsin. In order to better understand the hydrolysis of this protein, a time-resolved IVD was performed (supplemental Figure 5), showing that the hydrolysis had started already at the beginning of the gastric phase. Surprisingly, the β-lactoglobulin band was no longer visible on gel at the beginning of the intestinal phase (Figure 3); in addition, only peptides from certain regions of this protein were identified at this time (supplemental Figure

5), leading to the conclusion that β-lactoglobulin hydrolysis must occur rapidly once it is in contact with the pancreatic proteases.

4.4. Generation of free amino acids and small peptides after IVD

Generation of free amino acids and small peptides were analyzed with the OPA method after protein precipitation with perchloric acid. These first results show that WPI and pigeon peas released the highest values of glutamic acid equivalents. These values were in agreement with calculated protein digestibility-corrected amino acid scores (PDCAAS) and *in vivo* DIAAS values taken from literature (Table 3). Other authors reported sorghum's low digestibility (Butler et al., 1984; Chung et al., 1998; Duodu et al., 2002; Duodu et al., 2003; Nguz et al., 1998), as associated tannins present in the cereal bind to grain proteins and make them less susceptible to enzymatic action. Protein crosslinking between γ- and β-kafirins was also reported as one factor that negatively affected the digestibility of this cereal. Wheat protein digestibility in humans was reported between 85% and 95% for true ileal digestibility (Flambeau et al., 2017). The OPA results that we obtained for wheat cereals were much lower than expected and were better reflected by the PDCAAS values reported by Schafsma et al. (2000) and the DIAAS values from Mathai (2018). The negative effect of dietary fiber on protein digestibility has been reported earlier (Dégen et al., 2007; Lenis et al., 1996; Schulze et al., 1994). Schulze and his colleagues have shown that increasing the dietary fiber content led to a decreased apparent ileal protein digestibility due to increased ileal losses of both endogenous and exogenous protein (Schulze et al., 1994). These findings can explain our results considering the high fiber content (~15 %) present in the digested wheat bran cereals. Legume seeds contain protease and amylase inhibitors as well as lectins. They are considered anti-nutritional factors due to their ability to inhibit various digestive enzymes, including trypsin, chymotrypsin and amylase, compromising protein digestibility (Boye et al., 2010; Bressani, 1993). Although their activity is reduced or even eliminated after cooking (Shi et al., 2017), this could explain our results obtained in black bean samples. True digestibility of peanuts was reported as being higher than whole wheat (Arya et al., 2016), which is in agreement with our higher OPA results obtained for peanut samples compared to wheat cereals. Although bovine collagen hydrolysates have a high *in vitro* N-ileal digestibility (99. 8%) (Bindari et al., 2018), under the same conditions, native collagen was resistant to the attack by gastrointestinal proteases of the gastrointestinal tract because of its triple helical structure (Harkness et al., 1978). Harkness and her colleagues have shown that after *in vitro* incubation with pepsin at 37 °C and at pH 1.5, followed by trypsin or chymotrypsin incubation, only 40 % of collagen was converted into dialyzable material. This study supports our results that show very few free amino groups generated during *in vitro* digestion (Harkness et al., 1978).

Table 3. PDCAAS and DIAAS values taken from the literature.

	Sorghum	**Wheat**	**Black beans**	**Pigeon peas**	**Peanuts**	**Collagen**	**Whey protein**	**Zein**
PDCAAS	0.35 (Taylor & Taylor, 2011)	0.46 (Schaafsma, 2000)	0.534 (Nosworthy et al., 2017)	0.643 (Nosworthy et al., 2017) (yellow peas)	0.51 (Rutherfurd et al., 2015)	0 (Phillips, 2016)	1 (Rutherfurd et al., 2015)	0.01 (Sarwar, 1997)
DIAAS (Mathai, 2018)	45	0	43	57	0	0	103	0

Recently, the ileal digestibility of zein and whey proteins were determined in healthy volunteers (Calvez et al. 2019). These authors reported a low zein digestibility in human trials, compared to the high digestibility of whey proteins. Our results point in the same direction, considering that the OPA might possibly also detect non-absorbed peptides that were not precipitated by the perchloric acid and therefore overestimating the digestibility of zein. Zein's low digestibility may be attributed to its poor solubility in water (Shukla & Cheryan, 2001).

Taken together, the results obtained by IVD are in agreement with reported values on digestibility (Calvez et al., 2019; Mathai, 2018). As a next step toward *in vitro* DIAAS values, individual amino acids after IVD will be analyzed and compared to the *in vivo* data and will show if IVD experiments can be a useful tool for the prediction of the digestibility of different protein sources.

5. Conclusion

The harmonized IVD protocol was applied to eight different protein sources and protein hydrolysis was analysed at the different levels from intact proteins, to peptides and total free amino acids. No intact proteins were visible after the intestinal phase, indicating that all tested proteins were at least in part hydrolysed by the digestive proteases. However, digestion-resistant peptides were detected in all the substrates at the end of the intestinal digestion step, indicating that the protein hydrolysis was not complete. The different amounts of free amino acids and small peptides released from the eight substrates by IVD correlated with *in vivo* digestibility data. Similar to the *in vivo* findings, the highest amino acid release after IVD was found for whey protein and pigeon peas. However, more validation experiments on other protein sources will further confirm the utility of IVD for digestibility predictions for novel protein sources.

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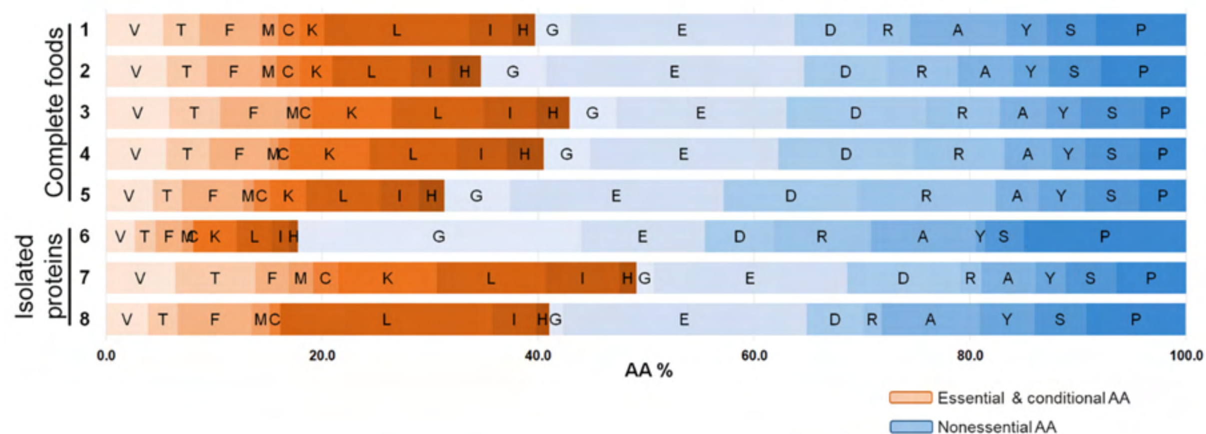
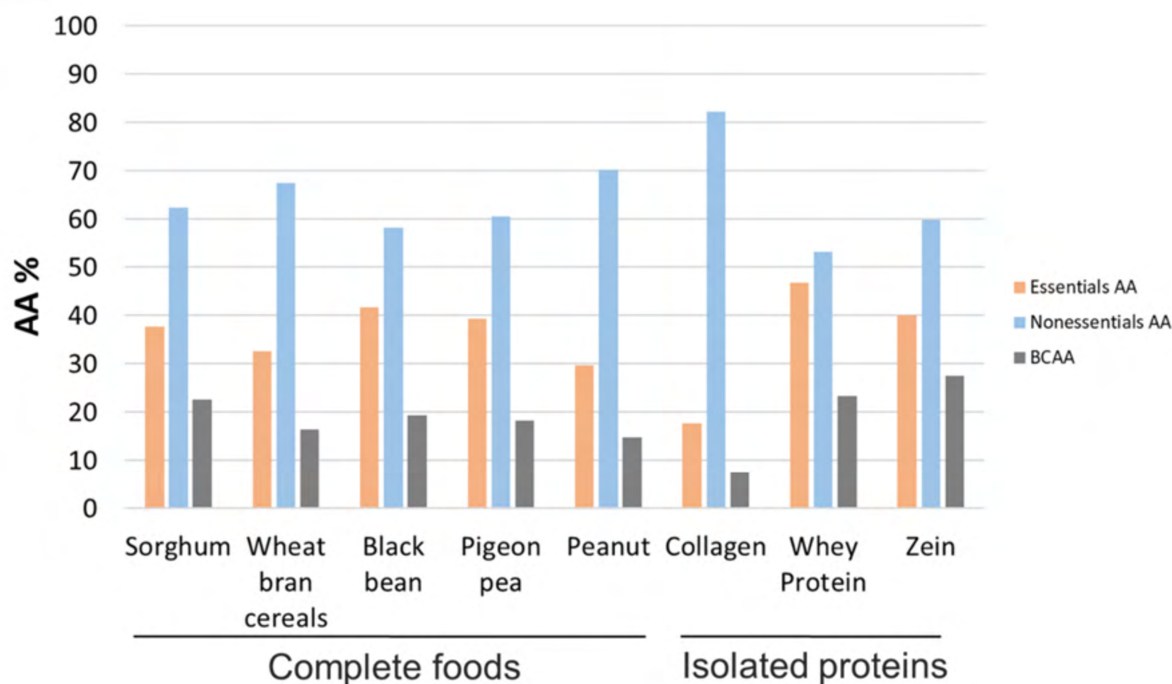
Supplementary material

Supplemental figure 1



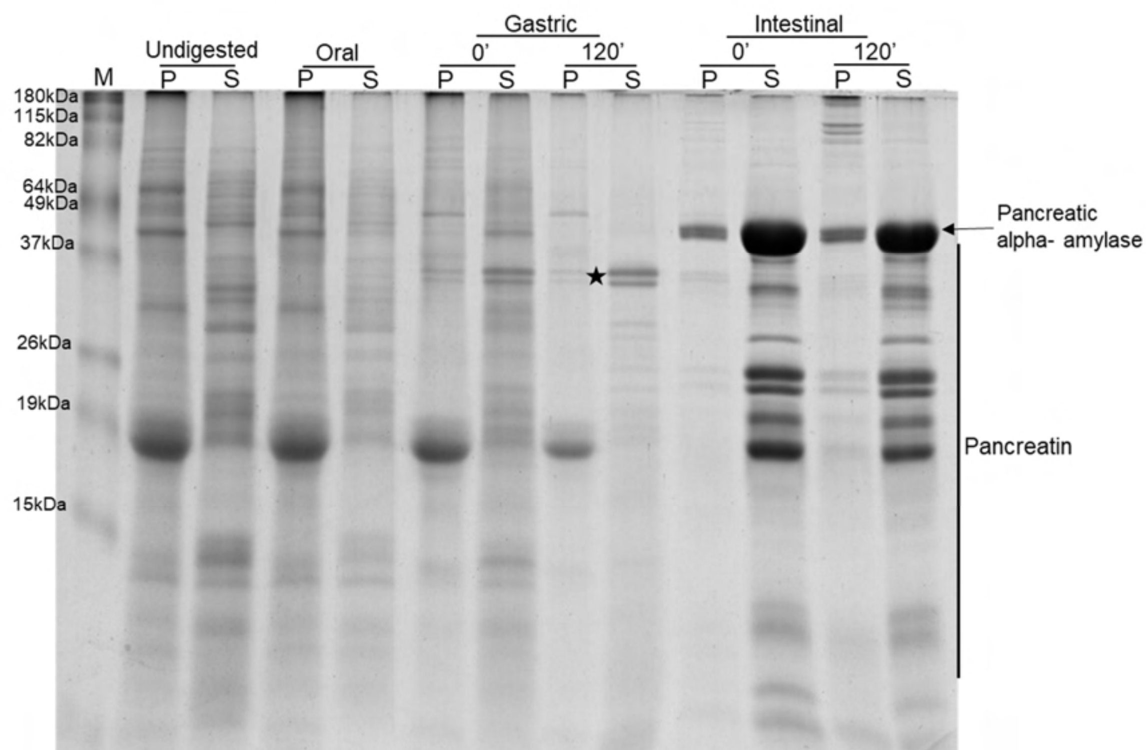
Suppl. figure 1. Relative amino acid distribution in % per g of protein in the product.

Supplemental figure 2

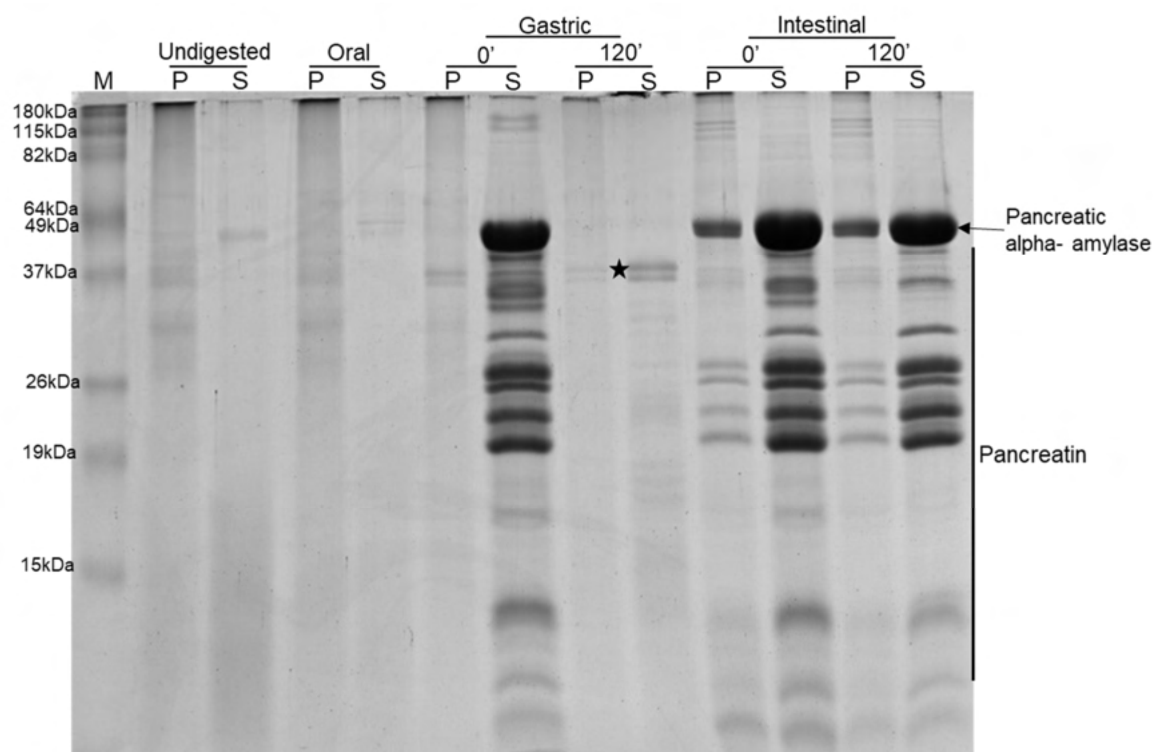


Suppl. figure 2. Relative distribution of essential, nonessential and branched chain (BC) amino acids in the different protein sources.

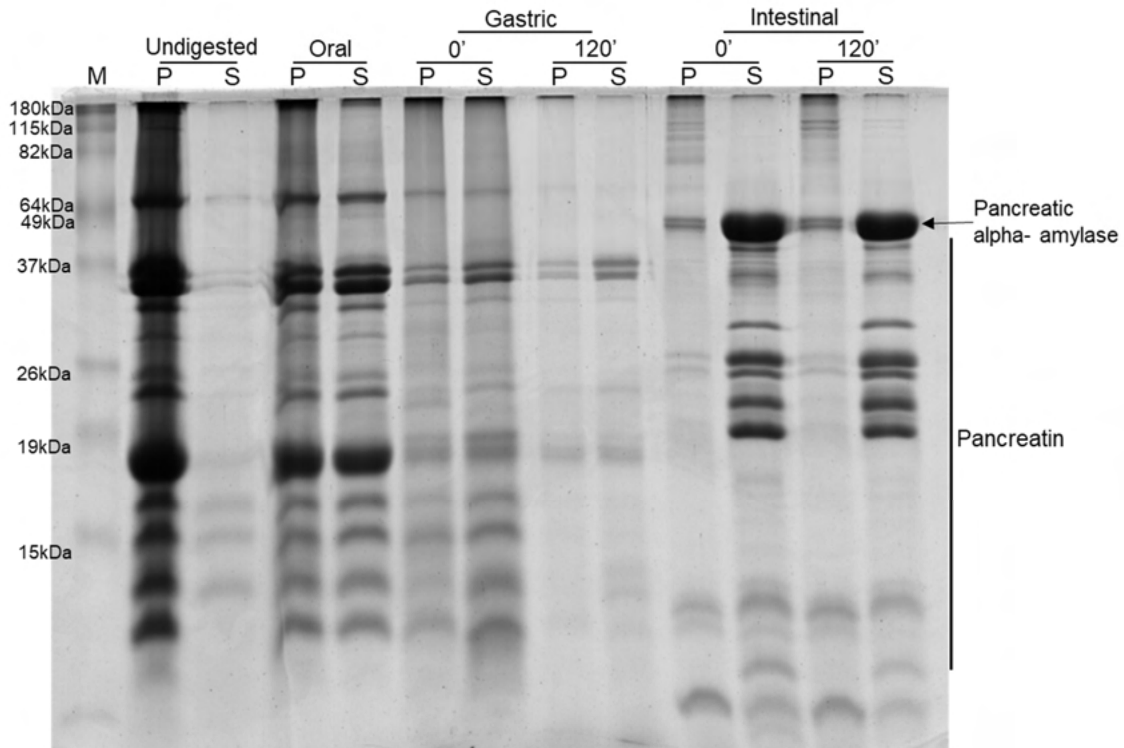
Supplemental figure 3



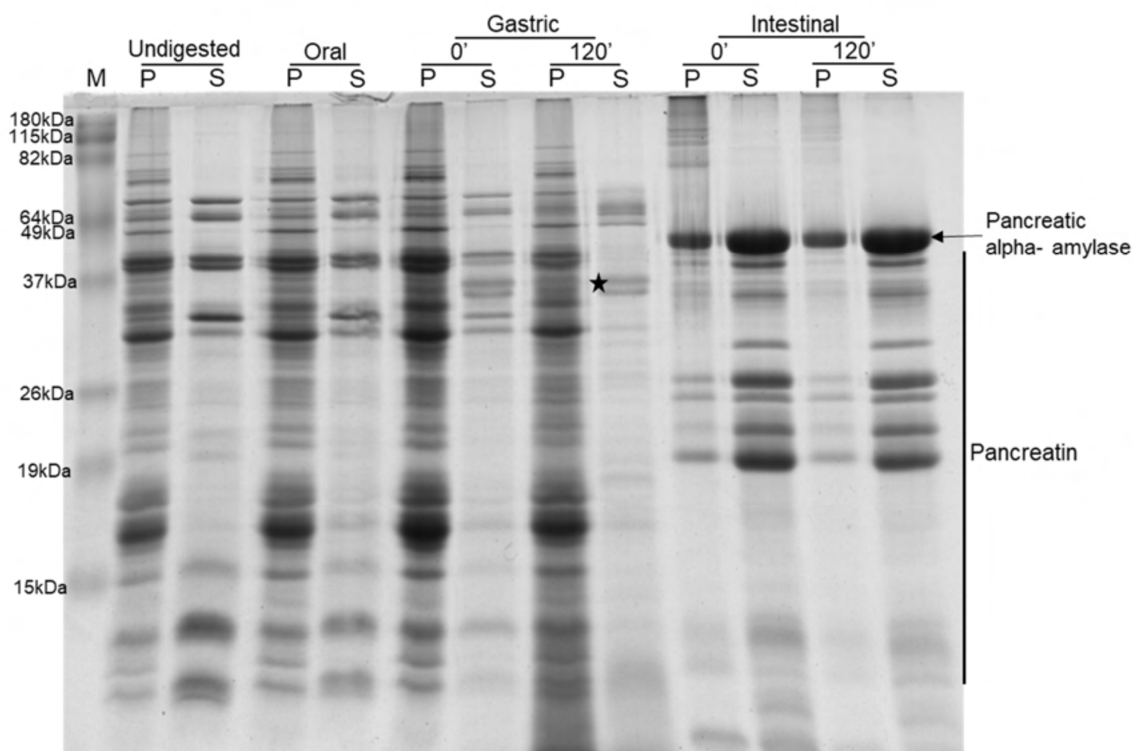
Suppl. figure 3a. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **sorghum**; asterisk: pepsin.



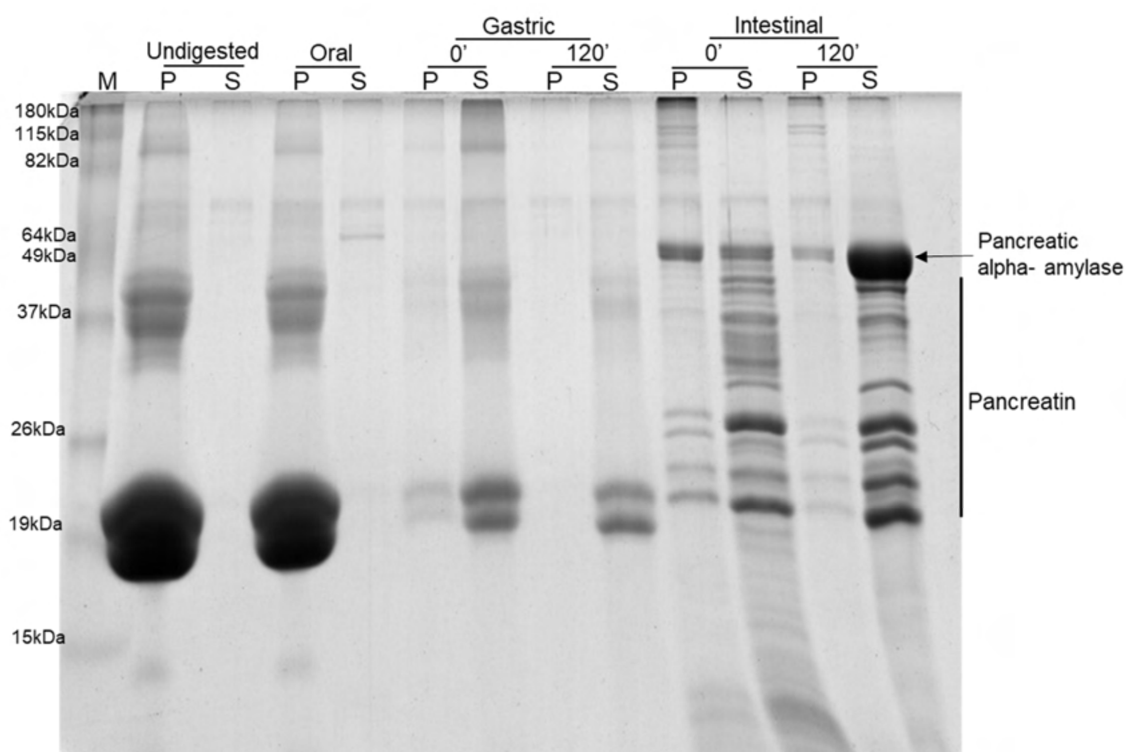
Suppl. figure 3b. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **wheat bran cereals**; asterisk: pepsin.



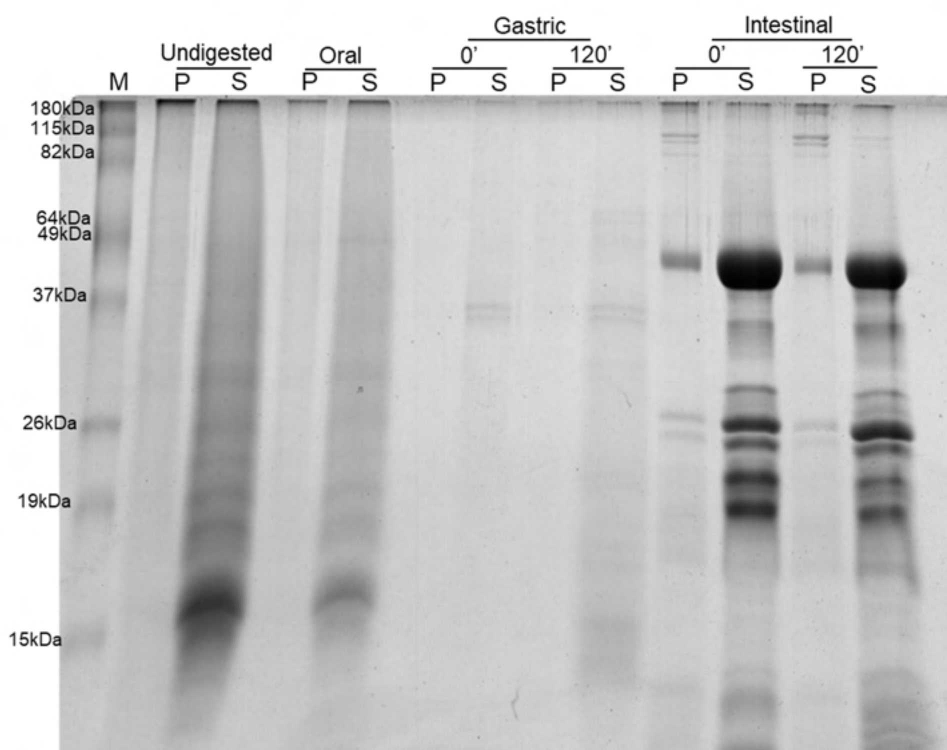
Suppl. figure 3c. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **pigeon peas.**



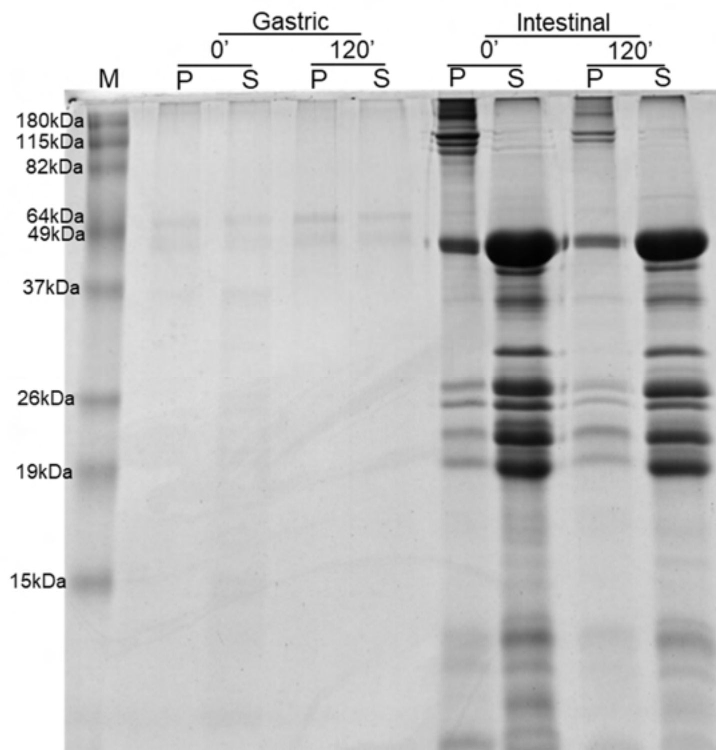
Suppl. figure 3d. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **peanuts**; asterisk: pepsin.



Suppl. figure 3e. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **zein.**

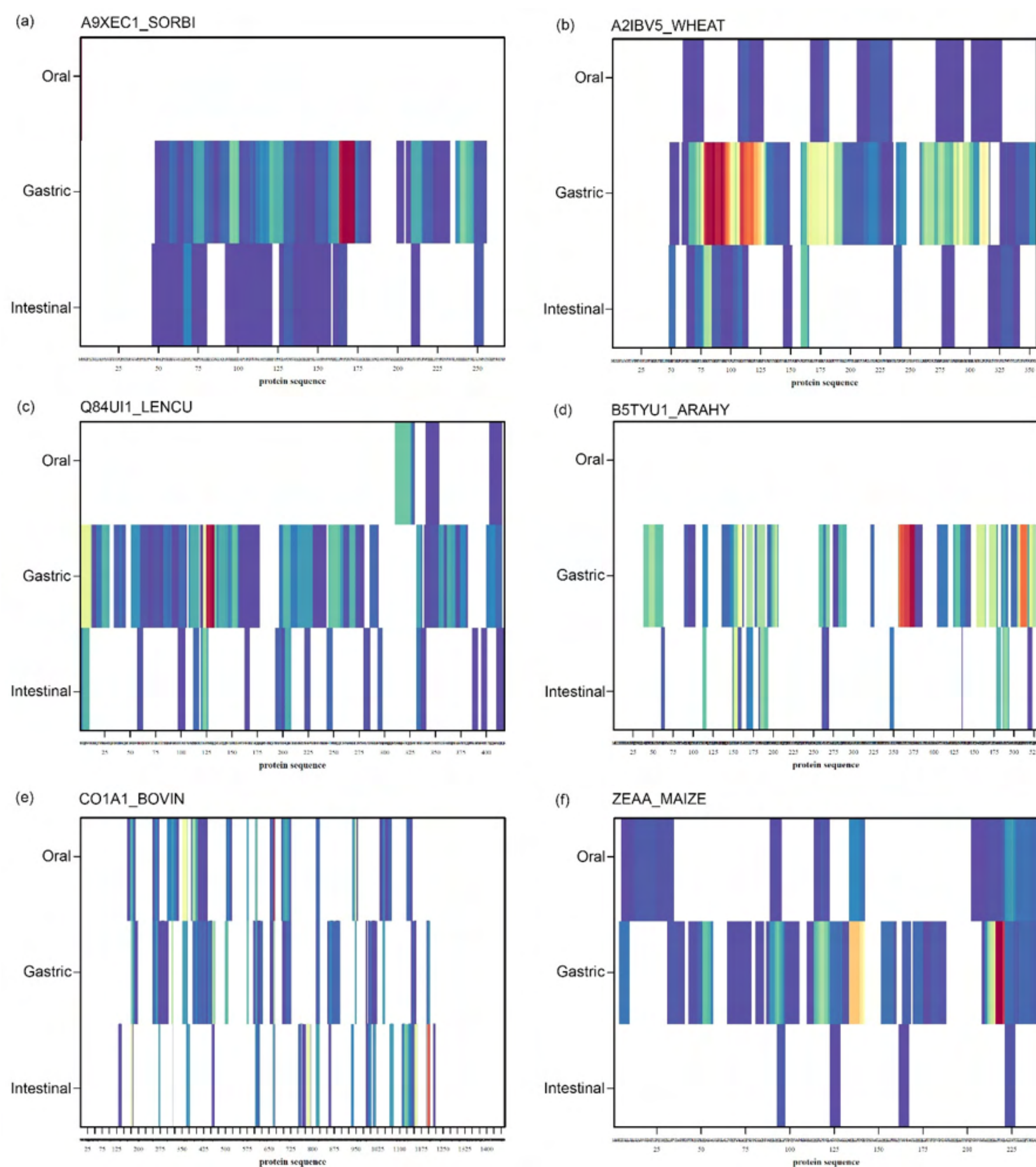


Suppl. figure 3f. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **collagen.**



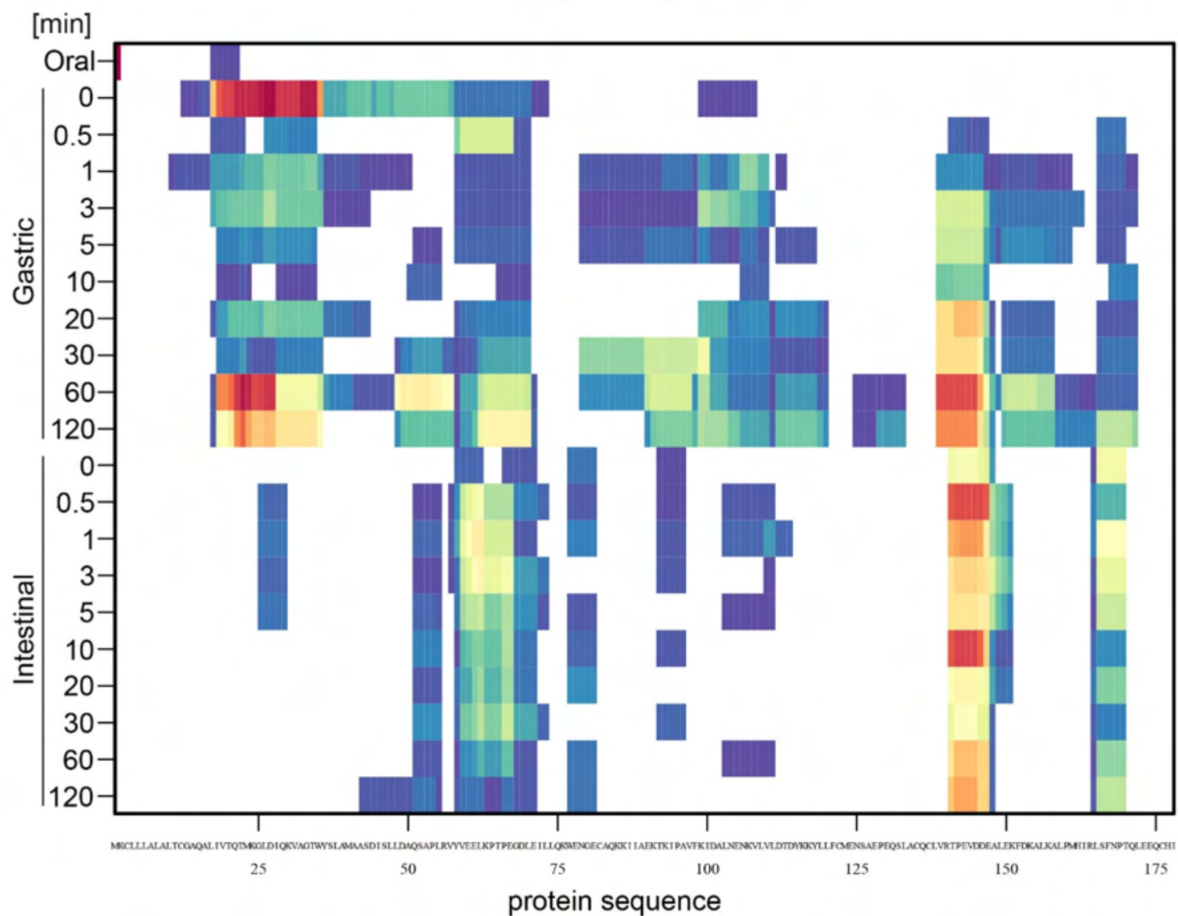
Suppl. figure 3g. SDS-PAGE of the supernatants (S) and pellets (P) of oral, gastric and intestinal phase from IVD of **the water blank**.

Supplemental figure 4



Suppl. figure 4. Peptide patterns from selected proteins of sorghum (a), wheat bran cereal (b), pigeon pea (c), peanut (d), collagen (e), and zein (f), respectively, after oral, gastric and intestinal IVD.

Supplemental figure 5



Suppl. figure 5. Time resolved IVD of α-lactoglobulin.

Chapter 2

Amendment of the INFOGEST *in vitro* digestion method and development of techniques to measure and calculate protein digestibility for the evaluation of protein quality and comparison with *in vivo* data

Manuscript 2

In vitro amino acid digestibility and *in vitro* DIAAS of protein sources established with the INFOGEST static protocol and validated using *in vivo* data

Raquel Sousa[1,2], Isidra Recio[2], Dominique Heimo[3], Sébastien Dubois[3], Paul J. Moughan[4], Suzanne Hodgkinson[4], Reto Portmann[1], and Lotti Egger[1]

[1]Agroscope, Schwarzenburgstrasse, 161, 3003 Bern, Switzerland
[2]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain
[3]Agroscope, Tioleyre 4, 1725 Posieux, Switzerland
[4]Riddet Institute, Massey University, Palmerston North, New Zealand

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Abstract

The FAO recommends the digestible indispensable amino acid score (DIAAS) to determine food protein quality. DIAAS, based on true ileal digestibility of individual indispensable amino acids and performed *in vivo* in humans or pigs, is the international gold standard, but these assays are costly and raise ethical concerns. Therefore, the FAO recommends the development of *in vitro* systems for amino acid (AA) digestibility assessment. The static INFOGEST *in vitro* digestion system, which gives robust and reproducible gastric and intestinal endpoints closely mimicking physiological digestion, is a good candidate for further development as a screening tool for assessing food protein digestibility and *in vitro* digestibility based DIAAS values. Here, food samples were digested *in vitro* using the static INFOGEST protocol and after MeOH precipitation, separated into an undigestible and digestible fraction. Total protein digestibility was determined and proxy DIAAS values calculated, either based on total nitrogen (Kjeldahl) analysis, or after acid hydrolysis based on total amino groups (*o*-phthalaldehyde method, OPA) or total amino acids (TAA; HPLC). Digestibilities of individual amino acids were also determined and *in vitro* digestibility based DIAAS calculated. Physiological relevance of the *in vitro* digestibility values was demonstrated by comparison with comparable *in vivo* true ileal digestibility values (mean values from humans and pigs) for WPI, zein, collagen, black beans, pigeon peas, and All-Bran® and from rats for peanuts. In general, the *in vitro* AA digestibility and *in vitro* DIAAS (correlation slope: 0.96, R^2: 0.89) values agreed well with *in vivo* values. A statistical method comparison between *in vitro* and *in vivo* values showed a mean bias of 1.2 % for total digestibility and 0.1 % for DIAA ratios. This *in vitro* digestibility protocol may be a complementary tool for screening and comparison of different protein sources.

Keywords: Digestibility; *in vitro* DIAAS; total amino acids; *in vitro* digestion; liquid chromatography-mass spectrometry; protein hydrolysis

Abbreviations:

AAA, aromatic amino acids, AA, amino acids; DIAAR, Digestible indispensable amino acid ratio; DIAAS, Digestible indispensable amino acid score, (lowest DIAAR); proxy *in vitro* DIAAR, DIAAR based on total digestibility; FAO, Food and Agriculture Organization of the United Nations; GLU, Glutamic acid; HPLC, High-performance liquid chromatography; IAA, indispensable amino acids; IVD, *In vitro* digestion; LC-MS, Liquid chromatography-mass spectrometry; MS, Mass spectrometry; OPA, *o*-phthalaldehyde; PDCAAS, protein digestibility-corrected amino acid scores; RT, Room temperature; SAA, sulphur containing amino acid; TAA, Total amino acids; UHPLC, Ultra-high-performance liquid chromatography; UV/VIS, Ultraviolet–visible; WPI, Whey protein isolate

1. Introduction

Digestibility is an important characteristic that partly determines the quality of a food protein. The nutritional quality of a protein depends on its amino acid composition, on the associated amino acid requirements, and on the digestibility of the amino acids in the upper gastrointestinal tract (Bessada et al., 2019; Havenaar et al., 2016). In the last three decades, the quality of dietary protein has been commonly evaluated using the Protein Digestibility Corrected Amino Acid Score (PDCAAS) method (FAO, 1991). Although the standardization of results has been significantly improved, this method estimates the bioavailability of all of the indispensable amino acids based on true fecal nitrogen digestibility, which can lead to overestimation or underestimation of the protein quality (Rutherfurd et al., 2014). This observation was further demonstrated by comparing DIAAS values with PDCAAS-like values based on the standardized total tract digestibility (STTD) of crude protein (CP) (Mathai et al., 2017).

In 2013, the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) introduced the Digestible Indispensable Amino Acid Score (DIAAS), which is based on the true ileal digestibility of each indispensable amino acid (FAO, 2013). Ileal digestibility is the most appropriate method for assessing AA digestibility because, unlike true fecal nitrogen digestibility, true ileal AA digestibility takes into account the dietary essential (indispensable) amino acids that are not absorbed in the ileum and are therefore lost in the colon via the activity of the intestinal flora (Schaafsma, 2012). Ideally, true ileal AA digestibility of foods should be determined in humans; for example, by sampling via a naso-ileal tube (Moughan & Wolfe, 2019). However, this approach is not compatible with the practical and ethical limits for routine studies; therefore, the current recommendation is to use ileum-fistulated growing pigs (Hodgkinson et al., 2002) or growing rats (Moughan & Wolfe, 2019) as animal models. The growing pig model has recently been validated in the PROTEOS project as a suitable *in vivo* model to establish human DIAAS values using substrates with expected variable digestibilities (Hodgkinson et al., 2022). Nevertheless, these invasive animal studies are also costly and raise ethical issues, especially for testing vegan food products. Therefore, the FAO/WHO (FAO, 2013) has recommended the development and validation of *in vitro* methods for predicting true ileal amino acid digestibility and bioavailability in humans. The feasibility of this strategy was demonstrated using a dynamic *in vitro* digestion (IVD) model (Havenaar et al., 2016). In 2014, a static model of *in vitro* digestion was developed by the COST Action INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014), with the aim of closely mimicking human physiology. This protocol was validated for its biological relevance with milk proteins fed to the growing pig model as the animal model (Egger et al., 2017), and by comparison with human jejunal effluents (Sanchón et al., 2018). Since its first publication, the INFOGEST protocol has been widely used to address multiple diet-related questions, such as

digestibility, bioavailability, release of bioactive compounds, and structural changes in food (Egger et al., 2017; Fernandes et al., 2020; Santos-Hernández et al., 2020).

The aim of the present work was to establish an *in vitro* workflow that would allow the determination of predicted true ileal AA digestibility and the calculation of *in vitro* DIAAS values using the INFOGEST static IVD protocol for seven protein sources for which *in vivo* AA digestibility values were available (PROTEOS project). The seven same protein sources were assessed for *in vitro* digestibility of individual AA, *in vitro* DIAAS values, and proxy *in vitro* DIAAS values. These protein sources included four foods (wheat bran cereal [All-Bran®], pigeon peas, black beans, and peanuts) and three isolated proteins (zein, whey protein isolated [WPI], and collagen) (Sousa et al., 2020).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and enzymes used in the present study were purchased from MERCK. The protein source origins are provided in Supplemental Table 1.

2.2. Sample preparation for in vitro digestion (IVD)

The seven protein sources (three isolated proteins [zein, WPI, and collagen] and four foods [peanuts, All-Bran® wheat bran cereal, pigeon peas, and black beans]; Supplemental Table 1) were prepared as previously described (Sousa et al., 2020) prior to IVD according to the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). Briefly, to simulate real ingested foods, 40 g of black beans and pigeon peas were soaked overnight (18 h), cooked (200 mL of water, 288 mg salt) for 20 min (black beans) or 10 min (pigeon peas), and ground. The All-Bran® and peanuts were ground before IVD, while collagen, WPI, and zein were used without further preparation. As a blank digestion, a protein-free cookie (Moughan et al., 2005), containing only fat and carbohydrates was digested in parallel to the test foods, as previously described (Sousa et al., 2020). The cookie was prepared from 40.8 g purified corn starch, 15.7 g sucrose, 4.9 g cellulose, 0.7 g baking powder, 0.5 g ground ginger, and 36.9 g margarine and baked at 175 °C in portions of ~35 g for 30 min. The protein sources were normalized according to their protein content, and 0.04 g of total protein per gram of food were used for *in vitro* digestion. The influence of other nutrients on *in vitro* digestion of protein was tested by mixing the collagen, WPI, and zein (normalized to 0.4 g protein) with different quantities of the ground cookie (0.1, 0.2, 0.25, 0.5 g) to simulate a meal composition, as previously described (Moughan et al., 2005).

2.3. Pancreatin suspension preparation

It was found that pancreatin formed a suspension with undissolved particles, resulting in non-reproducible measurements. Therefore, some modifications were made to the INFOGEST IVD protocol to improve the repeatability of the measurements. Trypsin activity was measured according to a previous protocol (Brodkorb et al., 2019) using a pancreatin suspension prepared as follows: Shortly before the digestion experiment (or for activity measurement), the pancreatin was first suspended by mixing in simulated intestinal fluid at a concentration of 100 U trypsin activity/mL of digest, then vortexed for 10 s, followed by ultrasound treatment (45 Hz, 130 W) at room temperature for 5 min. Thereafter, the suspension was centrifuged (2000 g, at RT, for 5 min), and the supernatant was transferred into a new tube, placed on ice, and immediately used for the digestion experiment (or for trypsin activity measurements).

2.4. In vitro digestion with the INFOGEST static model

Enzyme activities and bile concentrations were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). All substrates were digested *in vitro* using the INFOGEST protocol (Minekus et al., 2014) with the above described adaptation for pancreatin solubilization. In brief, the substrates were normalized to a protein content of 0.04 g, diluted to 1 mL with water, and then mixed with 1 mL simulated salivary fluid (pH 7, 37 °C) containing amylase (300 U/mL of digesta), for 2 min. A 2 mL volume of simulated gastric juice (pH 3, 37 °C) containing pepsin (2000 U/mL of digesta) was then added and the whole incubated at 37 °C for 120 min. A 4 mL volume of simulated intestinal juice (pH 7, 37 °C) containing pancreatin (100 U trypsin activity/mL of total digesta) and bile (10 mmol/L of total digesta) was then added and incubated at 37 °C for 120 min. The entire digestion was performed under constant gentle mixing on a rotating wheel. Digestion was stopped after 120 min of gastric digestion by increasing the pH to pH7 with NaOH (1 mol/L) and adding the protease inhibitor 4-(2 aminoethyl) benzensulfonylfluoride (AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland) after 120 min of the intestinal phase. All the samples were immediately snap frozen in liquid nitrogen. For each set of samples digested in each experiment, a protein-free enzyme blank (cookie) was digested in parallel (Figure 1).

2.5. Sample separation into digestible and indigestible fractions

After defrosting, the digested samples were separated into digestible and undigestible fractions by precipitation with MeOH (80 %) at -20 °C for 1 h and subsequent centrifugation (2000 g at 4 °C for 15 min). For both the foods and cookie blank, the supernatants (Fs, Cs) were collected in new tubes without taking the interface (a representative aliquot of the total), and the pellets (Fp, Cp) were washed twice with MeOH (100 %), centrifuged between the

washing steps (2000 g at 4 °C for 5 min), and then dried in a CentriVap (Labconco, Kansas City, Missouri USA) (Fig. 1). The volumes or weights of the total digests, the digests with added MeOH, and the dried pellets were monitored to allow the calculation of AA digestibility at the end of the experiment. The amino acids liberated from the protein-free cookie, corresponding to the enzyme background, were considered as the minimal amount; therefore, values found below the enzyme background (due to analytical bias) were set to zero.

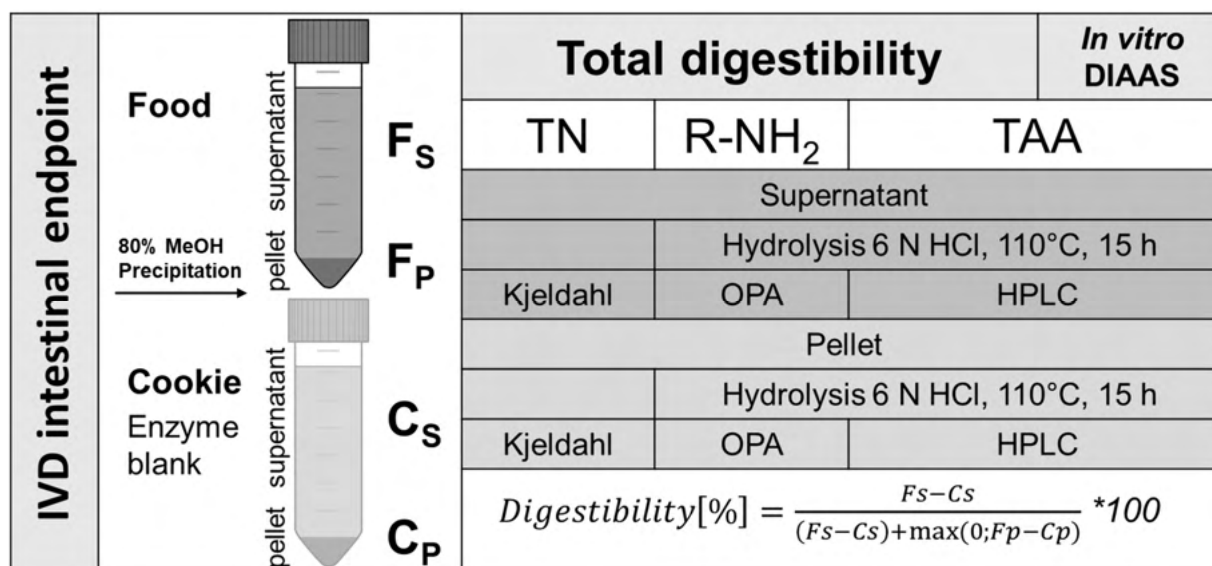


Figure 1. Sample preparation work flow. One protein-free cookie was digested in parallel with one or more foods in a set. After intestinal digestion, MeOH precipitation, and centrifugation, the samples were separated into supernatant (S) and pellet (P) and treated independently for analysis. Three different analytical endpoints were performed: Total N (TN) with Kjeldahl, total free primary amines ($R-NH_2$) with *o*-phthalaldehyde (OPA), and individual amino acids with HPLC. For OPA and HPLC, an acidic hydrolysis with 6 N HCl was performed for 15 h prior to analysis. Digestibilities were calculated with the same formula for all three methods. Fs = food supernatant, Fp = food pellet, Cs = cookie supernatant, Cp = cookie pellet

2.6. Size Exclusion Chromatography

The cut-off of the absorbable fraction after digestion was determined by high performance size exclusion chromatography (HPSEC), as described previously (Johns et al., 2011). Briefly, the HPSEC system was calibrated with 13 molecular mass markers (Supplemental Table 2). The non-precipitated digesta, supernatant, and the pellet resulting from the MeOH (80 %) precipitation were injected onto a Superdex Peptide 10/300 GL high performance gel filtration column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mobile phase was H_2O/ACN/TFA (700/300/1.00 (v/v)) at a flow rate of 0.35 mL/min. The

detection wavelength was 205 nm and the injection volumes were 10 µL for supernatant and pellet. The solid samples were resuspended in the mobile phase for injection.

2.7. Analysis of total nitrogen by Kjeldahl

The total nitrogen present in the pellet (P), and in the supernatant (S) after precipitation with MeOH 80 % was quantified using the Kjeldahl method, according to ISO 8968-3:2007/IDF 20-3: 2007 (ISO 8968-3, 2007). Solid pellet samples were quantitatively solubilized by addition of 2 mL of H_2SO_4 (96 %), followed by vigorous mixing for 1 min, subsequent addition of 2 mL of H_2O_2 and again followed by vigorous mixing until complete solubilization (Foods and Cookie: 1 min; WPI and Collagen 5-10 min; Zein 20 min). The addition of H_2O_2 leads to an exothermic reaction with foam production. In order to avoid sample loss, large vials were used.

2.8. Acid hydrolysis

Prior to the total amino acid (TAA) and total amino group (OPA) analyses, the samples were subjected to acid hydrolysis with 6 N HCl. Briefly, 220 µL of the supernatant was dried in glass vials in a CentriVap (Labconco, Kansas City, Missouri USA) and resuspended in 220 µL H_2O, 120 µL 3,3′-dithiodipropionic acid (DDP)/0.1 % NaOH (0.2 mol/L), 120 µL HCl (0.2 mol/L), 40 µL norvaline (NVa; 10 mmol/L), and 500 µL HCl (37 %). The whole digesta pellet was directly weighed into a vial and resuspended with 1760 µL H_2O, 960 µL DDP 0.1 %/NaOH (0.2 mol/L), 960 µL HCl (0.2 mol/L), 320 µL NVa (10 mmol/L) and 4 mL HCl (37 %). All the samples were placed in a 110 °C oven for 15 h.

2.9. Quantification of total amino groups (R-NH$_2$, OPA method)

The total amino groups (R-NH$_2$) in the supernatant and pellets of the precipitated samples after acid hydrolysis (2.8) were measured using the o-phthalaldehyde (OPA) method (Kopf-Bolanz et al., 2012). In brief, the samples were diluted 10 times with perchloric acid (0.5 mol/L) to precipitate proteins and longer peptides. After derivatization with OPA and in the presence of 2-mercapto-ethansulfonic acid, the resultant 1-alkylthio-2-alcylisonindol compounds were measured by UV/VIS photometry at 340 nm. The results were calculated based on a glutamic acid standard curve. A blank digestion (protein-free cookie) was used as the background.

2.10. Determination of individual amino acids in substrates

Sample preparation was based on a documented procedure (Waters, 2007). Briefly, hydrolyzed samples were obtained after 24 h of acid hydrolysis (6N HCl) at 110 °C. Then, 100 µL of the hydrolysate was evaporated to dryness and reconstituted with 20 mMol/L HCl, which contained α-aminobutyric acid (AAbA) as the internal standard. To convert the amino acids

into highly stable derivatives, 20 µL of the reconstituted sample was added to 60 µL of borate buffer and 20 µL of the derivatization reagent (6-aminoquinolyl-Nhydroxysuccinimidyl carbamate). The amino acid profile was analyzed using a Vanquish UHPLC system (Thermo Fisher Scientific, Reinach, Switzerland) equipped with an ultraviolet (UV) detector. Chromatographic separation was carried out on an AccQ-Tag Ultra analytical column (2.1 x 100 mm, 1.7 µm) (Waters, Baden, Switzerland) using the mobile phase and gradient described by Waters. Chromatographic conditions were: a flow rate of 0.7 mL/min, an injection volume of 0.5 µL a column temperature of 55 °C and and UV chromatograms were recorded at 260 nm. All analyses were done with at least two technical replicates. Cysteine and methionine were oxidized with perchloric acid before hydrolysis. Tryptophan content was quantified by HPLC (LC 1290 Infinity II LC System, Agilent Technologies, USA) after alkaline hydrolysis, according to ISO 13904.

2.11. Determination of individual amino acids of in vitro digesta

The TAA in the *in vitro* intestinal digests were analyzed with the adapted AOAC method 2018.06 for infant formula (Jaudzems et al., 2019). Briefly, after acid hydrolysis (Section 2.8), all the samples were derivatized with AccQ-Tag Ultra reagent (Waters, 2007), and the amino acid profile was determined by ultra-high-performance liquid chromatography (UHPLC) (Acquity UPLC BEH C18 2.1 × 150 mm, 1.7 µm, Waters) coupled with a UV detector (Vanquish, Thermo Scientific, Reinach, Switzerland). The UHPLC conditions were as follows: 2 µL injection volume, column temperature of 50 °C, UV detection at 260 nm, and a flow rate of 0.4 mL/min.

2.12. In vitro total digestibility, DIAAR, DIAAS, and proxy DIAAS calculation

Total digestibilities of the *in vitro* digested substrates were calculated using the formula shown in Fig. 1, by calculating the total amounts of N, R-NH_2, or AA in the supernatant and pellets and accounting for all dilution steps performed during the analysis. The amino acids in the supernatant and pellet of the protein-free cookie, corresponding to the enzyme background, were subtracted from the fractions of the food digests to account for the autolysis of the digestive enzymes. In addition, the amino acids from the protein-free cookie digest were set as the minimal amount; therefore, values below the enzyme background (due to analytical bias) were set to zero. The resulting digestible (supernatant) part was then divided by the total (supernatant + pellet).

The digestible indispensable amino acid (DIAA) per gram of food was calculated for each IAA by multiplying the mg of each indispensable amino acid per g of food protein by the respective digestibility of each indispensable amino acid obtained in the *in vitro* digestion. The DIAAR (%) = 100 × (mg of *in vitro* digestible dietary IAA in 1 g of the dietary protein) / (mg of

the same dietary IAA in 1 g of the reference protein (FAO, 2013). The DIAAS of a food corresponds to the lowest DIAA ratio (DIAAR).

Proxy *in vitro* DIAAR values were determined by using the total *in vitro* ileal digestibility obtained by TN, OPA, or TAA analysis (Formula Fig. 1), instead of the standardized total tract digestibility (%). As a reference protein, the FAO recommendation for preschool children (6 month to 3 years) was considered (FAO, 2013), and no truncation was applied for values higher than 100 %. For each indispensable amino acid, the digestible indispensable amino acid (DIAA) per gram of food was calculated by multiplying the mg of indispensable amino acid per g of food protein by the total ileal digestibility value obtained in the *in vitro* digestion. The proxy *in vitro* DIAAR (%) = total digestibility (obtained with TN, OPA, or TAA, according to Fig. 1) × mg of *in vitro* digestible dietary IAA in 1 g of the dietary protein / mg of the same dietary IAA in 1 g of the reference protein (FAO, 2013).

3. Results

3.1. Characterization of the bioavailable fraction and enzyme background by size exclusion chromatography

The calculation of food AA digestibility required definition of the bioavailable fraction of the tested food after completion of IVD and separation of this fraction from the background enzymes and the undigested AA. The background enzyme material was determined by digesting the protein-free cookie alone and in parallel with the food sources (see Figure 1). The cookie digest was preferred over a water blank digest because previous experiments with water had shown a high autolysis of enzymes, which led to an underestimation of food digestion (data not shown). After the intestinal phase of IVD, the digesta from the food or cookie were precipitated with MeOH (80 %), centrifuged, and separated into a supernatant (S) containing free amino acids and short peptides and a pellet (P) containing longer peptides and proteins. The peptide size distribution in both fractions was determined using SEC. Standard peptides of known size (Figure 2a, Supplemental Table 2) were injected in parallel to verify that the estimated size of peptides in the supernatant of the digesta was maximally 1000 Da, which was considered to be the absorbable peptide fraction. This limit was approximately between angiotensin with a molecular weight of 1046 Da and the peptide D-Ala^2-deltrophin with a molecular weight of 783 Da, corresponding to a SEC column elution time of 40 min that was estimated with the equation based on the standards (Figure 2b).

Analysis of the supernatant (S) and pellets (P) of the intestinal digests showed that the peaks present in the supernatant of the food digests could be almost completely attributed to peptides < 1000 Da (Figure 2c; the black line indicates the 40 min corresponding to 1000 Da cutoff), whereas the pellet contained mostly fragments with molecular weights > 1000 Da

(Figure 2c). The SEC chromatogram of the cookie digest (Figure 2c, cookie) is shown as a baseline for the digestive enzymes. An additional water digest confirmed that the main peak in the chromatogram of the cookie supernatant (Figure 2c, cookie) was not from proteins of the digestive enzymes, because it was absent in the supernatant of the water digest (Suppl. Figure 1).

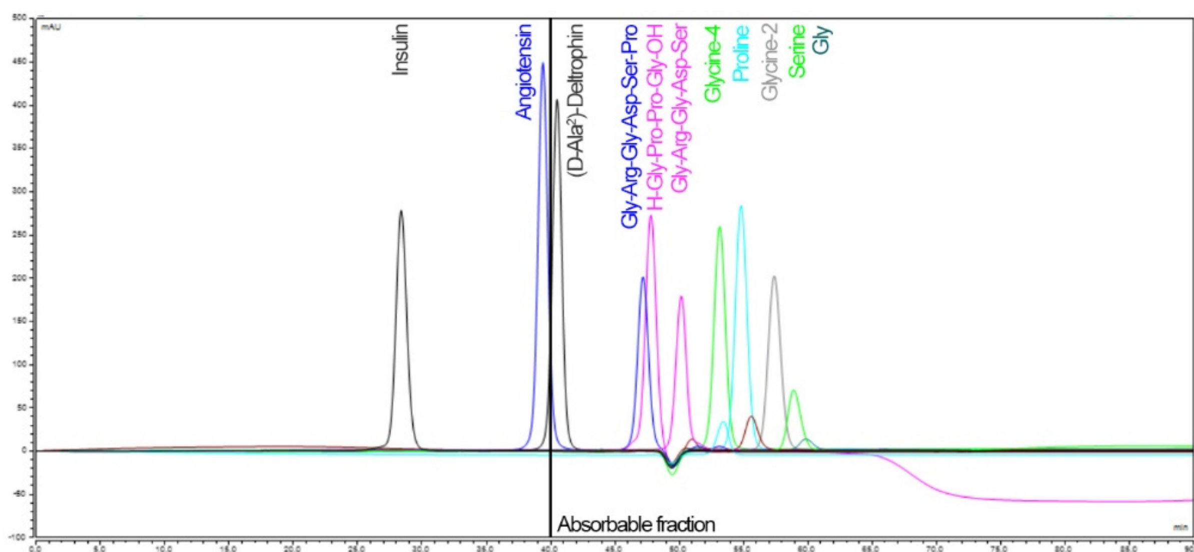


Figure 2a. Size exclusion chromatogram of standard peptides (listed in supplemental table 2) used for the estimation of peptide size distribution in the absorbable fraction after *in vitro* digestion. A line corresponding to a cutoff of approximatively 1000 Da (between 782.88 Da (D-Ala^2)-Deltrophin and 1046.18 Da (Angiotensin)) was set arbitrarily, corresponding to 40 min of elution time.

3.2. Digestibility of protein and individual amino acids

For the AA and protein digestibility calculations, supernatants and pellets from MeOH precipitated intestinal digests were analyzed using three different analytical approaches: total nitrogen (TN) by Kjeldahl, total primary amines (R-NH2) by OPA after acid hydrolysis, and AA by HPLC after acid hydrolysis. For all three analytical endpoints, total digestibility was calculated using the formula given in Figure 1 and described in section 2.11. The *in vitro* digestibility results were compared with *in vivo* digestibility data obtained from growing pigs cannulated at the terminal ileum (T-cannula) and from adult human ileostomates (PROTEOS project), for the same protein sources from the same batches of material (Hodgkinson et al., 2022). In the PROTEOS study, there was no statistically significant effect of species, so the *in vivo* values are means of the human and pig data (Hodgkinson et al., 2022). Digestibility of peanut protein was not reported in the PROTEOS *in vivo* study because of a high variability in digestibility. Therefore instead, AA digestibility and DIAAS values established in a rat ileal

digestibility model were taken for *in vivo* comparison of the peanut data (Rutherfurd et al., 2014).

Among the substrates, WPI, zein, and collagen, with different expected AA digestibilities, had been chosen for the *in vivo* experiments. No differences in digestibility were observed *in vitro*, as all three substrates were highly digestible when digested as pure proteins (Figure 3 a, bars 0 g of cookie). However, *in vivo*, these substrates had not been given alone but had been combined with other macronutrients, so the final protein concentration had been only 10 % of the total food fed to the subjects. In order to mimic a whole meal, the pigs were also fed additional fat, carbohydrates (starch and sugars), vitamins, and minerals in their diet, while the human subjects had received 25 g of proteins and a protein-free cookie (Moughan et al., 2005). Therefore, in the present study, a better simulation of the *in vivo* digestion of pure proteins was provided by digesting the three isolated protein powders (0.04 g protein) *in vitro* together with different amounts of the same protein-free cookie used for the enzyme blanks, to simulate a whole meal (Figure 3 a). In the “meal” (0.04 g protein), 0.25 g of cookie were added, such that, 11 % of the calories originated from proteins, 39 % from carbohydrates, and 50 % from fat (Supplemental Figure 3).

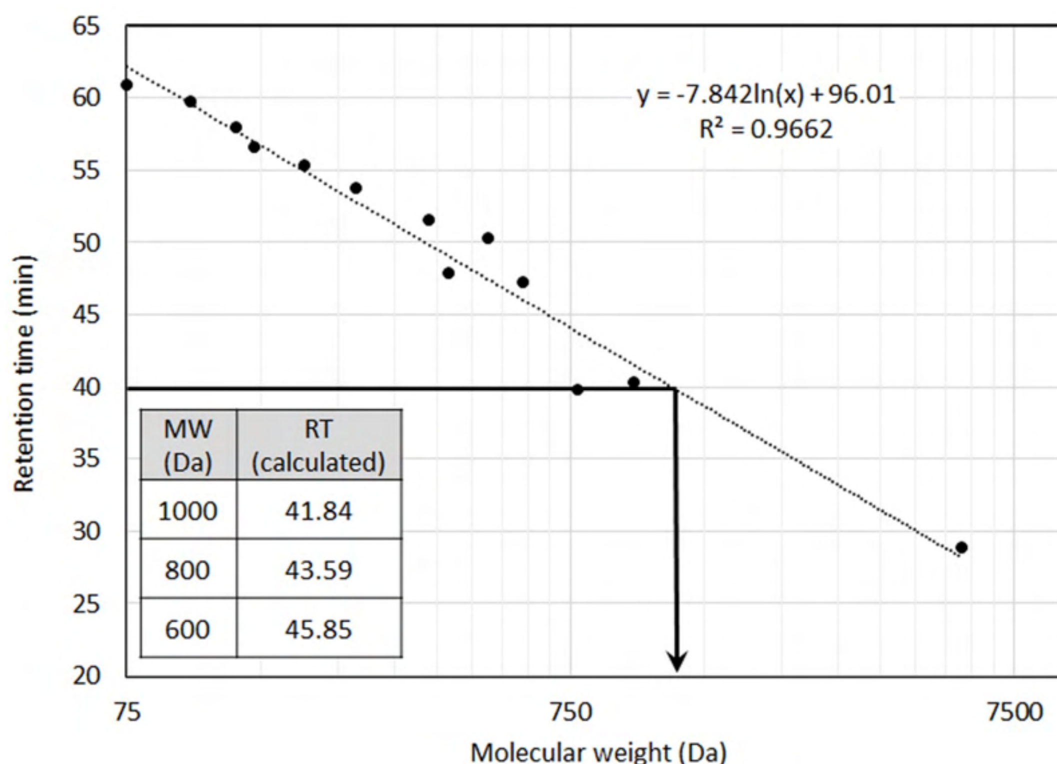


Figure 2b. Relation between retention time and the molecular weights of protein standards (Supplemental Table 2) analyzed by size exclusion chromatography. The retention times for 600, 800, and 1000 Da were calculated using the formula describing the best relationship. The arrow at a retention time of 40 min indicates the estimated limit for absorbable fragments of

approximately 1000 Da. The x-axis in logarithmic scale shows the molecular weight, and the y-axis shows the retention time.

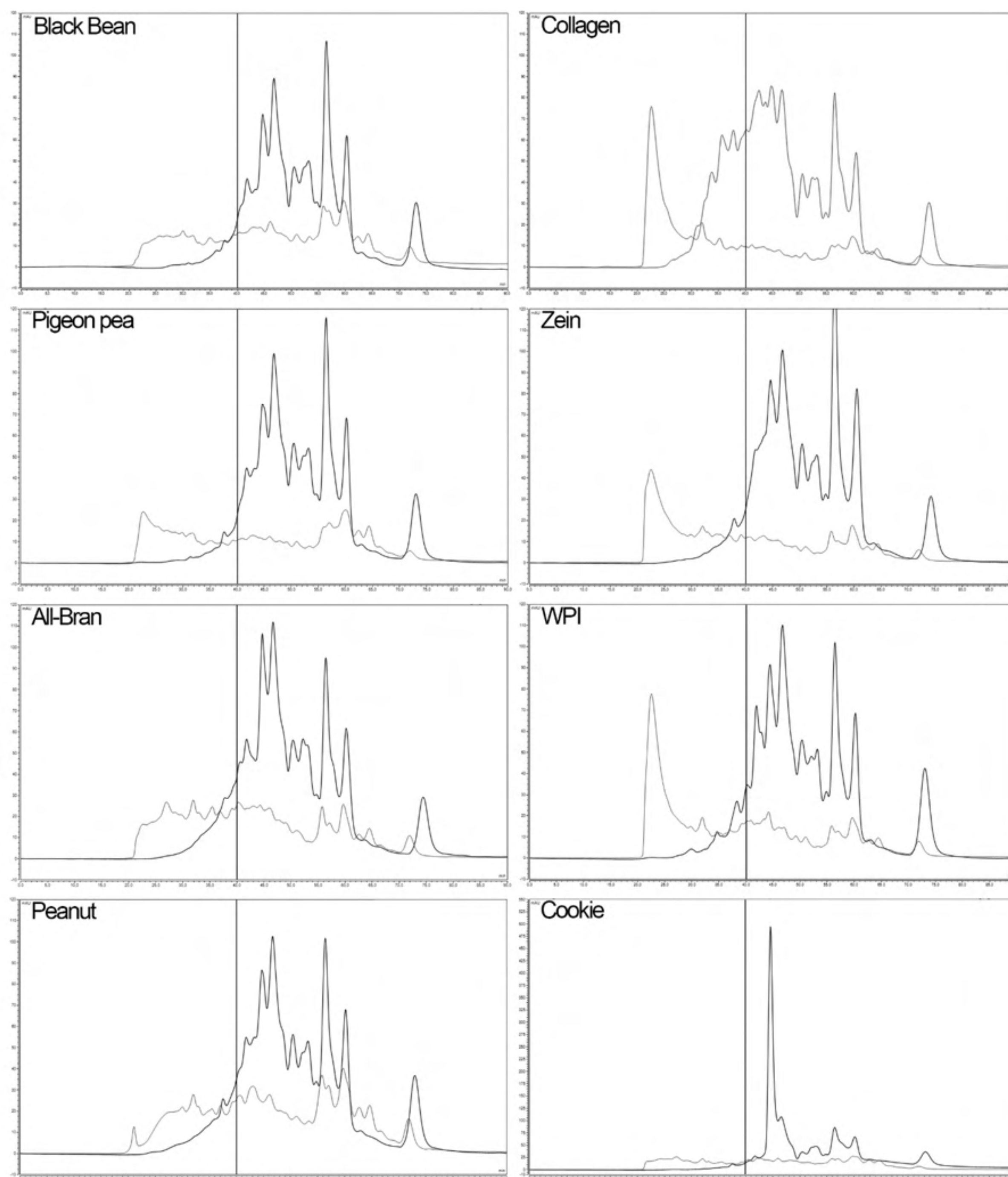
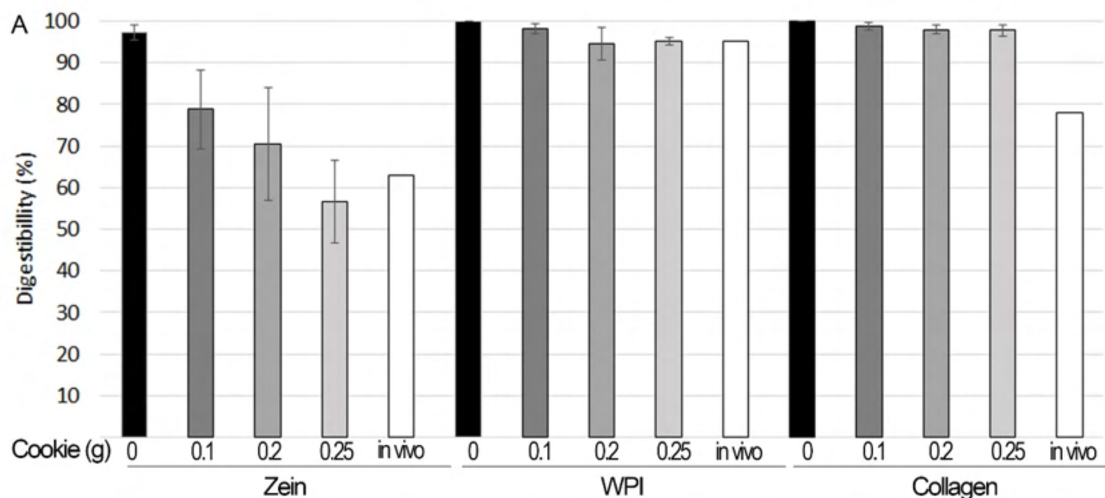


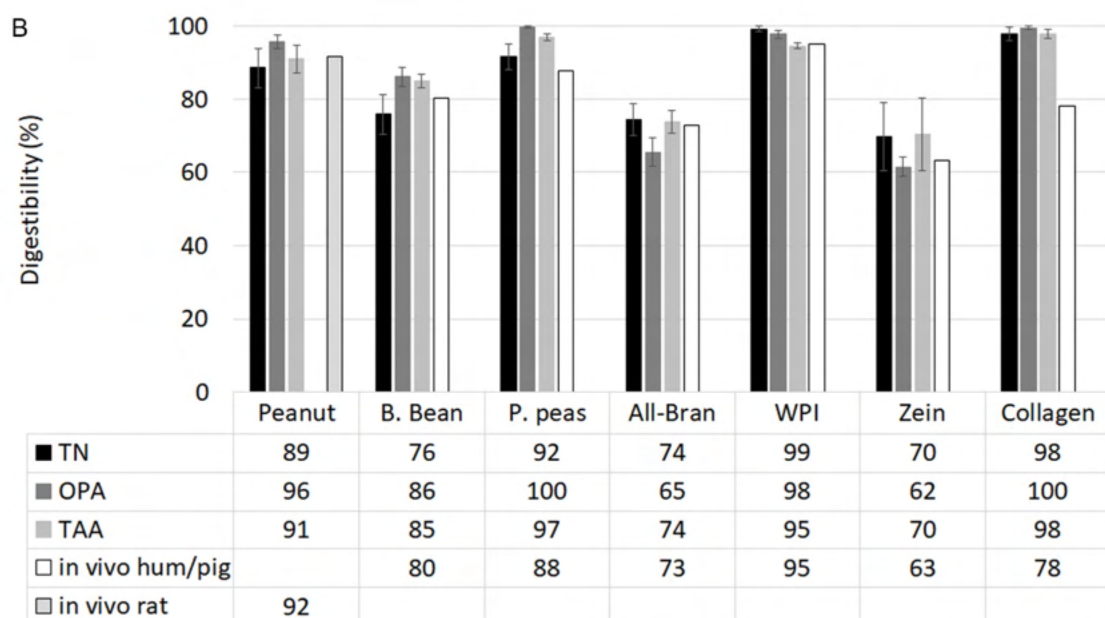
Figure 2c. Size exclusion chromatograms of the supernatants after intestinal IVD of the seven protein substrates and the cookie. The black line indicates the estimated cutoff at 40 min corresponding to a molecular weight between approximatively 800 and 1000 Da. The gray chromatogram corresponds to the pellet, and black corresponds to the supernatant.

The results clearly showed a decrease in the *in vitro* total AA digestibility of zein, a poorly soluble protein with a low *in vivo* digestibility with increasing amounts of cookie in the digestion. By contrast, digestibility of WPI and collagen, which were both highly digestible *in vivo,* remained high. Therefore, for subsequent experiments, the isolated proteins (0.04 g) were combined with 0.25 g of protein-free cookie.

The *in vitro* digestibility of TN, R-NH_2 and TAA was subsequently calculated for all the foods, and yielded results that were generally in line with the respective *in vivo* digestibilities obtained in the PROTEOS project in humans and pigs (Figure 3 b) (Hodgkinson et al., 2022). In addition, the results for analysis of TAA by HPLC were used to calculate the digestibilities for all individual amino acids for each substrate, according to the formula in Figure 1; the *in vitro* digestibilities are given in Table 3. For each substrate, *in vitro* values were compared to the *in vivo* mean of the human and pig values (or rats for peanut, respectively) (see methods) and are compiled in Supplemental Figure 2. No *in vivo* digestibility data from humans and pigs were available for glycine, and proline.

A method comparison (Bland & Altman, 1986) was also performed to show the average *in vitro* and *in vivo* digestibilities (x-axis) versus the differences between *in vitro* and *in vivo* digestibilities (y-axis) for each individual amino acid for the seven substrates (Figure 3 c). The *in vitro* digestibilities were compared with data from humans and pigs (average digestibility) (Hodgkinson et al., 2022) or rats (triangles) (Rutherfurd et al., 2014). The mean bias between methods was 1.2 % and the upper and lower limits indicated ±2 * standard deviations of the average difference between methods (Figure 3 c).





	Peanut	B. Bean	P. peas	All-Bran	WPI	Zein	Collagen
TN	89	76	92	74	99	70	98
OPA	96	86	100	65	98	62	100
TAA	91	85	97	74	95	70	98
in vivo hum/pig		80	88	73	95	63	78
in vivo rat	92						

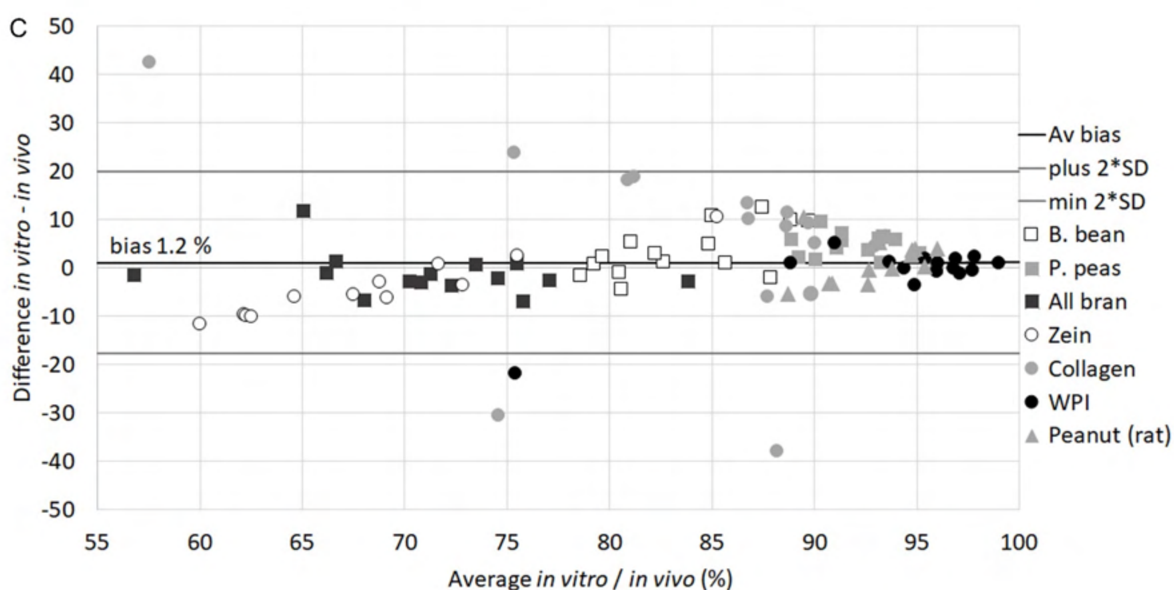


Figure 3. Total Protein digestibility of the pure protein sources (zein, whey protein isolate [WPI], and collagen; 0.04 g protein) in the absence (0) or presence of increasing amounts (0.1, 0.2, 0.25 g) of protein-free cookie. All samples were analyzed in at least three independent experiments, and error bars represent the standard error of the mean (SEM). *In vivo*: mean digestibility in humans and pigs (Hodgkinson et al., 2022) (A). Total protein digestibility was calculated based on released TN (Kjeldahl), R-NH_2 (o-phthalaldehyde [OPA] method), and total amino acids (TAA; HPLC) and the values shown are the means of the three methods. The *in vitro* results were compared with *in vivo* (mean human and pig values) results from the PROTEOS study (Hodgkinson et al., 2022) and rat values for peanuts (Rutherfurd et al., 2014). All *in vitro* samples were analyzed in at least three independent experiments, and the error bars represent SEM (B). Method comparisons between *in vitro* and *in vivo* results were performed on foods and isolated proteins + 0.25 g of cookie, according to previous work (Bland & Altman, 1986) and show the average digestibility (x-axis) versus the differences in total digestibilities (y-axis) of all individual amino acids. The mean bias between methods was 1.2 %, and the upper and lower limits indicate ±2 * standard deviations of the average difference (C).

Table 3: *In vitro* digestibility of the individual amino acids. Pure protein substrates (zein, WPI, and collagen; 40 mg) were digested together with 0.25 g protein-free cookie.
* Amino acid is not present or present only in trace amounts.

Digestibility	N	HIS	SD	ILE	SD	LEU	SD	LYS	SD	CYS	SD	MET	SD	TYR	SD	PHE	SD	THR	SD	TRP	SD	VAL	SD	ALA	SD	ARG	SD	ASP	SD	GLU	SD	GLY	SD	PRO	SD	SER	SD
Peanut	3	**98**	3	**92**	7	**88**	11	**100**	0	**100**	0	**87**	12	**96**	7	**89**	9	**100**	0	**100**	0	**99**	2	**94**	6	**85**	10	**97**	2	**83**	11	**99**	1	**91**	8	**99**	3
B. bean	3	**90**	3	**77**	2	**79**	3	**76**	1	**81**	0	**89**	2	**99**	1	**84**	3	**86**	5	**100**	0	**80**	4	**82**	5	**86**	3	**96**	7	**87**	2	**99**	2	**79**	4	**85**	8
P. pea	3	**100**	0	**91**	4	**92**	1	**94**	4	**100**	0	**95**	9	**100**	0	**96**	3	**99**	2	**100**	0	**95**	4	**97**	3	**96**	4	**100**	0	**98**	2	**100**	0	**98**	2	**99**	1
All-Bran	7	**74**	4	**68**	7	**69**	4	**55**	14	**67**	8	**75**	7	**72**	12	**76**	6	**68**	6	**61**	21	**68**	6	**65**	5	**69**	8	**77**	7	**81**	7	**64**	12	**79**	4	**70**	5
Zein	3	**69**	8	**59**	8	**48**	11	*		**85**	20	**78**	4	**26**	19	**72**	24	**52**	24	*		**62**	10	**52**	3	**63**	41	**96**	7	**52**	4	**100**	0	**45**	22	**66**	4
WPI	3	**99**	2	**95**	2	**97**	2	**97**	1	**79**	0	**91**	15	**53**	32	**100**	0	**96**	1	**100**	0	**95**	3	**97**	2	**96**	3	**97**	1	**94**	1	**100**	0	**97**	2	**90**	3
Collagen	3	**100**	0	**95**	8	**97**	5	**100**	0	**54**	19	**50**	18	**44**	0	**82**	32	**99**	2	*		**97**	5	**99**	1	**97**	2	**100**	0	**100**	0	**99**	1	**98**	1	**99**	0

3.3. Calculation of in vitro DIAAR values and comparison with in vivo DIAAR

The DIAAR and DIAAS values based on using the *in vitro* AA digestibilities were calculated based on the digestibility of each individual indispensable amino acid, the amount of that AA in the food and the reference requirement values for that AA for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). The *in vitro* values were compared to values based on *in vivo* true ileal digestibility data from pigs and humans (mean value across species, white bars) (Hodgkinson et al., 2022), or rats (peanut) (Rutherfurd et al., 2014) (Figure

4 a-g, Supplemental table 3). DIAAR values for the same foods but for the reference group of older children, adolescents, and adults are given in Supplemental Figure 4. The lowest *in vitro* and *in vivo* DIAA ratios for preschool children (6 month to 3 years) for each investigated substrate were in agreement between the different models, where available (*in vivo* SAA data are missing at this time point), and are listed in Table 4. A correlation graphic of *in vitro* (x-axis) versus *in vivo* (y-axis) digestibility based DIAAR values was calculated by comparing the *in vitro* data for all essential amino acids in each substrate with *in vivo* data (mean values from human and pig) or rat (triangles) data, yielding a slope of 0.96 and an R2 of 0.89. The DIAAR values obtained *in vitro* were also compared statistically with *in vivo* data for each essential amino acid for all the substrates and were represented as a Bland-Altman graph (Bland & Altman, 1986) (Figure 4 i). The average difference (*in vitro* – *in vivo*, bias) between the two methods was 0.1 %, indicating that the *in vitro* system slightly overestimated the *in vivo* DIAAR values. It has to be mentioned that at this point, the DIAA values, as well as the correlation and the statistical comparison for the sulphur containing amino acid cysteine no *in vivo* data were available, therefore SAA values could not be reported.

A 250 B. bean

200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL

B 250 Collagen

200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL

C 250 Pigeon Pea

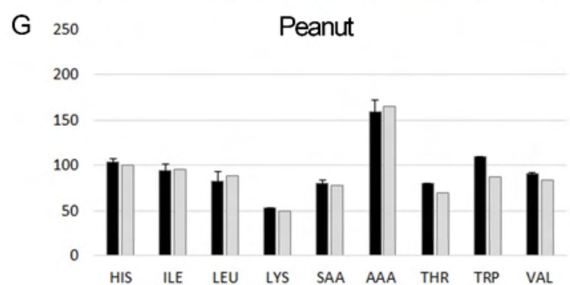
200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL

D 250 Zein

200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL



F 250 WPI

200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL

G 250 Peanut

200 150 100 50 0

HIS ILE LEU LYS SAA AAA THR TRP VAL

Fig. 4 A-G

in vitro

in vivo

in vivo (rat)

Fig. 4 H, I

B. bean

P. peas

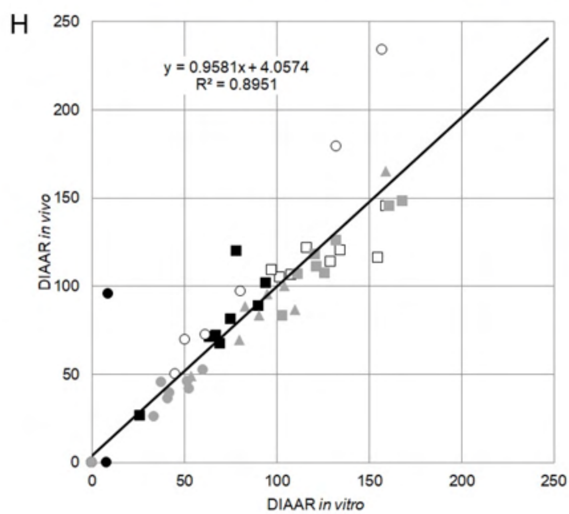
All-Bran

Peanut

Zein

Collagen

WPI



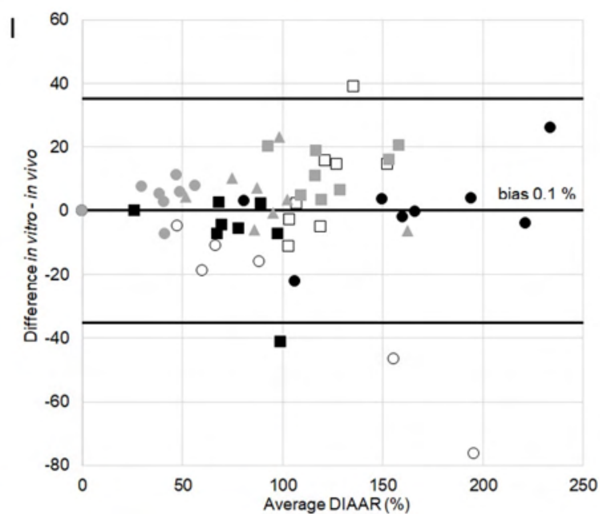


Figure 4. *In vitro* AA digestibility based DIAAR values compared to *in vivo* AA digestibility based data from pigs and humans (average values, white) for black bean, pigeon pea, All-Bran® wheat cereal, collagen, zein, and whey protein isolate (WPI) (Hodgkinson et al., 2022), or from rats for peanut (Rutherfurd et al., 2014). Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. Isolated proteins (collagen, zein, and WPI) were digested together with 0.25 g of a protein-free cookie to simulate a whole meal. Samples were analyzed at least in triplicate, and the error bars represent standard deviations (SD) (A-G). Correlation of *in vitro* DIAAR values with average in vivo data from pigs and humans for black bean, pigeon pea, All-Bran®, collagen, zein, and WPI or from rats for peanut (Rutherfurd et al., 2014) (G). Statistical comparison between *in vitro* and *in vivo* DIAAR results (Suzanne M. Hodgkinson et al., 2022), according to previous work (Bland & Altman, 1986), show the average DIAAR (x-axis) versus the differences between *in vitro* and *in vivo* DIAAR (y-axis) of all essential amino acids of the same comparisons, as described in Fig. 3c (H). The mean bias between methods was 0.2 % and upper and lower limits indicate ±2 * standard deviations of the average difference (I). Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. (H, I).

Table 4. Lowest *in vitro* and *in vivo* DIAA ratio (DIAAS) (Hodgkinson et al., 2022; Rutherfurd et al., 2014) for the seven investigated substrates for preschool children (6 month - 3 years), according to FAO (FAO, 2013). *In vivo* SAA data could not be calculated due to missing *in vivo* cysteine values. n.d.= not determined.

DIAAS	**Peanut**	**B. bean**	**P. peas**	**WPI**	**Zein**	**All-bran®**	**Collagen**
limiting AA	LYS	SAA	SAA	HIS	LYS	LYS	TRP
in vitro	54	72	67	82	0	26	0
in vivo Av		n.d.	n.d.	99	0	31	0
in vivo rat	49						

3.4. Calculation of proxy in vitro DIAAR and comparability with actual in vivo DIAAR

The proxy *in vitro* DIAAR values for the essential amino acids were calculated in a manner similar to that described by (Mathai et al., 2017) using the FAO reference protein for preschool children (FAO, 2013), but with an adaptation. Total ileal digestibility values from all three analytical approaches (TAA, R-NH_2 (OPA), and TN) were used instead of STTD, and no truncation to 100 % was applied (Supplemental table 4). *In vitro* DIAAR values and proxy *in vitro* DIAAS values were compared with *in vivo* data (Hodgkinson et al., 2022) (Supplemental Figure 5). Correlation lines between *in vivo* DIAAR (mean human and pig) (y-axis) versus *in*

vitro DIAAR (slope 0.96, R^2 0.89, white circles) or proxy *in vitro* DIAAR values, based on TAA (slope 1.02, R^2 0.95, black), OPA (slope 0.96, R^2 0.96, gray), or TN (slope 0.98, R^2 0.97, white) (x-axis), were calculated (Supplemental Figure 6).

4. Discussion

4.1. Definition of the bioavailable fraction

DIAAS values are based on true ileal AA digestibility which is usually determined *in vivo* by determining the fraction of the ingested food that remains in the terminal ileum after absorption of free amino acids and small peptides through the brush border cells. In humans, these values are obtained in clinical studies with ileostomy patients or via naso-ileal intubation (Moughan & Wolfe, 2019). Recently, the ileal cannulated pig model was validated within the PROTEOS project (Hodgkinson et al., 2022). With the *in vivo* methods, the bioavailable fraction is calculated by determining the total consumed proteins minus the part remaining at the terminal ileum corrected for gut endogenous protein. The addition of an indigestible marker gives the total fluxes, thereby enabling the calculation of digestibilities and DIAAS values. The static *in vitro* model used here lacks the absorption step, and thus all food components, both digested and undigested, remain in the reaction vessel. This model also lacks the brush border enzymes that are responsible for additional hydrolysis in the small intestine (Holmes & Lobley, 1989).

Therefore, one of the first goals for validation of the *in vitro* method for protein digestibility by comparison with *in vivo* data was to define and characterize the fraction that can be considered bioavailable after intestinal *in vitro* digestion of the food. This was done by SEC analysis of the *in vitro* digested samples at the end of the intestinal digestion. Standards of known sequence and molecular weights were correlated according to their retention time, and the resulting linear curve showed that a retention time of around 40 min corresponded to a molecular weight slightly above 1000 Da, which is the approximate size of angiotensin (8 amino acids in length). Comparison of the SEC standards and the SEC profiles of the supernatant and pellets of the intestinal digests after MeOH precipitation revealed that most of the peptides <1000 Da were in the supernatant. The size of the peptides absorbed *in vivo* is still debated and remains a matter of ongoing research (Ozorio et al., 2020; van der Wielen et al., 2017; Wang et al., 2019). Protein digestion in the gut lumen, resulting in the release of free amino acids is reported to be incomplete, representing only 20–30 % of the total nitrogen at the level of the ileum (Adibi & Mercer, 1973; Santos-Hernández et al., 2020). The major part is reportedly present as oligopeptides, which are further cleaved by the proteases of the brush border membrane (Tobey et al., 1985) prior to absorption. However, the amounts of oligopeptides that are processed and finally absorbed are not well known (Ozorio et al., 2020).

For the *in vitro* system, the supernatant after MeOH precipitation, which contained mainly peptides with fewer than 6–8 amino acids according to the SEC profile, was considered the bioavailable fraction, assuming that, *in vivo*, these peptides would be further cleaved by brush border enzymes and rendered bioaccessible. If substantial amounts of "limit peptides" are present in the digesta (e.g. heated foods), this assumption may not be valid.

An additional difficulty regarding the *in vitro* models is the quantification of the background protein, which consists of enzymes. In the *in vivo* experiments within the PROTEOS project, the pigs received a protein-free diet, while the human subjects were fed a protein-free cookie, and the AA's measured at the terminal ileum after ingestion of these products were considered to represent the baseline for endogenous material. In agreement with this, a protein-free cookie was digested *in vitro* in parallel and used as an enzyme blank for all foods. The digestion of a water blank was also tested but without any substrate. In the latter case, the hydrolysis of proteins (i.e., those present in pancreatin) and autolysis of digestive enzymes was higher, leading to an underestimation of digestibility compared to the *in vivo* data (data not shown).

4.2. Digestibility of proteins and individual amino acids

After the intestinal digestion, three different analytical approaches were performed and used to calculate the digestibilities of total proteins and individual amino acids. The *in vitro* digestibility based on TN, primary amines (OPA, R-NH_2), and TAA showed similar tendencies compared to the equivalent *in vivo* digestibilities. A trend to overestimate the total digestibility of pure proteins was evident, as shown in Fig. 3a, where pure proteins without the cookie had a digestibility of 100 %. For the *in vivo* experiments, the pure proteins were combined with diets containing fat, carbohydrates, sugars, and other nutrients as used in the pig trials and with a protein-free cookie as used in the human experiments (Hodgkinson et al., 2022). Consequently, this was simulated *in vitro* by adding the same protein-free cookie used as a baseline to the protein sources (Figure 3 a) to provide a closer simulation of the macronutrient composition of a complete meal (Supplemental Figure 3).

Interestingly, the digestibility of zein, a poorly soluble protein that has a significantly lower digestibility *in vivo* (Calvez et al., 2019; Hodgkinson et al., 2022), was clearly reduced with increasing amounts of cookie. This effect was much less pronounced for WPI and collagen (Figure 4 a), where digestibilities remained high even in the presence of increasing amounts of cookie. Therefore, the *in vitro* digestion of pure proteins or ingredients should always be measured in combination with other nutrients representing a food or even a whole meal, as recommended in the protocol (Brodkorb et al., 2019).

In vitro digestibility of collagen overestimated the *in vivo* digestibility to some extent. Earlier studies with rats showed that collagen had a digestibility of 95 % (Laser-Reuterswärd

et al., 1982). One possible explanation for the observed differences between *in vitro* and *in vivo* experiments could be protein solubilization during digestion, as this is dependent on gastric pH which has a strong influence on digestibility (Reuterswärd & Fabiansson, 1985). Nevertheless, evaluating all the investigated substrates together showed a high comparability between *in vivo* and *in vitro* digestibilities of individual amino acids in the seven substrates, with an average bias of 1.2 % according to the Bland-Altman comparison (Bland & Altman, 1986) (Figure 3 c).

4.3. In vitro DIAAR and proxy in vitro DIAAR comparability with in vivo experiments

The *in vitro* DIAAR values for all of the essential amino acids in the seven investigated substrates were highly correlated with the *in vivo* data, with a mean bias between *in vitro* and *in vivo* data of 0.1 %, according to the Bland-Altman plot (Bland & Altman, 1986) (Figure 4 i). This analysis showed that > 95 % of all the data points lay between ±2 * SD of the average of the two methods. The calculation of proxy *in vitro* DIAAR values with the adapted calculation from Mathai et. al. (Mathai et al., 2017), using the total in vitro ileal digestibility instead of STTD could partially overcome earlier described limitations of the PDCAAS (Mathai et al., 2017; Schaafsma, 2012) (e.g., the overestimation due to consideration of fecal instead of ileal digestibility and the truncation to 100 % (FAO, 1991). However, our results confirm previous observations that proxy *in vitro* DIAAS, especially when based on TN or OPA values, are crude estimates (Mathai et al., 2017), neglecting differences in the digestibility of individual amino acids, and not considering the FAO recommendation to treat each indispensable amino acid as individual nutrient (FAO, 2013). Moreover, if TAA were analyzed, the calculation of *in vitro* DIAAR considering the digestibilities of individual indispensable AA should be preferred over the proxy DIAAR based on overall TAA digestibility.

5. Conclusion

The *in vitro* AA digestibility and *in vitro* digestibility predicted DIAAS values of seven foods were established with the INFOGEST static IVD protocol in parallel with human and pig i*n vivo* experiments. Overall, the *in vitro* digestibilities, *in vitro* DIAAR, and *in vitro* DIAAS showed a high correlation with *in vivo* results, with a tendency toward overestimation with the *in vitro* approach. The digestibilities of pure proteins should be assessed in combination with other nutrients to simulate the digestion of a real food. The present data clearly show that the INFOGEST static IVD protocol has great potential as a tool for the calculation of protein and AA digestibility to determine *in vitro* DIAAS values. However, at present, only seven substrates were investigated. More *in vitro* and *in vivo* comparability data will be needed in the future to further validate this newly developed *in vitro* workflow and allow its implementation as a robust

and reproducible method for digestibility and DIAAS predictions. The protocol also needs further testing with highly transformed products, such as extruded proteins or highly heated foods. Nevertheless, once broadly validated, this protocol could represent an ideal tool for the screening of new products and could be helpful for producers when evaluating and screening protein sources at the level of product development. An additional advantage of *in vitro* digestibility systems is their fast adaptation to conditions of special age groups or health conditions, such as infants, the elderly, or health-impaired persons, for which *in vivo* studies are not possible due to ethical constraints.

Acknowledgments

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Supplementary material

Supplemental table 1

Product	Origin
Wheat bran cereals	All Bran®, Kelloggs®
Peanuts	Commercial product, US
Black beans	Harvest North, Henshall, Canada
Pigeon peas	Davis Food Ingredients, New Zealand
Zein	Sigma, US
Collagen	Dat-Schaub, Poland
Whey protein isolate	Fonterra, New Zealand

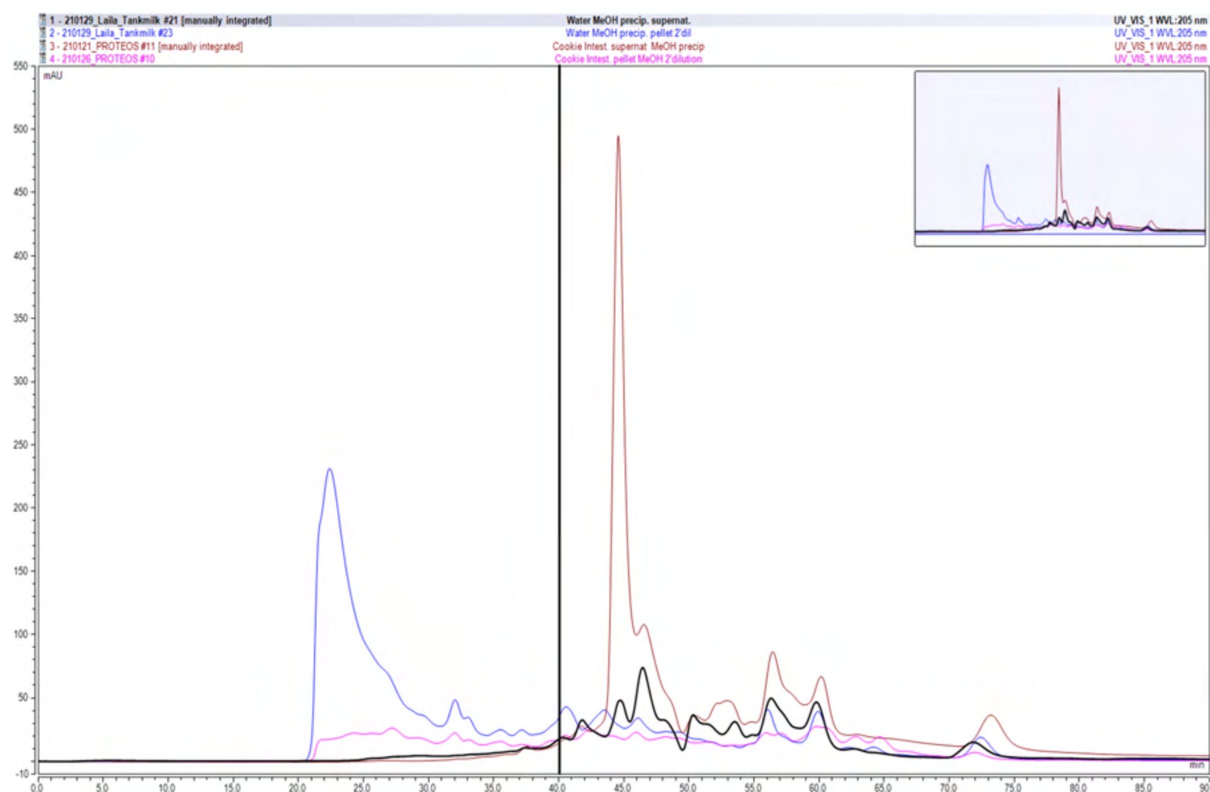
Suppl. table 1. Origin of the protein sources.

Supplemental table 2

Molecular mass marker	Mass (Da)	Molecular mass marker	Mass (Da)
Gly	75	H-Gly-Pro-Pro-Glu-OH	398.42
Serine	105	[D-Ala^{2}]-Deltrophin II	782.88
Gly-Gly	132.12	Gly-Arg-Gly-Asp-Ser	490.47
Lysine	146	Gly-Arg-Gly-Asp-Ser-Pro	587.58
Gly-Gly-Gly	189.17	Angiotensin II	1046.18
Gly-Gly-Gly-Gly	246.2	Insulin	5733.5
Gly-Gly-Gly-Gly-Gly-Gly	360.3		

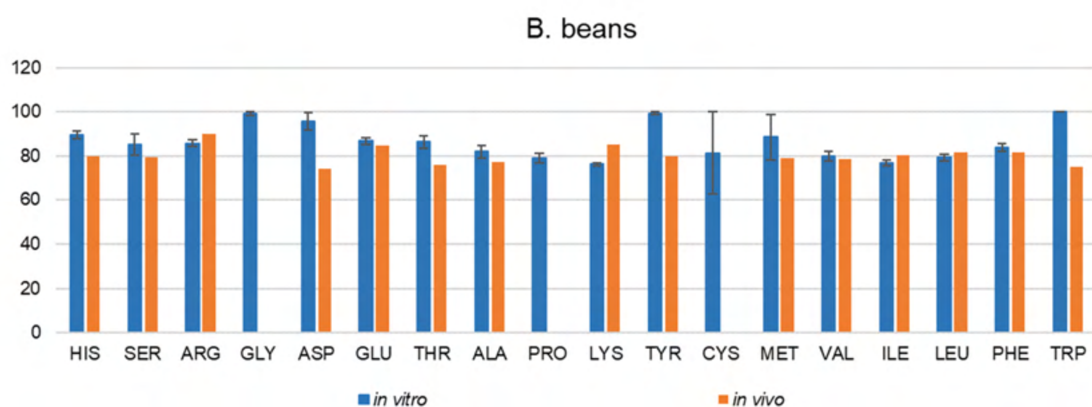
Suppl. table 2. Molecular mass markers used for SEC system calibration.

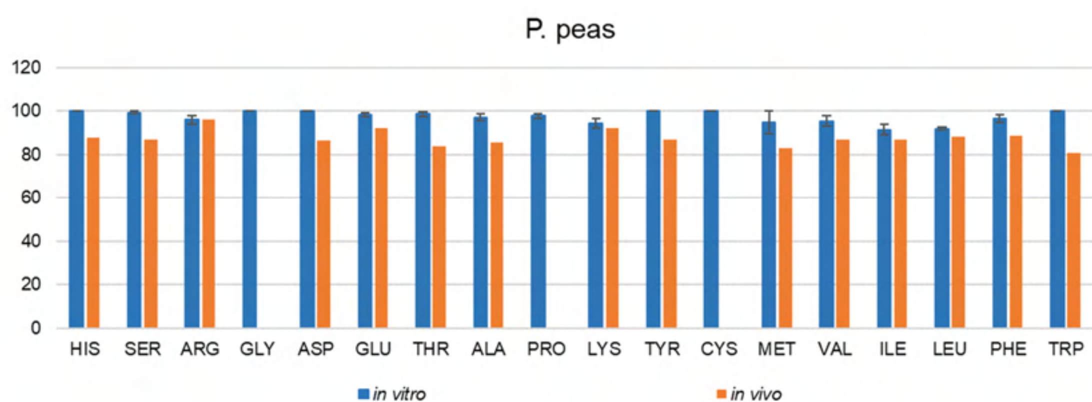
Supplemental figure 1. Size exclusion chromatogram (SEC) of intestinal water digest

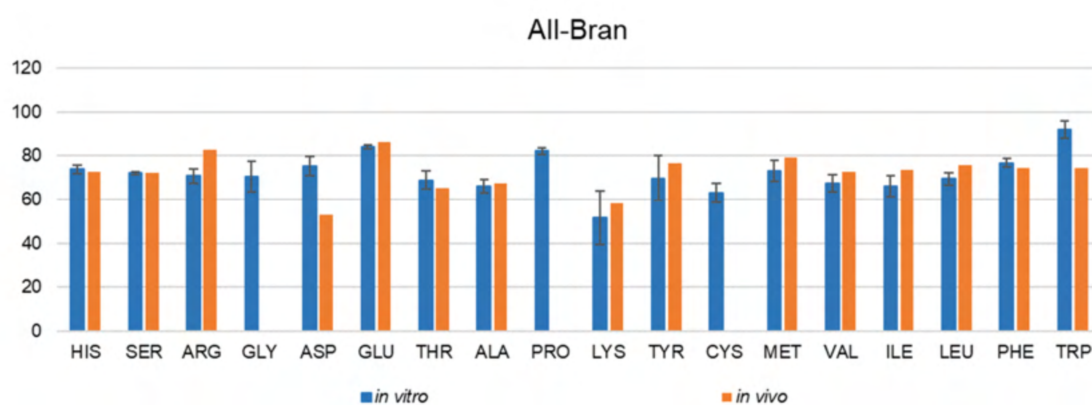


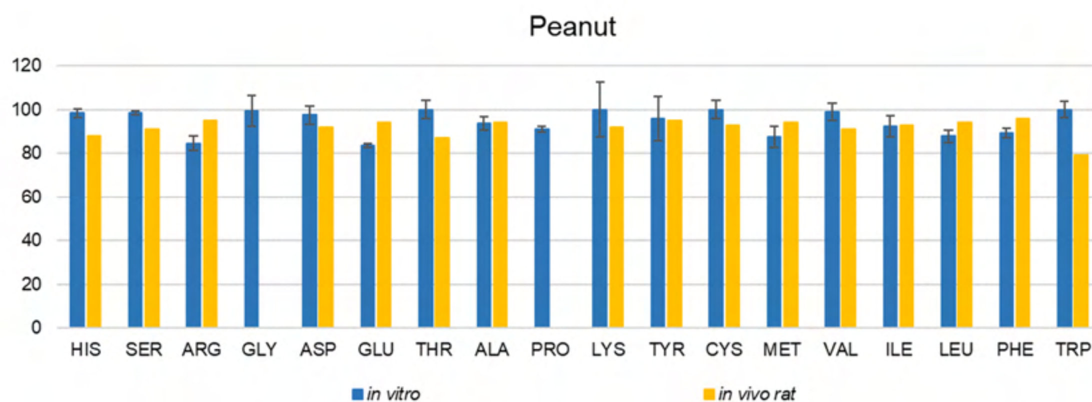
Suppl. figure 1. SEC profile of intestinal cookie and water digests. Black: water supernatant, Blue: water pellet, Brown: cookie supernatant, Pink: cookie pellet.

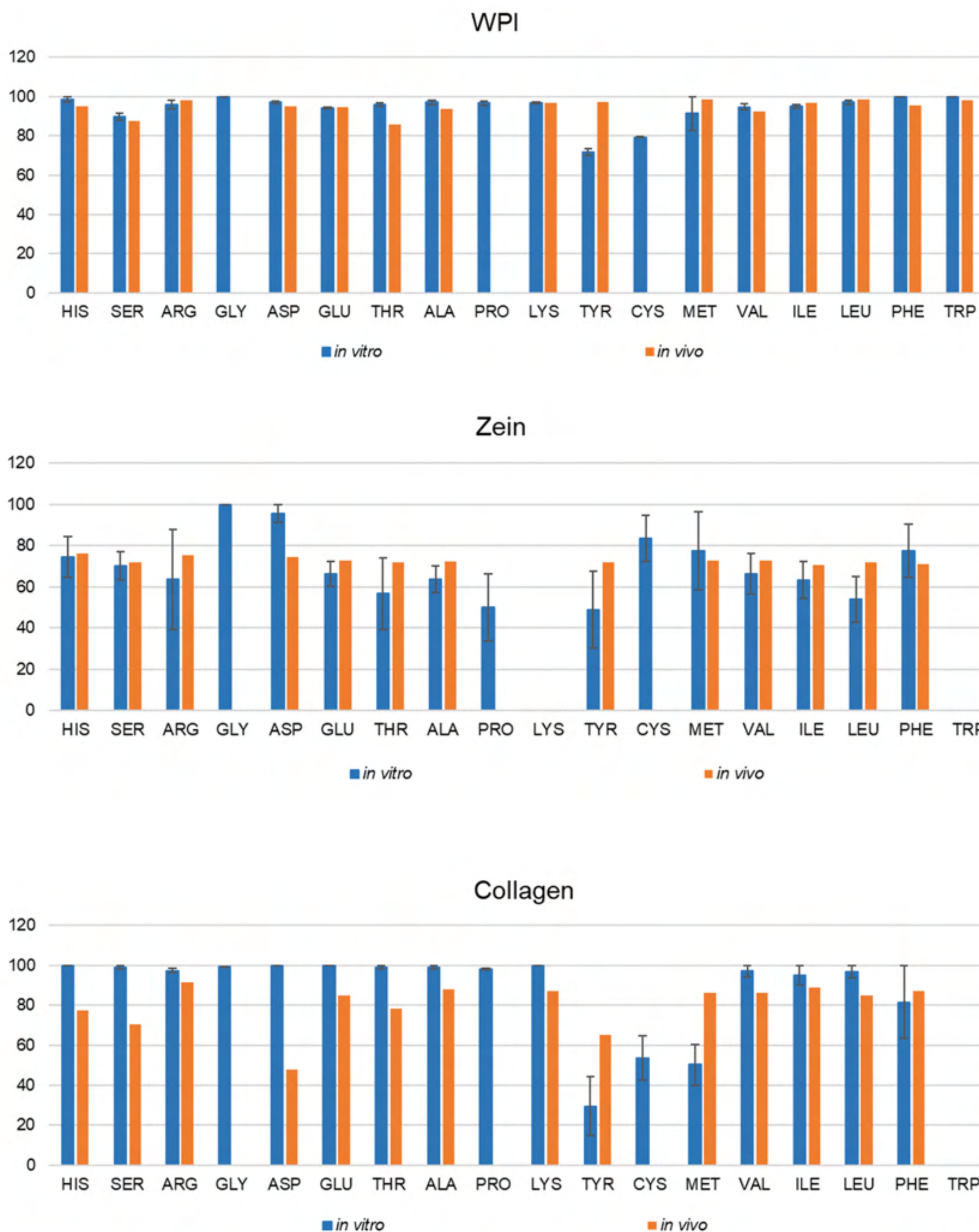
Supplemental figure 2. Digestibility of individual amino acids in the seven substrates after IVD





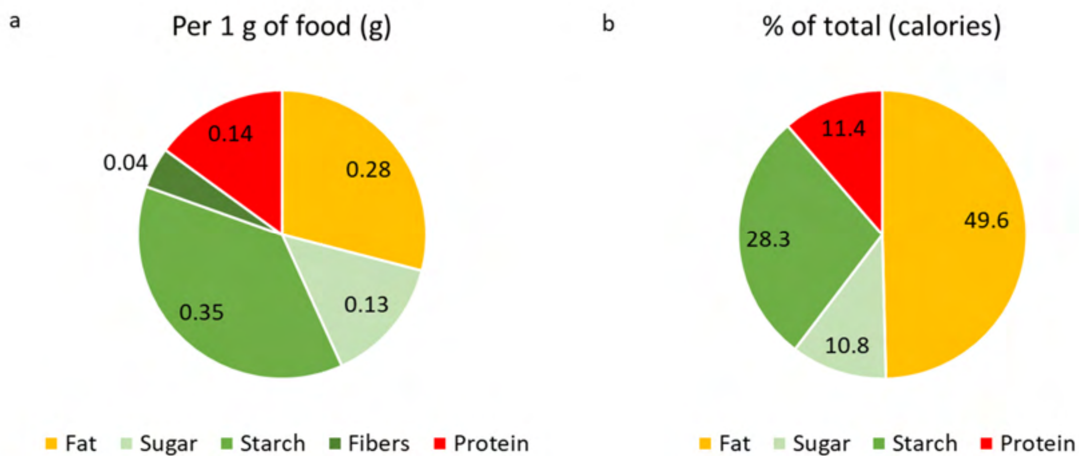






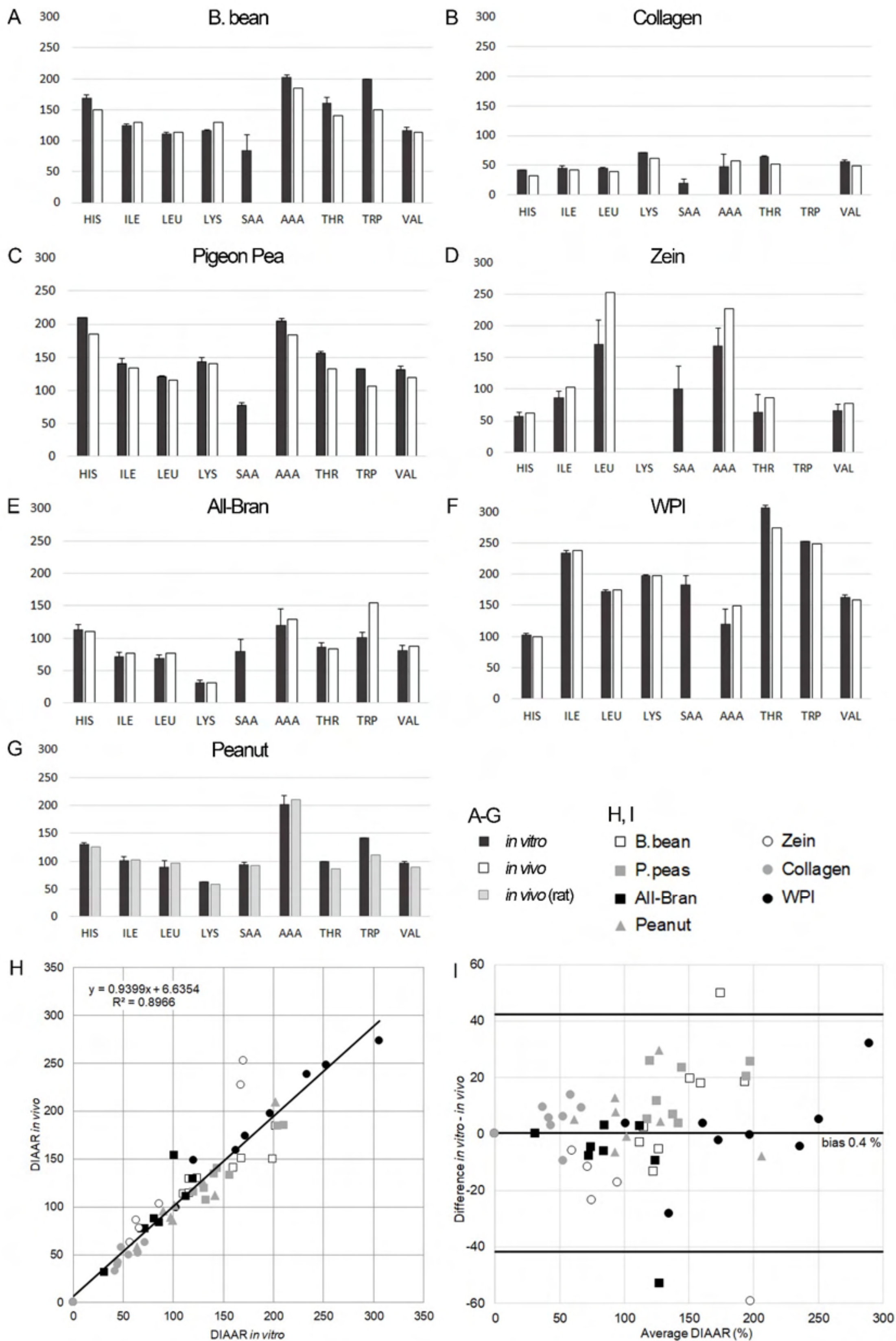
Suppl. figure 2. *in vitro* digestibility of individual amino acids (blue) after IVD compared with *in vivo* data (mean pig and human values, orange) (Hodgkinson et al., 2022), or rat (yellow). Error bars are SEM of three individual *in vitro* experiments.

Supplemental figure 3. "Meal" composition with pure protein sources + 0.25 g Cookie



Suppl. figure 3. Macronutrient composition of combined pure protein sources (40 mg of protein) with 0.25 g cookie in a "meal", distribution in gram (a) and in % (b) of the total, respectively.

Supplemental figure 4. In vitro AA digestibility based DIAAR values compared to in vivo AA digestibility based data from pigs and humans for older children, adolescents, and adults.



Suppl. figure 4. *In vitro* AA digestibility based DIAAR values for older children, adolescents, and adults (FAO, 2013), compared to *in vivo* AA digestibility based data from pigs and humans (average values, white) for black bean, pigeon pea, All-Bran® wheat cereal, collagen, zein, and whey protein isolate (WPI) (Hodgkinson et al., 2022), or from rats for peanut (Rutherfurd et al., 2014). Isolated proteins (collagen, zein, and WPI) were digested together with 0.25 g of a protein-free cookie to simulate a whole meal. Samples were analyzed at least in triplicate, and the error bars represent standard deviations (SD) (A-G). Correlation of *in vitro* DIAAR values with average *in vivo* data from pigs and humans for black bean, pigeon pea, All-Bran®, collagen, zein, and WPI or from rats for peanut (Rutherfurd et al., 2014) (G). Statistical comparison between *in vitro* and *in vivo* DIAAR results (Hodgkinson et al., 2022), according to previous work (Bland & Altman, 1986), show the average DIAAR (x-axis) versus the differences between *in vitro* and *in vivo* DIAAR (y-axis) of all essential amino acids of the same comparisons, as described in Fig. 3c (H). The mean bias between methods was 0.4 % and upper and lower limits indicate ±2 * standard deviations of the average difference (I). Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values.

Supplemental table 3. Total amino acids per gram of product

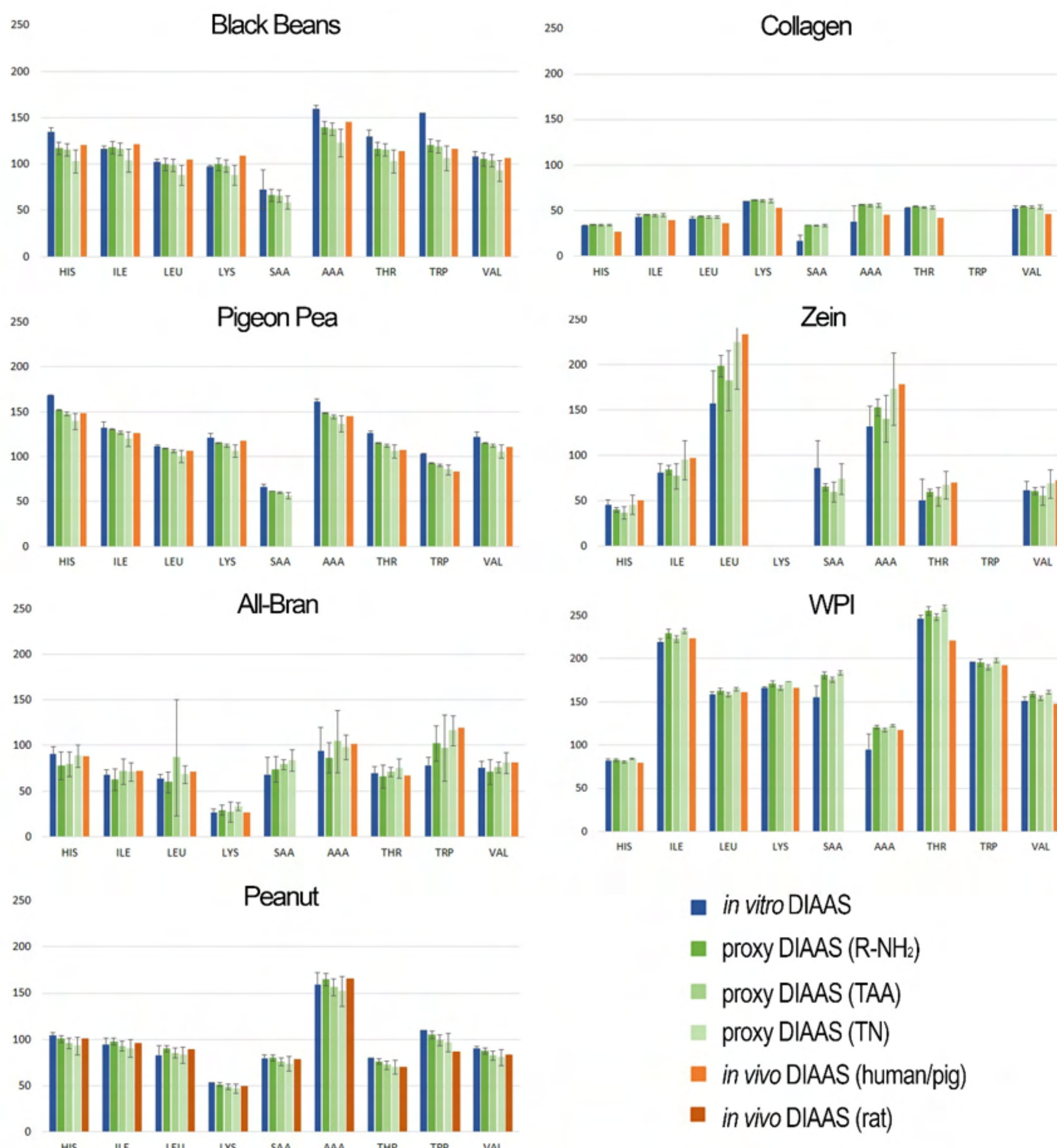
Total AA per gram product (mg AA/g product)							
	Peanut	B.bean	P.peas	WPI+C	Zein+C	All-Bran	Collagen+C
HIS	6.5	2.1	2.3	14.4	12.0	3.3	7.2
ILE	10.2	3.3	3.2	63.6	40.3	4.3	15.2
LEU	19.3	5.8	5.5	93.1	197.5	8.5	29.9
MET	2.8	0.8	0.5	20.9	16.6	1.7	9.1
CYS	4.2	0.8	0.7	21.5	9.8	2.5	0.5
PHE	15.9	4.2	3.8	28.8	68.8	5.8	22.9
TYR	11.9	2.2	2.1	25.7	51.0	3.8	7.9
THR	7.7	3.2	2.7	68.6	27.6	4.4	17.6
TRP	2.9	0.9	0.6	14.4	1.0	1.9	0.1
VAL	12.2	4.0	3.8	59.2	39.1	6.6	24.5
LYS	9.4	5.0	5.0	84.4	0.0	3.5	36.8
ALA	11.2	2.9	3.0	46.4	91.3	6.1	89.3
ARG	36.0	4.7	5.7	18.4	16.7	7.7	82.6
ASP	35.0	8.8	8.6	96.9	52.8	9.0	59.0
GLU	55.8	10.7	11.8	165.8	226.7	28.0	105.7
GLY	17.2	2.9	3.0	14.7	12.7	7.1	241.4
SER	14.2	4.0	3.4	43.5	48.0	5.7	33.2
PRO	12.3	2.6	2.9	59.9	92.4	9.2	138.4
total AA	284.7	69.0	68.8	940.1	1004.5	119.3	921.3
TN	49.5	11.0	11.0	138.0	147.0	22.0	171.0
Prot (TN*6.25)	309.4	68.8	68.8	862.5	918.8	137.5	1068.8
DIAAR (%) for infant (birth - 6 month)							
	Peanut	B.bean	P.peas	WPI+C	Zein+C	All-Bran	Collagen+C
DIAAR %	n=3	n=3	n=3	n=3	n=3	n=6	n=3
HIS	99	128	160	78	43	86	32
ILE	55	68	77	128	47	39	25
LEU	57	70	77	109	108	44	28
LYS	44	81	100	137	0	22	50
SAA	65	59	55	127	70	56	14
AAA	88	88	89	52	73	52	21
THR	56	91	89	174	36	49	37

TRP	55	77	52	98	0	39	0
VAL	71	85	95	118	48	59	41
SD %							
HIS	3	4	0	2	5	6	0
ILE	4	2	4	2	6	3	2
LEU	7	2	1	2	25	3	2
LYS	0	1	4	1	0	3	0
SAA	3	17	2	11	25	13	5
AAA	7	2	2	10	12	11	9
THR	0	5	2	3	17	4	1
TRP	0	0	0	0	0	3	0
VAL	1	4	4	3	8	5	2
DIAAR (%) for preschool children (6 month – 3 years)							
	Peanut	B.bean	P.peas	WPI+C	Zein+C	All-Bran	Collagen+C
DIAAR %	n=3	n=3	n=3	n=3	n=3	n=6	n=3
HIS	104	134	168	82	45	91	34
ILE	95	116	132	219	81	67	42
LEU	83	102	112	159	157	64	41
LYS	54	98	121	166	0	26	60
SAA	80	72	67	155	86	68	17
AAA	159	160	161	95	132	94	38
THR	80	129	126	247	51	69	53
TRP	110	155	103	196	0	78	0
VAL	90	108	122	151	61	75	52
SD %							
HIS	3	4	0	2	6	6	0
ILE	7	3	6	4	11	6	4
LEU	10	3	1	3	36	4	2
LYS	0	1	5	1	0	3	0
SAA	4	21	3	13	30	16	6
AAA	13	3	3	18	22	20	17
THR	0	7	2	4	23	6	1
TRP	0	0	0	0	0	7	0
VAL	2	5	5	4	10	7	3
DIAAR (%) for older children, adolescents, and adults							

	Peanut	B.bean	P.peas	WPI+C	Zein+C	All-Bran	Collagen+C
DIAAR %	n=3	n=3	n=3	n=3	n=3	n=6	n=3
HIS	n=3	n=3	n=3	n=3	n=3	n=6	n=3
ILE	130	168	210	103	57	113	42
LEU	101	124	141	234	86	72	45
LYS	90	110	121	172	170	69	45
SAA	64	116	144	197	0	31	72
AAA	94	85	78	182	101	80	20
THR	202	202	205	120	168	119	48
TRP	99	160	157	306	63	86	65
VAL	142	200	133	253	0	101	0
SD %							
HIS	4	6	0	2	7	8	0
ILE	8	4	7	4	11	6	4
LEU	11	4	2	3	39	5	2
LYS	0	1	6	2	0	4	0
SAA	4	25	3	15	35	19	7
AAA	16	4	4	23	28	25	21
THR	0	9	3	4	29	7	1
TRP	0	0	0	0	0	8	1
VAL	2	5	6	4	11	7	3

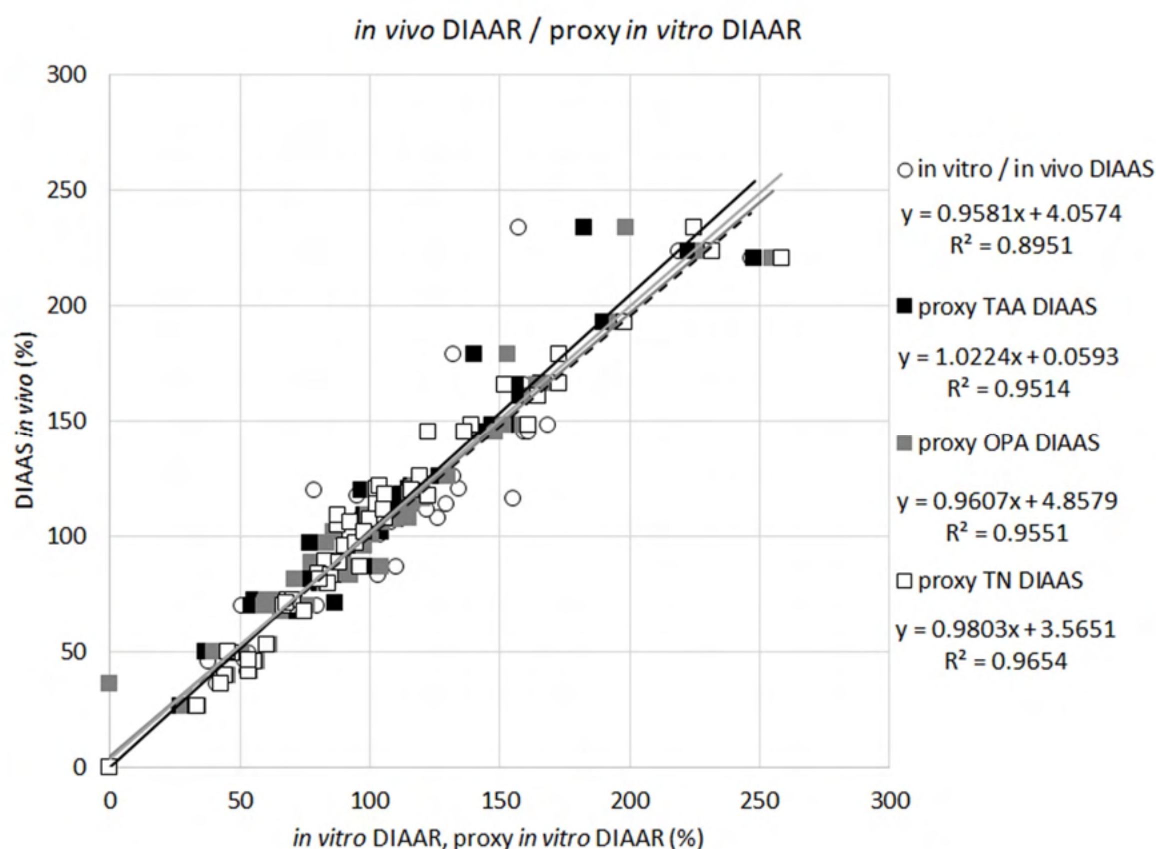
Suppl. table 3. Total AA per gram product (mg/g), *in vitro* DIAAR values for the seven food sources for the three different age groups defined by the FAO (FAO, 2013).

Supplemental figure 5. Proxy in vitro DIAAS values calculated based on total digestibility compared with in vitro and in vivo DIAAS values



Suppl. figure 5. *In vitro* proxy DIAAR values calculated based on $R\text{-}NH_2$ (OPA), TAA, and TN values and compared to *in vivo* data (mean from pig and human, for black bean, pigeon Pea, All-Bran®, collagen, zein, and WPI and from rat for peanut. Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. Isolated proteins (Collagen, Zein and WPI) were digested together with 0.25 g of cookie, to simulate a whole meal. Samples were at least analyzed in triplicates; error bars represent standard deviations.

Supplemental figure 6. Correlation of proxy in vitro DIAAS and in vitro DIAAS with in vivo DIAAS



Suppl. figure 6. Correlation of proxy *in vitro* DIAAR and *in vitro* DIAAR (x-axis) with *in vivo* DIAAR from human and pig experiments for preschool children (6 month – 3 years) (Hodgkinson et al., 2022) (y-axis). *In vitro* DIAAR versus *in vivo* DIAAR (white circles, dashed black line); proxy *in vitro* DIAAR based on TAA (black squares, black line); proxy *in vitro* DIAAR based on OPA (R-NH_2, gray squares, gray line); proxy *in vitro* DIAAR based on TN (white squares, light gray line) versus *in vivo* DIAAR (Hodgkinson et al., 2022), respectively. Each data point represents an essential amino acid from one of the seven investigated substrates (black bean, pigeon pea, All-Bran®, peanut, collagen, zein, and WPI). Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. The comparisons are shown in detail in Supplemental Fig. 4.

Supplemental table 4

Proxy DIAAR (TAA, %)									
	HIS	**ILE**	**LEU**	**LYS**	**SAA**	**AAA**	**THR**	**TRP**	**VAL**
Peanuts	95	93	85	48	76	156	72	99	83
Black	115	116	98	98	65	137	115	119	103
Pigeon	147	127	106	112	60	144	112	90	112
All-Bran	94	219	158	166	167	127	240	184	155
Collage	66	146	290	26	114	223	116	39	104
Zein	79	71	86	27	79	104	71	97	76
Whey	34	45	43	62	34	57	54	0	55
SD (%)									
Peanuts	6	6	5	3	5	9	4	6	5
Black	3	3	3	3	2	4	3	3	3
Pigeon	2	2	2	2	1	2	2	1	2
All-Bran	25	39	21	22	47	10	55	40	18
Collage	9	39	57	66	32	45	64	71	26
Zein	14	14	64	11	6	34	5	36	6
Whey	0	0	0	0	0	0	0	0	0
Proxy DIAAR (OPA, %)									
Average	**HIS**	**ILE**	**LEU**	**LYS**	**SAA**	**AAA**	**THR**	**TRP**	**VAL**
Peanuts	100	98	90	51	80	165	76	104	87
Black	116	117	100	99	66	139	116	120	105
Pigeon	151	130	109	115	61	148	115	93	115
All-Bran	84	232	165	173	183	123	259	198	161
Collage	60	126	300	0	98	231	89	0	91
Zein	78	62	60	29	73	86	66	102	71
Whey	34	45	43	62	34	57	54	0	55
SD (%)									
Peanuts	4	4	3	2	3	6	3	4	3
Black	6	6	5	5	4	8	6	7	6
Pigeon	0	0	0	0	0	0	0	0	0
All-Bran	1	4	3	3	3	2	4	3	3
Collage	6	13	32	0	10	25	9	1	10
Zein	15	12	11	6	14	16	13	20	14
Whey	0	0	0	0	0	0	0	0	0
Proxy DIAAR (TN, %)									
Average	**HIS**	**ILE**	**LEU**	**LYS**	**SAA**	**AAA**	**THR**	**TRP**	**VAL**
Peanuts	93	90	83	47	74	152	70	96	80
Black	103	104	88	88	58	123	103	106	92
Pigeon	139	119	100	106	56	136	106	85	106
All-Bran	82	227	161	169	180	120	253	194	158
Collage	62	130	309	0	101	238	92	0	94
Zein	88	71	68	33	83	98	75	116	81
Whey	34	45	43	61	34	56	54	0	54
SD (%)									
Peanuts	10	10	9	5	8	16	7	10	9
Black	13	13	11	11	7	15	13	13	11
Pigeon	9	8	7	7	4	9	7	6	7
All-Bran	4	11	8	8	9	6	12	9	8
Collage	7	14	33	0	11	25	10	1	10
Zein	12	10	9	5	12	14	10	16	11
Whey	0	1	1	1	0	1	1	0	1

Suppl. table 4. Proxy *in vitro* DIAAR values calculated based on TAA, OPA (R-NH_2), and TN values for preschool children (6 month – 3 years). Isolated proteins (collagen, zein and WPI) were digested together with 0.25 g of cookie, to simulate a whole meal. Samples were at least analyzed in triplicates.

Additional results

Amount of substrate in the system

The INFOGEST protocol (Minekus et al., 2014) was designed focusing on physiological enzyme activities and had as main goal to improve the comparability of experimental data between labs. The protocol was validated by inter-laboratory studies using skim milk powder (SMP) where a clear harmonization of the data obtained when compared with previous experiments with individual *in vitro* digestion protocols was achieved (Egger et al., 2016). For this inter-laboratory study, 5 mL of a 1/10 dilution of SMP in H2O (w:v) were used, which corresponds to 0.04 g of protein per digest. The protocol also proved its physiological relevance by comparing *in vivo* data with *in vitro* results where protein hydrolysis obtained by the *in vitro* digestion was similar to *in vivo* protein hydrolysis in pigs at the gastric and intestinal endpoints (Egger et al., 2017).

Taking both studies as a starting point, we decided to normalize the protein input for our *in vitro* digestions to 0.04 g protein as well. Nevertheless, to ensure our decision, different amounts of protein input were tested (Figure 1). Different *in vitro* digestions where performed with three different amounts of protein (40 mg, 60 mg and 80 mg) and as can be seen in Figure 1, the higher the protein input, the lower the protein digestibility, which was more accentuated in the substrates with expected lower digestibility.

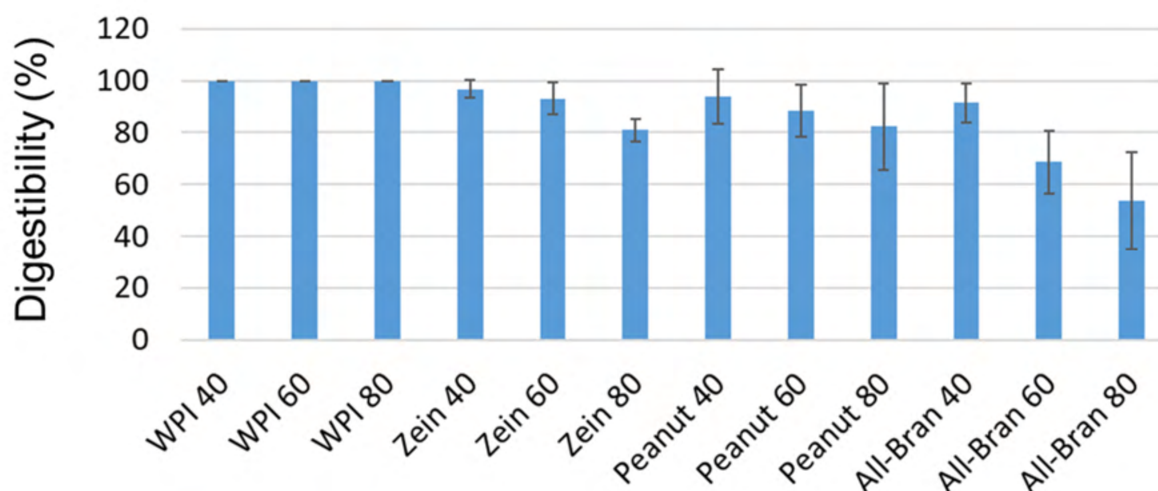


Figure 1. Protein digestibility of whey protein isolate (WPI), zein, peanut and All-Bran®. The different substrates were digested *in vitro* with different protein inputs (40 mg, 60 mg and 80 mg). The experiment was at least performed three times and error bars represent standard deviations.

These results show that for concentrations of 60 mg and 80 mg of protein, the *in vitro* system was overloaded and the amount of enzymes present was not enough to guarantee the enzymatic reactions to their full extent. Besides that, it is also possible to see that the standard deviation of the results increased with the increase of protein input. Thus, these results supported the decision of normalizing the *in vitro* digestion system to 40 mg of protein input.

Pancreatin solubilization

It was found that pancreatin formed a suspension with undissolved particles, resulting in non-reproducible measurements. Therefore, some modifications were made to the INFOGEST *in vitro* digestion protocol to improve the repeatability of the measurements. As first attempt, the pancreatin was dissolved and centrifuged (2000 g, at RT, for 5 min), and only the supernatant was taken. However, the enzyme activity of the supernatant was much lower than the activity of the complete enzyme suspension. Consequently, a new attempt was made to dissolve the pancreatin, subject the suspension to 5 min of ultrasound (45 Hz, 130 W) and then centrifuge it (2000 g, at RT, for 5 min) to allow only the clear supernatant to be collected. Trypsin, pancreatic amylase and pancreatic lipase activities were measured according to a previous protocol (Brodkorb et al., 2019). As seen in the graphs (Figure 2) trypsin (Figure 2a) and pancreatic amylase (Figure 2b) activities slightly decreased with the ultrasounds and centrifugation treatment. Nonetheless, however pancreatic lipase (Figure 2c) has a more accentuated loss of activity, none of the *p-values* was inferior of 0.05 so we assumed this differences as not significant (Trypsin *p*-value = 0.60; pancreatic amylase *p*-value = 0.35; Lipase *p*-value = 0.08).

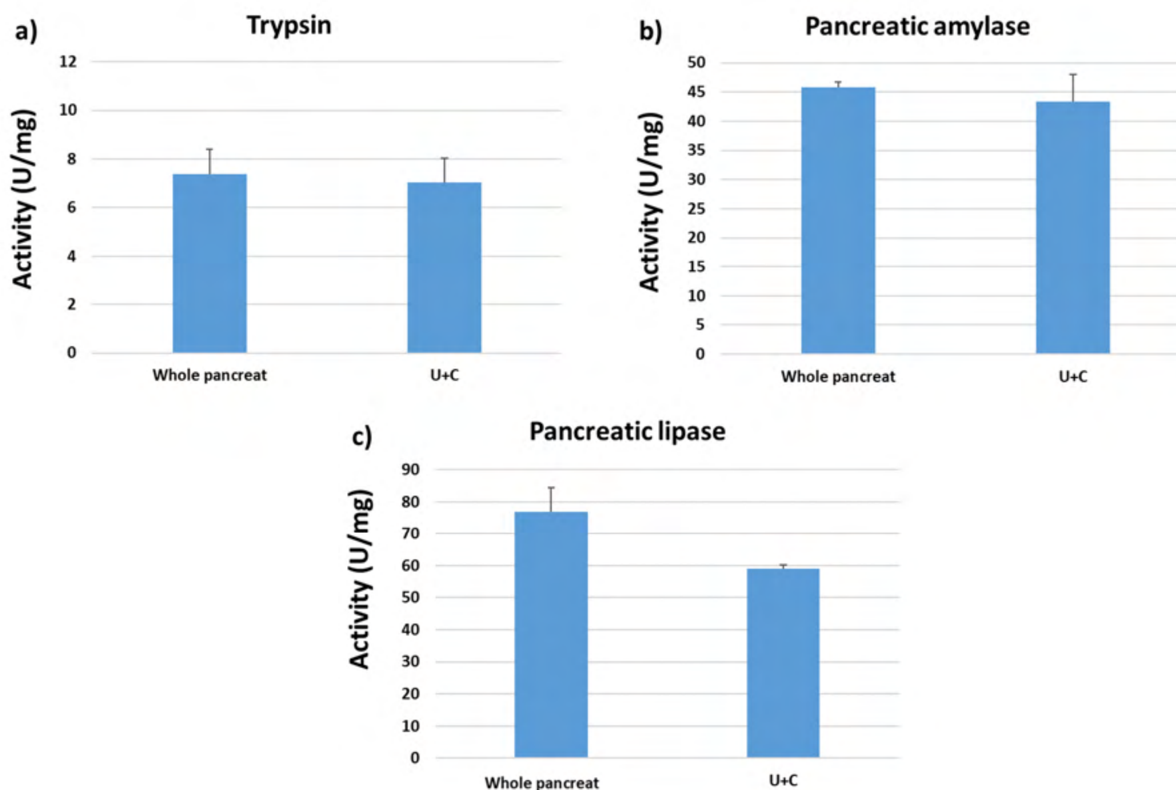


Figure 2. Trypsin (a), amylase (b) and lipase (c) pancreatic activities determined for the untreated enzymatic suspension (whole pancreat) and for the ultra-sonicated and centrifuged enzymatic suspension (U+C). All the measurements were performed at least three times and error bars represent standard deviations.

Precipitation methods for isolating absorbable fraction

Our *in vitro* model does neither include the absorbable step nor the brush border enzymes, and both undigested/non-bioavailable and digested/bioavailable fractions remain in the reaction vessel at the end of the intestinal digestion phase. Therefore, additional steps, such as precipitation and centrifugation of the digesta, are needed to separate the bioavailable fraction from the non-digested products. Ideally, the supernatant resulting from the precipitation corresponds to the bioavailable fraction.

Several different precipitation agents were tested, such as perchloric acid, trichloroacetic acid (TCA), tannic acid (TA), acetone, methanol/formic acid (MeOH/FA), and methanol (MeOH) (Figure 3). Additionally, 3K filters were also tested (Figure 4). On Figure 3, the chromatograms of the supernatant resulting from the precipitation of the WPI digesta with the different agents are depicted. Surprisingly, all the tested agents showed a similar molecular weight cut-off, however, it is possible to see that all the precipitation agents, except MeOH, give additional peaks (indicated with the arrows). 70 % MeOH/ FA 1% and 80 % MeOH seemed to be the best precipitation conditions. These two conditions have a similar elution

pattern and a good recovery of the small molecules in the supernatant since their peaks overlap with the peaks corresponding to small molecules from the non-precipitated digest.

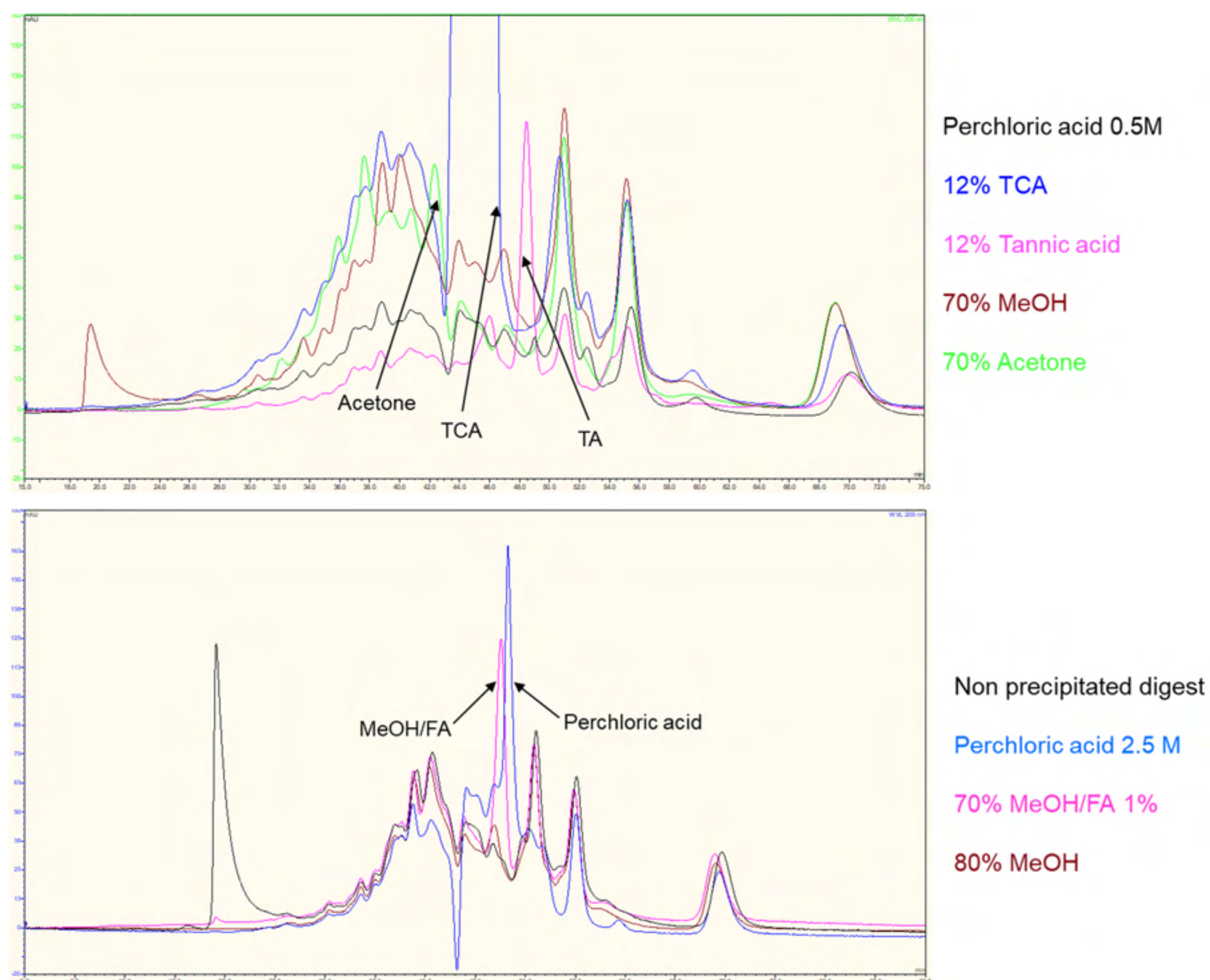


Figure 3. SEC chromatograms of the supernatant resulting from the different precipitations of the WPI digest. Arrows point the additional peaks produced by the precipitation agents.

However, 70% MeOH/ FA 1% give a major additional peak in the middle of the chromatogram. This peak is probably derived from the formic acid, since the same is not visible on the 80 % MeOH chromatogram.

SEC chromatograms of the filtered and retained fraction of the WPI digesta after filtration with 3K filters are shown in Figure 4. It is possible to see that the filtration is not optimal since many peptides and amino acids are retained in the filter leading to a significant decrease in the yield of the method. In consequence, MeOH 80 % has proven to be the best condition for precipitation of undigested proteins and big peptides, since there are no additional peaks on the chromatogram derived from the precipitation agent and the peaks corresponding to low molecular weights are eluted in a very similar pattern to that observed in the non-precipitated sample, indicating a good recovery (Figure 3).

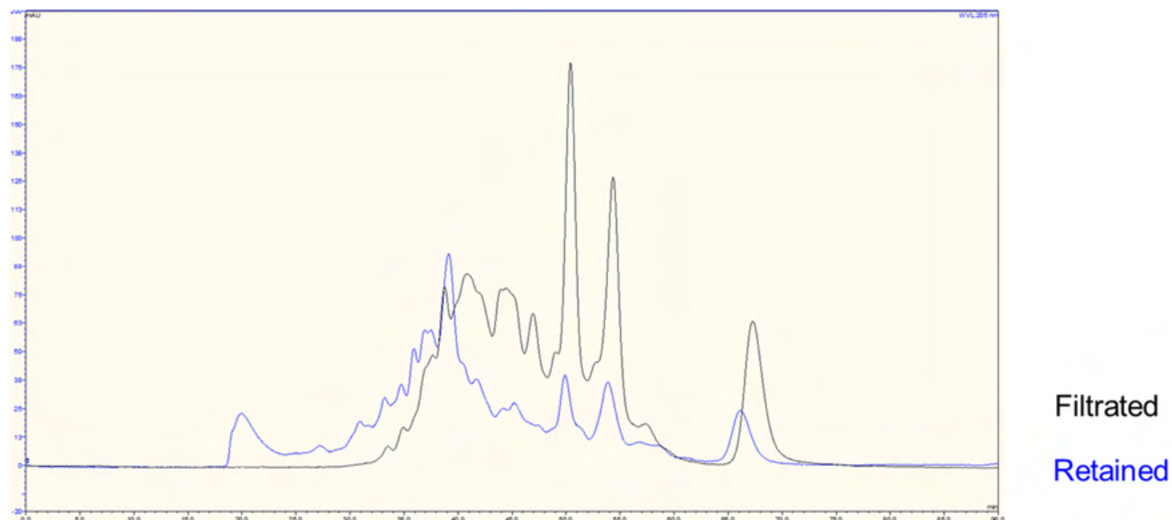


Figure 4. SEC chromatogram from the filtrated fraction (black) and the retained fraction (blue) resulting from the filtration of WPI digest with 3K filters.

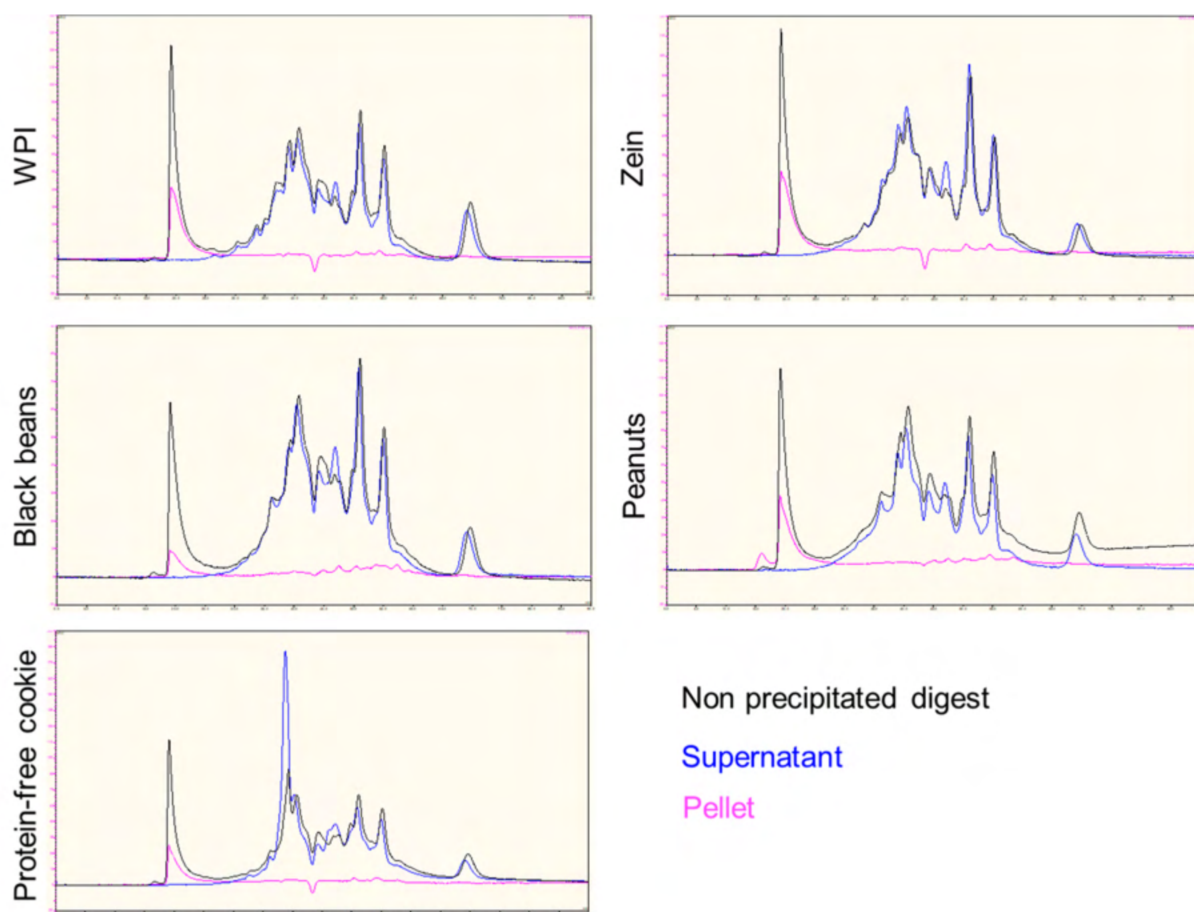


Figure 5. SEC chromatograms of digests from different foods precipitated with 80 % MEOH.

Definition of the bioavailable fraction

Ideally, the bioavailable fraction would correspond to the peptides remaining in the supernatant after the precipitation. The size of the peptides absorbed *in vivo* is still debated but there are studies showing that peptides with 1600 Da can be absorbed (Caira et al., 2016; Ozorio et al., 2020). In addition, the protocol used for *in vitro* digestion lack the brush border enzymes. These enzymes can hydrolyse poly- and oligopeptides into di- and tripeptides, and free amino acids, and *in vivo* they are responsible for the final stage of peptide digestion, before absorption into the enterocytes (Hooton et al., 2015).

In order to characterize the bioavailable fraction after intestinal *in vitro* digestion, the size exclusion chromatography (SEC) system was calibrated using peptides and small proteins of known sequence and molecular weights (standard compounds). By correlating the molecular weights of the standards with their retention times, the resulting linear curve showed that a retention time of around 40 min corresponded to a molecular weight of approximately 1000 Da, which is the approximate size of angiotensin (8 amino acids in length). SEC profiles of the supernatant and pellets from the intestinal digests after 80 % MeOH precipitation were compared with the SEC chromatograms of the standard compounds.

As shown in Figure 5, the peaks in the supernatants of the digested foods (blue) eluted after 40 min, corresponding to a MW <1000 Da and nicely overlapped with the peaks of the small peptides in the un-precipitated foods. In contrast, the peak in the pellet (pink) eluted very early (< 30 Min) and overlapped with the peak from undigested food at high molecular weight. Taking all together, we could confirm that the supernatant contained mainly peptides < 1000 Da, corresponding to free amino acids and peptides up to 10 amino acids.

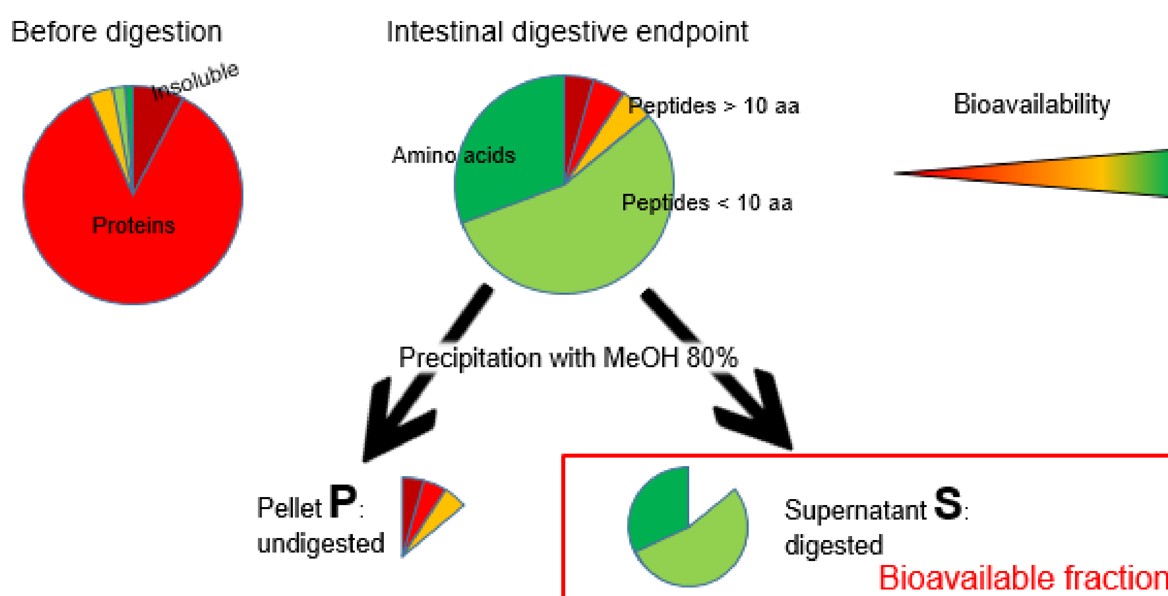


Figure 6. Scheme for defining and obtaining the absorbable fraction.

Enzymatic background subtraction

Initially, our enzymatic background was a blank digestion, containing all the exact same components as the real digests but adding water instead of the tested protein sources. However, and due to the high enzymatic auto-digestion, the release of free AA and $R\text{-}NH_2$ after deducing the background led to an underestimation of protein hydrolysis. Therefore, a digestion of a more realistic food, such as a protein-free cookie containing only fat and carbohydrates was used as background in order to reduce the auto-digestion of the digestive enzymes (Moughan et al., 2005). In order to reduce the inter-experimental variability together with each set of tested samples, this protein-free cookie was digested in parallel. *In vitro* protein digestibility was calculated according to the formula shown in Figure 7, where the soluble fraction of the protein-free cookie is deduced from the soluble fraction of the sample and subsequently divided by the total, corresponding to the soluble fraction of the protein-free cookie minus the soluble fraction of the sample plus the insoluble fraction of the cookie minus the insoluble fraction of the sample.

The protein-free cookie pellet was defined as minimal pellet, because, in case the insoluble fraction of the food sample was smaller than the insoluble fraction of the enzyme blank (protein-free cookie), this would result in a negative value in the pellet. Therefore this term was set to zero, according to the term max (0;Fp-Cp) in the formula in Figure 8.

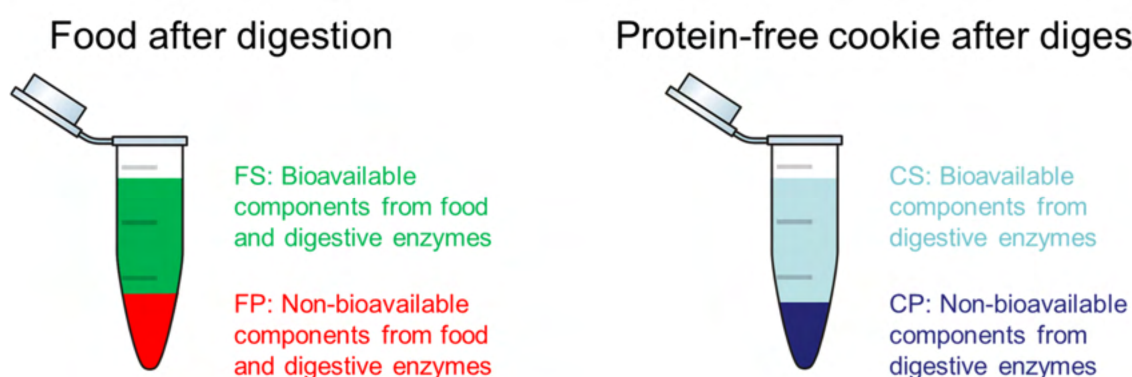


Figure 7. Definition of the enzymatic background.

In vitro protein and amino acids digestibility

Total Nitrogen (Kjeldahl), R-NH_2 (OPA method), and total amino acids (TAA, HPLC) were determined on both soluble and insoluble fractions of the different digests after precipitation with MeOH. Total *in vitro* protein digestibility was calculated using the data resulting from Kjeldahl, OPA, or TAA according to the formula present bellow (Fig. 8). The *in vitro* digestibility of each individual amino acid was calculated using the data from HPLC measurements according to the very same formula.

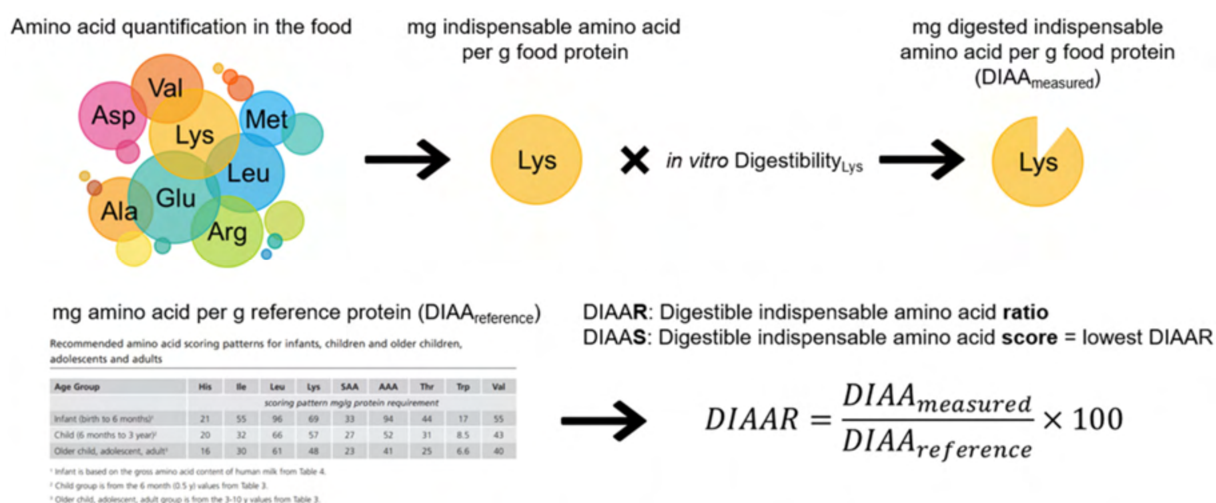
$$\textit{in vitro}\ \text{digestibility}\ [\%] = \frac{F_S - C_S}{(F_S - C_S) + max(0; F_P - C_P)} \times 100$$

Figure 8. *In vitro* digestibility calculation.

How to calculate *in vitro* DIAAR

DIAAS determination requires the absolute protein content and levels of indispensable amino acids for a given food and uses the ileal digestibility of each amino acid as a constituent of food to determine the true ileal digestibility of the indispensable amino acids. By assuming that the *in vitro* amino acids digestibility would correspond to the *in vivo* ileal amino acid digestibility, we calculated the *in vitro* DIAAS as following:

For each indispensable amino acid, the digestible indispensable amino acid (DIAA) per gram of food was calculated by multiplying the mg of indispensable amino acid per g of food protein by the *in vitro* total ileal digestibility value, instead of the standardized total tract digestibility (%). *Proxy* DIAAR were calculated by dividing the $DIAA_{measured}$ by the $DIAA_{reference}$ provided by FAO (Fig. 9) (FAO, 2013) multiplied by 100 (%). The DIAAS of a food corresponds to the lowest DIAAR, and no truncation was applied for values higher than 100 %.



Recommended amino acid scoring patterns for infants, children and older children, adolescents and adults

Age Group	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
	scoring pattern mg/g protein requirement								
Infant (birth to 6 months)[1]	21	55	96	69	33	94	44	17	55
Child (6 months to 3 year)[2]	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult[3]	16	30	61	48	23	41	25	6.6	40

[1] Infant is based on the gross amino acid content of human milk from Table 4.
[2] Child group is from the 6 month (0.5 y) values from Table 3.
[3] Older child, adolescent, adult group is from the 3-10 y values from Table 3.

Figure 9. *In vitro* DIAAR and *in vitro* DIAAS calculation scheme.

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Chapter 3

Application of the *in vitro* digestibility protocol to evaluate the protein quality of alternative sources of highly transformed plant proteins

Manuscript 3

Digestibility of meat analogues and influence of processing

Raquel Sousa[1,2], Reto Portmann[1], Isidra Recio[2], Lotti Egger[1]

[1]Agroscope, Schwarzenburgstrasse, 161, 3003 Bern, Switzerland
[2]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain

Abstract

Protein is an essential macronutrient found throughout the body and virtually every body part or tissue. It is commonly found in animal products, meat, eggs and dairy, although it is also present in other sources, such as nuts, beans, grains and legumes. The livestock sector is a major contributor to greenhouse gas (GHG) emissions worldwide, having a negative impact on the environment and driving to a climate change at a rate unprecedented in human history. Animal-based food production tends to have higher GHG emissions than plant-based food production –and especially red meat and dairy stand out for their disproportionate impact. Therefore, plant-based meat alternatives are needed in order to minimize the agriculture impact in the environment. Many plant-based proteins are not complete proteins. However, incomplete proteins can be combined to create complete proteins that meet human body requirements. In the present work beef burger known as excellent source of proteins, was compared to highly transformed veggie burgers made from soy as single plant source (soy burger) and from combined protein sources (pea-faba burger), were digested according to the INFOGEST *in vitro* digestion protocol. The effect of grilling was also evaluated. As expected, beef burger had the highest DIAAS values, but both plant protein based burgers reached DIAAS values that could be rated as good (pea-faba burger) or even excellent (soy burger) source of proteins according to FAO. The texturing process did not significantly affect protein digestibility, and DIAAS values and grilling only led to a decrease in pea-faba burger but no in soy burger and beef burger. Total protein digestibility was determined, either based on total nitrogen (Kjeldahl) analysis, or after acid hydrolysis based on total amino groups (*o*-phthalaldehyde method, OPA) or total amino acids (TAA; HPLC). Digestibilities of individual amino acids were also determined and *in vitro* digestibility based DIAAS calculated.

Keywords: Digestibility; *in vitro* DIAAS; total amino acids; *in vitro* digestion; liquid chromatography; protein hydrolysis; plant-based protein; meat alternatives

Abbreviations:

AA, amino acid; BCA, Bicinchoninic acid; DIAA, Digestible Indispensable Amino Acid; DIAAR, Digestible Indispensable Amino Acid Ratio; DIAAS, Digestible Indispensable Amino Acid Score; DTT, Dithiothreitol; EAA, Essential Amino Acids; FAO, Food and Agriculture Organization of the United Nations; GHG, Greenhouse gas; HPLC, High-Performance Liquid Chromatography; IVD, *in vitro* digestion; LC-MS, Liquid Chromatography-Mass Spectrometry; MS, Mass Spectrometry; NEAA, Nonessential Amino Acids; OPA, *o*-phthalaldehyde; RT, Room Temperature; SDS, Sodium Dodecyl Sulfate; SDS-PAGE, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; TAA, total amino acids; TN, total nitrogen; UHPLC, Ultra-High-Performance Liquid Chromatography; UV, Ultraviolet; UV/VIS – Ultraviolet/Visible

1. Introduction

The current livestock sector uses about 70 % of global agricultural land (FAO, 2009) being responsible for approximately 14.5 % of global greenhouse gas emissions and resulting in negative impacts on the environment, global health, and water and land resources (Gerber et al., 2013; McMichael et al., 2007). Animal-based proteins provide a significant portion of the human diet and, and meat consumption has risen markedly over the past century. In addition, to the negative environmental impact, high consumption of meat, specially read and processed meat, have been extensively associated with health problems (increased risk of cardiovascular diseases, cancer and diabetes type 2) (Zhang et al., 2022). In contrast, vegetarian and meat-reduced diets can help to overcome critical environmental, animal welfare, and health challenges in the food system (Dinu et al., 2017). Therefore, a shift towards a higher consumption of plant proteins is needed. New protein sources have emerged in recent years to support the transition toward more sustainable food production dedicated to human nutrition. Due to their similar appearance, texture, and taste to that of animal products, plant-based meat analogues have become well accepted, and the market is rapidly expanding to meet growing consumer demand (Beardsworth & Keil, 1991). Soy protein is historically the most used raw ingredient in the preparation of meat analogues, making it the best-known alternative to animal protein (Zhang et al., 2021). Although, sources like chickpeas, faba beans, rice, and green peas are also gaining popularity (Bohrer, 2019).

Unlike animal proteins, plant proteins may not contain all essential amino acids in the required proportions to meet human nutritional need, and a strict vegan diet might lead to possible nutritional deficiencies (Elorinne et al., 2016). In addition, plant proteins are also linked to low protein digestibility values (Bohrer, 2019). Together, this can constitute a challenge for the replacement of animal proteins by plant proteins. Food producers are trying to understand how plant proteins can partially or fully replace traditional animal protein ingredients by alternative plant-based foods to provide optimal nutrition, taste and functionality. However, at this moment, little is known about the gastrointestinal behaviour of the new developed meat analogues when compared to real meat products (Lee et al., 2020). This knowledge is important because the digestion and absorption of these products affects their nutritional profile and their impact on human health (Ogawa et al., 2018), and therefore more attention should be paid to the nutritional quality of new and alternative protein sources.

The aim of the present work was to determine the effect of food transformations (protein purification, drying, extrusion) and grilling on protein quality and digestibility. Therefore, soy burger, pea-faba burger and beef burger were digested according to the INFOGEST *in vitro* digestion protocol (Brodkorb et al., 2019), before and after grilling. Together with the burgers, their highly transformed ingredients (soy concentrate, texturized soy, pea isolate, faba bean concentrate and extruded pea-faba) were also digested and analyzed.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and enzymes used in the present study were purchased from MERCK.

2.2. Sample preparation for in vitro digestion (IVD)

The ten protein sources were digested according to the INFOGEST protocol. Briefly, minced bovine meat was shaped into a burger without addition of spices or other ingredients. The two plant based burgers, pea-faba burger and soy based burger, as well as the beef meat burger were grilled for 3 min on each side at 70 % of power of the stovetop without addition of fat. Both grilled and raw burgers were cut into pieces of 2-3 mm in order to mimic the chewing process. The protein sources were normalized according to their protein content and 0.04 g of total protein per mL of digest were used for *in vitro* digestion. As a blank digestion, a protein-free cookie (Moughan et al., 2005), containing only fat and carbohydrates was digested in parallel to the test foods, as previously described (Sousa et al., 2020). The cookie was prepared from 40.8 g purified corn starch, 15.7 g sucrose, 4.9 g cellulose, 0.7 g baking powder, 0.5 g ground ginger, and 36.9 g margarine and baked at 175 °C in portions of ~35 g for 30 min. The influence of other nutrients on *in vitro* digestion was tested by mixing the single proteins (soy concentrate, texturized soy, pea isolate, faba bean concentrate and extruded pea-faba) (normalized to 0.4 g protein) with 0.25 g of the ground cookie to simulate a meal composition, as previously described (Moughan et al., 2005).

2.3. Pancreatin suspension preparation

When in suspension, pancreatin forms undissolved particles, leading to non-reproducible measurements. Therefore, some modifications have been done to the INFOGEST IVD protocol to improve the repeatability of the measurements. Trypsin activity was measured according to a previous protocol (Brodkorb et al., 2019), using a pancreatin suspension prepared as described in (Sousa et al., 2022, submitted). Right before the digestion experiment, the pancreatin was dissolved with simulated intestinal fluid at a concentration of 100 U trypsin activity/mL of digest, then vortexed for 10 s, followed by ultrasound treatment (45 Hz, 130 W) at room temperature for 5 min. The suspension was then centrifuged (2000 g, at RT, for 5 min), and the supernatant was transferred into a new tube, placed on ice, and immediately used for the digestion experiment.

2.4. In vitro digestion with the INFOGEST static model

Enzyme activities and bile concentrations were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). All substrates were *in vitro* digested using the INFOGEST protocol (Brodkorb et al., 2019) with the adaptation for

pancreatin solubilization described above. Briefly, the substrates were normalized to a protein content of 0.04 g, diluted to 1 mL with water, and then mixed with 1 mL simulated salivary fluid (pH 7, 37 °C) containing amylase (300 U/mL of digesta), for 2 min. Then, 2 mL volume of simulated gastric juice (pH 3, 37 °C) containing pepsin (2000 U/mL of digesta) was added to the reaction tube and incubated at 37 °C for 120 min. As next, 4 mL volume of simulated intestinal juice (pH 7, 37 °C) containing pancreatin (100 U trypsin activity/mL of total digesta) and bile (10 mmol/L of total digesta) was added and incubated at 37 °C for 120 min. The whole digestion experiment was performed under constant gentle mixing on a rotating wheel. Gastric digestion was stopped after 120 min by increasing the pH to pH7 with NaOH (1 mol/L) and intestinal phase was stopped by adding the protease inhibitor 4-(2 aminoethyl) benzensulfonylfluoride (AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland). All the samples were immediately snap frozen in liquid nitrogen. For each set of samples digested, a protein-free enzyme blank (cookie) was digested in parallel.

2.5. Sample separation into digestible and indigestible fractions

After thawing, the digested samples were separated into digestible and indigestible fractions by precipitation with MeOH (80%) at -20 °C for 1 h, followed by centrifugation (2000 g at 4 °C for 15 min) as described in (Sousa et al., 2022, submitted). For every digested sample, a representative aliquot of the supernatants (Fs, Cs) were collected into new tubes. The pellets (Fp, Cp) were washed twice with MeOH (100 %), centrifuged between the washing steps (2000 g at 4 °C for 5 min), and then dried in a CentriVap (Labconco, Kansas City, Missouri USA). The volumes or weights of the total digests, the digests with added MeOH, and the dried pellets were monitored to allow the calculation of digestibility or *in vitro* DIAAS at the end of the experiment. The amino acids liberated from the protein-free cookie was set as the enzyme background, considered as the minimal amount and therefore, values below the enzyme background (due to analytical bias) were set to zero.

2.6. Protein extraction and gel electrophoresis

Proteins from undigested samples were extracted by dissolving 100 mg of sample with 200 µL SDS/Tris 1% (pH 7.4) and sonicate six times for 5 sec (on ice). Then, 1 mL of acetone was added to the suspension, vortexed and centrifuged at 13 000 g for 10 min (4 °C). The supernatant was discarded and the pellet was left to dry for at least for 4 h. 400 µL SDS/Tris 1% (pH 7.4) were added and the samples were left to incubate for 1 h at room temperature. The samples were sonicated until complete dissolution and then centrifuged at 13 000 g for 10 min (4 °C). The supernatant was collected into a new Eppendorf tube and used for protein quantification with the bicinchoninic acid (BCA) protein determination kit (Pierce®, Thermo Scientific, Basel, Switzerland). Equal amounts of the protein were diluted with a 6× sample

buffer (Tris–HCl, 350 mM, pH 6.8, SDS 10%, DTT 100 mM, glycerol 50%) and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide). A molecular weight marker (Benchmark™, Invitrogen, Basel, Switzerland) was included on each gel. The gels were stained with colloidal Coomassie (Kang et al., 2002).

2.7. Peptide mass fingerprinting

Proteins were identified by peptide mass fingerprinting (Egger et al., 2019; Saraswathy & Ramalingam, 2011). Shortly, polyacrylamide gel pieces were manually excised from the protein bands. The gel pieces were washed three times, alternating between a 100 µL distaining buffer (ammonium bicarbonate 25 mmol/L, acetonitrile 50% v/v) and 100 µL digestion buffer (ammonium bicarbonate 25 mmol/L). The gel pieces were then digested with 2 µL trypsin (4 mg/L) in 20 µL of digestion buffer at 37 °C overnight. After tryptic in-gel digestion, the peptides were separated using high-performance chromatography (HPLC) (Rheos 2200, Flux Instruments) equipped with an XTerra MS C18 column (3.5 mm, 1.0 mm 3,150 mm, Waters). By means of an electron spray ionization interface, the HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland). Protein identifications were performed by submitting the fragmentation data to the Mascot search engine (Matrix Science, London, UK) using UniProt (July 2020) with the following search parameters: enzyme: trypsin; maximum miscleavages: 1; peptide and MS/MS tolerance: 0.8; variable modifications: deamidated (NQ), Gln->pyro-GLU (N-term Q), oxidation (M); significance threshold: $p < 0.05$; ions score cut-off: 20. All the identifications were manually validated according to the following criteria: protein score above 40, peptide score above 25, identification of at least two different peptides, and identification of at least three consecutive fragmentation ions per peptide.

2.8. Analysis of total nitrogen by Kjeldahl

The total nitrogen present in the pellet (P), and in the supernatant (S) after precipitation with MeOH 80 % was quantified with the Kjeldahl method, according to ISO 8968-3:2007/IDF 20-3: 2007 (ISO 8968-3, 2007).

2.9. Acid hydrolysis

The samples were subjected to acid hydrolysis with 6 N HCl in order to measure the total amino acids (TAA) and total amino groups (OPA) contents. Shortly, 220 µL of the supernatant was directly in the glass vials using a CentriVap (Labconco, Kansas City, Missouri USA) and resuspended in 220 µL H_2O, 120 µL 3,3′-dithiodipropionic acid (DDP)/0.1 % NaOH (0.2 mol/L), 120 µL HCl (0.2 mol/L), 40 µL norvaline (NVa; 10 mmol/L), and 500 µL HCl (37 %). The whole digesta pellet was directly weighed into a vial and resuspended with 880 µL

H_2O, 480 µL DDP 0.1 %/NaOH (0.2 mol/L), 480 µL HCl (0.2 mol/L), 160 µL NVa (10 mmol/L) and 2 mL HCl (37 %). All the samples were incubated for 15h at 110 °C.

2.10. Quantification of total amino groups (R-NH₂, OPA method)

After acid hydrolysis (section 2.9) the total amino groups (R-NH_2) in the supernatant and pellets of the precipitated samples were measured using the *o*-phthalaldehyde (OPA) method (Kopf-Bolanz et al., 2012). Briefly, in order to precipitate proteins and longer peptides, the hydrolyzed samples were diluted 10 times with perchloric acid (0.5 mol/L). After derivatization with OPA and in the presence of 2-mercapto-ethansulfonic acid, the produced 1-alkylthio-2-alcylisonindol compounds were measured by UV/VIS photometry at 340 nm. The results were calculated based on a glutamic acid standard curve. A blank digestion (protein-free cookie) was used as background.

2.11. Determination of total amino acids

The total amino acids (TAA) of each indigested substrate were determined as described in ISO 13903:2005 (ISO 13903, 2005). The TAA in the digests were analyzed with the adapted AOAC method 2018.06 for infant formula (Jaudzems et al., 2019). In brief, once hydrolyzed (Section 2.9), every sample was derivatized with AccQ-Tag Ultra reagent (Waters, 2007). The amino acid pattern was determined by ultra-high-performance liquid chromatography (UHPLC) (Acquity UPLC BEH C18 2.1 × 150 mm, 1.7 µm, Waters) coupled with a UV detector (Vanquish, Thermo Scientific, Reinach, Switzerland). The UHPLC conditions were as follows: 2 µL injection volume, column temperature of 50 °C, UV detection at 260 nm, and a flow rate of 0.4 mL/min.

2.12. Determination of individual amino acids of in vitro digesta

The TAA in the *in vitro* digests were measured using the adapted AOAC method 2018.06 for infant formula (Jaudzems et al., 2019). Shortly, once hydrolyzed (Section 2.9), all the samples were derivatized with AccQ-Tag Ultra reagent (Waters, 2007), and the amino acid pattern was evaluated using ultra-high-performance liquid chromatography (UHPLC) (Acquity UPLC BEH C18 2.1 × 150 mm, 1.7 µm, Waters) coupled with a UV detector (Vanquish, Thermo Scientific, Reinach, Switzerland). The UHPLC conditions were as follows: 2 µL injection volume, column temperature of 50 °C, UV detection at 260 nm, and a flow rate of 0.4 mL/min.

2.13. In vitro total digestibility, DIAAR, DIAAS, and proxy DIAAS calculation

As described in Figure 1 from Sousa et al., (submitted manuscript, Chapter 2), total digestibilities of the *in vitro* digested substrates were calculated by calculating the total amounts of N, R-NH_2, or AA in the supernatant and pellets and accounting for all dilution steps

performed during the analytical process. The amino acids in the supernatant and pellet of the protein-free cookie digest, set as the enzyme background, were subtracted from the fractions of the food digests to consider the autolysis of the digestive enzymes. In addition, the amino acids amount from the protein-free cookie digest were set as the minimal. Therefore, values below the enzyme background (resulting from analytical bias) were set to zero. The digestible fraction (supernatant) was then divided by the total (supernatant + pellet).

By multiplying the mg of each indispensable amino acid per g of food protein by the respective digestibility of each indispensable amino acid obtained in the *in vitro* digestion was possible to calculate the digestible indispensable amino acid (DIAA) per gram of food was calculated for each IAA. The DIAAR was calculated as follows: DIAAR (%) = 100 × (mg of *in vitro* digestible dietary IAA in 1 g of the dietary protein) / (mg of the same dietary IAA in 1 g of the reference protein (FAO, 2013). The lowest DIAA ratio (DIAAR) corresponds to the DIAAS.

Proxy *in vitro* DIAAR values were determined by using the total *in vitro* ileal digestibility obtained by TN, OPA, or TAA analysis, instead of the standardized total tract digestibility (%) (Sousa et al., submitted manuscript, Chapter 2). Preschool children (6 month to 3 years) was considered as reference, as recommended by FAO (FAO. 2013), and no truncation was applied for values higher than 100 %. For each indispensable amino acid, the digestible indispensable amino acid (DIAA) per gram of food was calculated by multiplying the mg of indispensable amino acid per g of food protein by the total ileal digestibility value obtained in the *in vitro* digestion.

3. Results

3.1. Substrates composition

All the samples were analyzed for their composition in protein, fat, carbohydrates and moisture (Table 1), as well as the amino acid distribution per g of kg of protein source (Figure 2). The substrates were also characterized in their composition of individual proteins. Therefore, the proteins were extracted and separated by SDS-PAGE for further identification using liquid chromatography-mass spectrometry (LC-MS) after tryptic in-gel digestion. The individual proteins present in the substrates are shown in supplemental figures 1 and 2, and 74 identified modified INFOGEST protocol (Sousa et al., 2022, submitted manuscript, Chapter 2).

To allow the comparability of protein hydrolysis between the samples, all the digestions were normalized according to a protein content of 0.04 g, which was based on a conversion factor of 6.25 for all sources.

Table 1. Composition of the protein sources in protein, fat, carbohydrates and moisture. n.d. = not determined.

(g/100g)	Protein (TN x 6.25)	Fat (OICC)	Carbohydrates (by difference)	Moisture (Oven)
Faba bean concentrate	54.6	3.3	15.43	7.5
Pea isolate	78.6	9.1	0	5.8
Extruded pea & faba	28.7	3.1	2.2	61.1
Pea & faba burger (raw)	18.5	16.8	4.0	55.9
Pea & faba burger (grilled)	20.3	n.d	n.d	n.d
Soy concentrate 70%	62.5	n.d	n.d	n.d
Soy protein based meat analog	23.8	64	< 0.3	6.2
Soy concentrate	64.4	0.26	0	6.0
Texturized soy	27.3	0.31	1.3	65.6
Soy burger (raw)	12.9	13.3	1.6	65.2
Soy burger (grilled)	13.9	n.d	n.d	n.d
Beef meat (raw)	20.7	n.d	n.d	n.d
Beef burger (grilled)	24.1	n.d	n.d	n.d

The substrates had a variable composition, with protein contents between 12.9 g and 78.6 g. Fat and carbohydrates values reached 16.8 g and 15.4 g, respectively. The content of individual amino acids for each sample was analyzed by UHPLC, not considering tryptophan because of its destruction during acid hydrolysis (maximal bias <2 %).

As expected the isolated/concentrated protein powders used as ingredients for the vegan burgers and the meat analogue had higher protein and amino acid contents than the finished products (table 1 and Figure 1). Despite the difference in amino acid content between

the ingredients and the final products, all of them have very similar and high essential/nonessential amino acids ratio (between 3.2 and 3.7) (supplemental table 1).

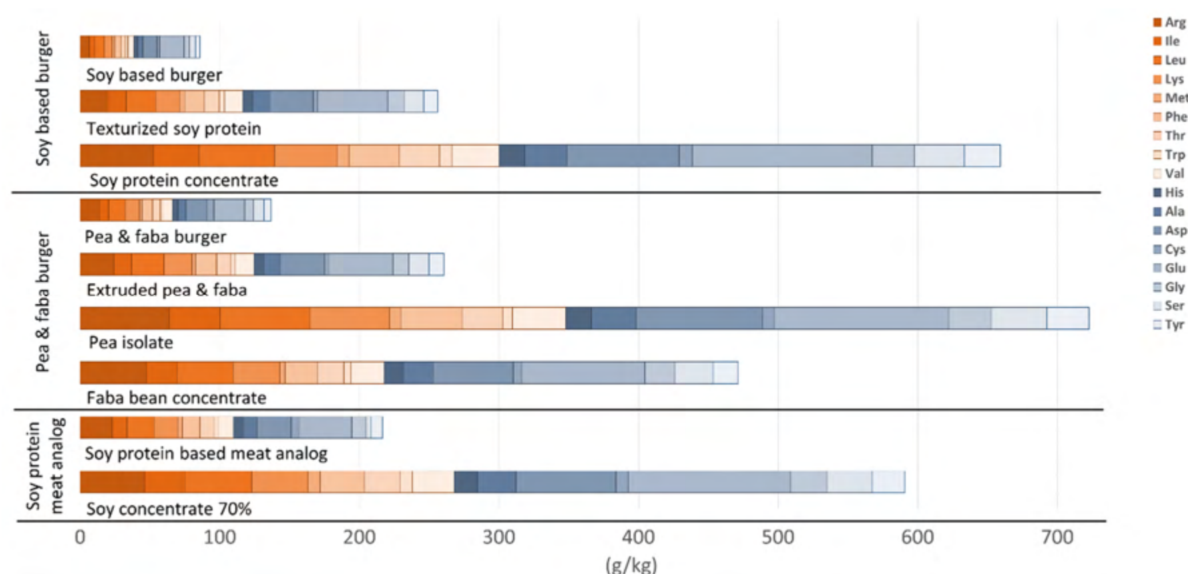


Figure 1. Amino acid composition (g/kg of protein source) of the ingredients and the finished products. Orange: essential AA, blue: non-essential AA.

3.2. Protein digestibility

Amino acid and protein digestibilities were calculated using three different analytical approaches: total nitrogen (TN) by Kjeldahl, total primary amines ($R\text{-}NH_2$) by OPA, and AA by HPLC. For OPA and HPLC measurements the supernatants and pellets from MeOH precipitated intestinal digests were first hydrolyzed with 6 (mol/L) HCl at 110 °C for 15 h.

For all three methods, total digestibility was calculated using the formula given by Figure 1 in Sousa et al., (submitted manuscript, Chapter 2) and described in section 2.13. All the ingredients (soy concentrate 70 %, faba bean concentrate, pea isolate, pea-faba extruded, soy concentrate and texturized soy) were digested together with 0.25 g of cookie to mimic a real meal regarding the macronutrients content. The finished products (soy meat analog, soy burger, and pea-faba burger) and beef were digested alone (Sousa et al., 2022). When raw, the tested plant-based protein sources (ingredients and final products) had a good digestibility with values around 85 % or higher for all the methodologies (Figure 2). Nevertheless, beef meat had a much higher protein digestibility (100 %) compared with the plant-based protein sources. Grilling had no impact on meat protein digestibility, however, it negatively affected the digestibility of both vegan burgers. This decrease on protein digestibility was greater for the pea-faba burger compared to the soy burger. When comparing the three different methods, in general OPA ($R\text{-}NH_2$, blue bars) gave lower values for protein digestibility while Kjeldahl (TN, yellow bars) gave the highest values, with HPLC (TAA, green bars) being in between.

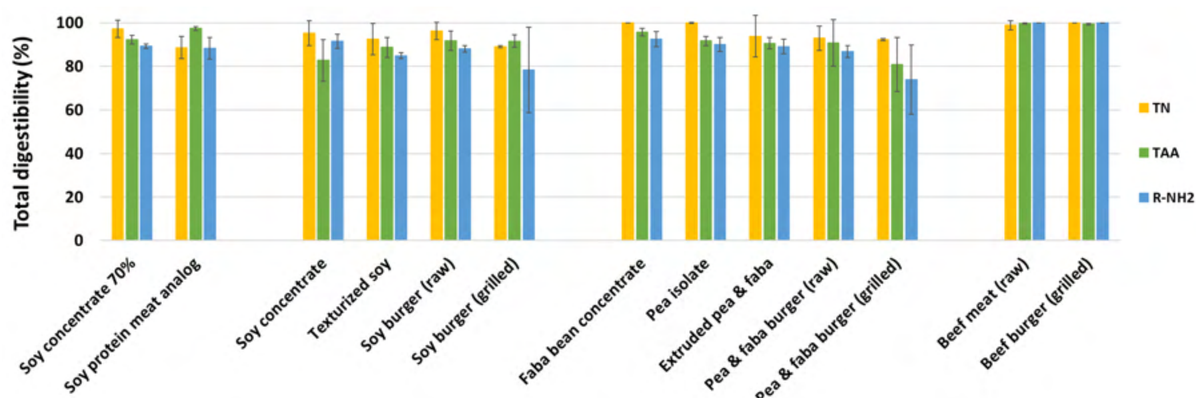


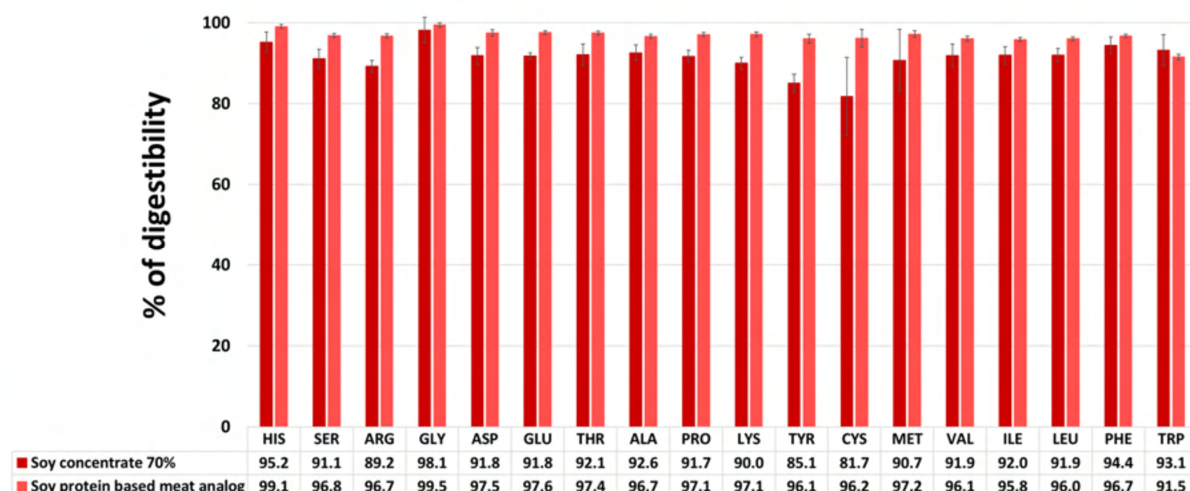
Figure 2. Total digestibility of the ingredients and the finished products. All substrates were analyzed by three different methods. At least three independent experiments were performed, and error bars represent the standard error of the mean (SEM). Release of total amino acids (green), release of primary amines (blue), and total nitrogen (yellow). All the ingredients (soy concentrate 70 %, faba bean concentrate, pea isolate, pea-faba extruded, soy concentrate and texturized soy) were digested together with 0.25 g of cookie to mimic a real meal regarding macronutrients content.

3.3. Individual amino acids digestibility and effect of grilling

The results of the total amino acids analysis by HPLC were used to calculate the digestibilities of each individual amino acid for each substrate. These calculations were made according to the Figure 1 in Sousa et al., (submitted manuscript, Chapter 2) and as described in section 2.13.

Digestibilities of individual amino acids were calculated for ingredients plus cookie and for the finished products in comparison with beef meat burger raw and grilled, respectively. In figures 3, 4 and 5 digestibilities of ingredients together with cookie and the corresponding finished product are compared.

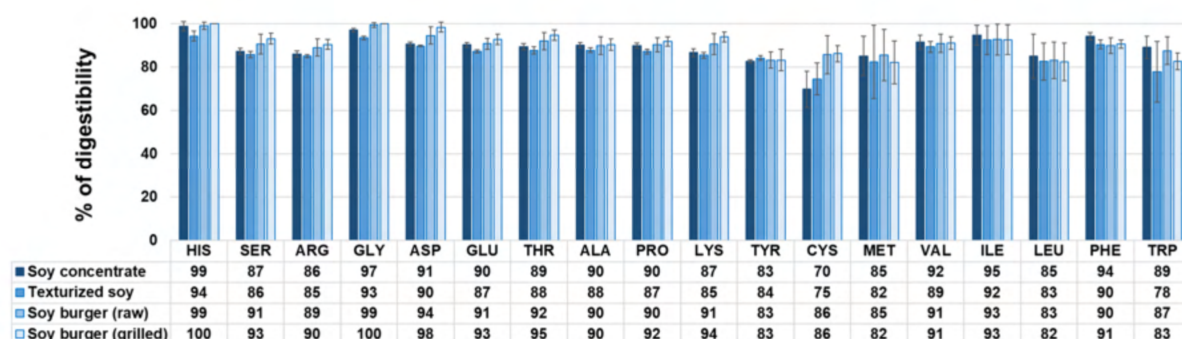
Figure 6 shows the comparison of the digestibility of the individual amino acids between the plant-based burgers (pea-faba burger and soy burger) with beef meat burger under raw and grilled conditions. As shown Figure 3 an improvement in amino acid digestibility was observed when comparing the ingredient (soy concentrate 70 %) with the final product (soy meat analog), indicating a positive effect of the extrusion/production process on digestibility.



	HIS	SER	ARG	GLY	ASP	GLU	THR	ALA	PRO	LYS	TYR	CYS	MET	VAL	ILE	LEU	PHE	TRP
Soy concentrate 70%	95.2	91.1	89.2	98.1	91.8	91.8	92.1	92.6	91.7	90.0	85.1	81.7	90.7	91.9	92.0	91.9	94.4	93.1
Soy protein based meat analog	99.1	96.8	96.7	99.5	97.5	97.6	97.4	96.7	97.1	97.1	96.1	96.2	97.2	96.1	95.8	96.0	96.7	91.5

Figure 3. Digestibility of individual amino acids of the ingredient soy concentrate 70 % together with cookie (dark red) and the finished product soy protein based meat analog (light red), calculated as in (Sousa et al., submitted manuscript, Chapter 2). The error bars are the SD of triplicate analysis.

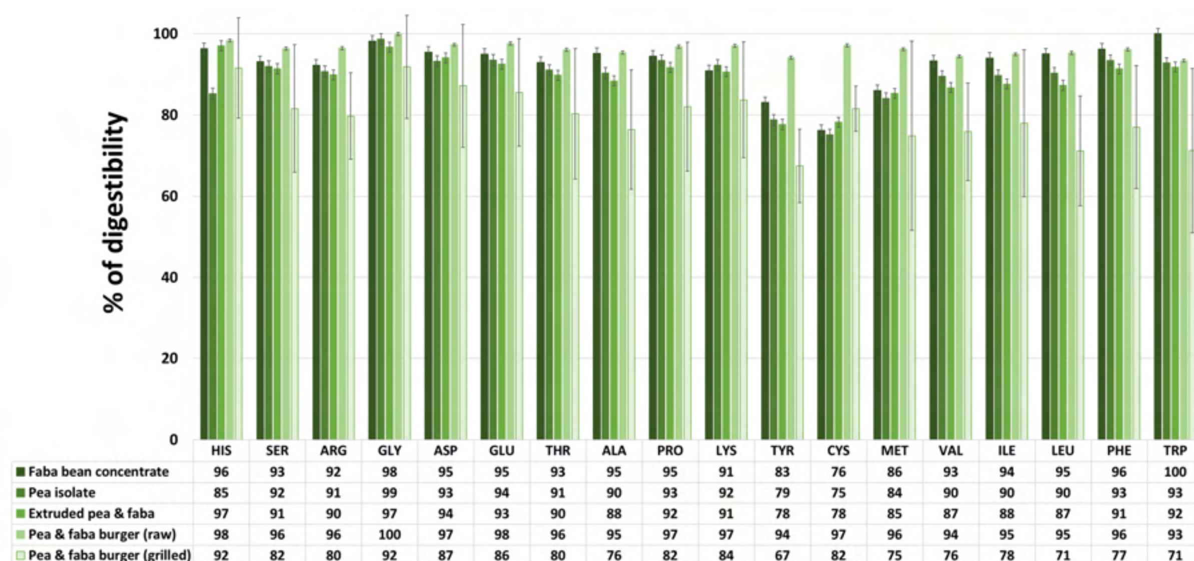
When comparing the results for soy concentrate and texturized soy, the texturizing process seemed not to significantly affect the amino acid digestibility (Figure 4). The finished product (soy burger raw and grilled) presented higher values for amino acid digestibility than its ingredients (soy concentrate and texturized soy). This increase in digestibility was not observed for the amino acids methionine, valine, isoleucine, leucine, phenylalanine and tryptophan, which are virtually unchanged. The effect of grilling was not clear for the soy burger.



	HIS	SER	ARG	GLY	ASP	GLU	THR	ALA	PRO	LYS	TYR	CYS	MET	VAL	ILE	LEU	PHE	TRP
Soy concentrate	99	87	86	97	91	90	89	90	90	87	83	70	85	92	95	85	94	89
Texturized soy	94	86	85	93	90	87	88	88	87	85	84	75	82	89	92	83	90	78
Soy burger (raw)	99	91	89	99	94	91	92	90	90	91	83	86	85	91	93	83	90	87
Soy burger (grilled)	100	93	90	100	98	93	95	90	92	94	83	86	82	91	93	82	91	83

Figure 4. Digestibility of individual amino acids of the ingredients soy concentrate (dark blue) and texturized soy protein (medium blue) together with cookie, and the finished product soy burger raw (light blue) and grilled (very light blue), calculated as in (Sousa et al., submitted manuscript, Chapter 2). The error bars are the SD of triplicate analysis.

In Figure 5, the amino acid digestibilities for the ingredients (faba bean concentrate, pea isolate and extruded pea-faba) and the finished product (pea-faba burger) are shown. Among the ingredients faba bean concentrate is the one having the highest amino acid digestibilities values. For all the amino acids (except tryptophan), the pea-faba burger, had higher digestibility values in comparison to its ingredients. There is no clear effect of the extrusion process on amino acid digestibility. A negative impact of cooking was clear and evidenced by the decrease in digestibility of all amino acids when comparing the values of the raw burger and the grilled burger.



	HIS	SER	ARG	GLY	ASP	GLU	THR	ALA	PRO	LYS	TYR	CYS	MET	VAL	ILE	LEU	PHE	TRP
Faba bean concentrate	96	93	92	98	95	95	93	95	95	91	83	76	86	93	94	95	96	100
Pea isolate	85	92	91	99	93	94	91	90	93	92	79	75	84	90	90	90	93	93
Extruded pea & faba	97	91	90	97	94	93	90	88	92	91	78	78	85	87	88	87	91	92
Pea & faba burger (raw)	98	96	96	100	97	98	96	95	97	97	94	97	96	94	95	95	96	93
Pea & faba burger (grilled)	92	82	80	92	87	86	80	76	82	84	67	82	75	76	78	71	77	71

Figure 5. Digestibility of individual amino acids of ingredients faba bean concentrate (dark green), isolated pea protein (semi-dark green), and extruded pea-faba proteins (medium green) together with cookie, and the finished product pea-faba burger raw (light green) and grilled (very light green) were calculated as in (Sousa et al., submitted manuscript, Chapter 2). The error bars are the SD of triplicate analysis.

In agreement with what was shown in Figure 2, the individual amino acid digestibilities of beef burger were higher than the amino acid digestibility of its vegan analogues (Figure 6). Once more, it the impact of grilling on the amino acid digestibility of the pea-faba burger was clearly visible. This negative impact was not observed for beef and soy burgers.

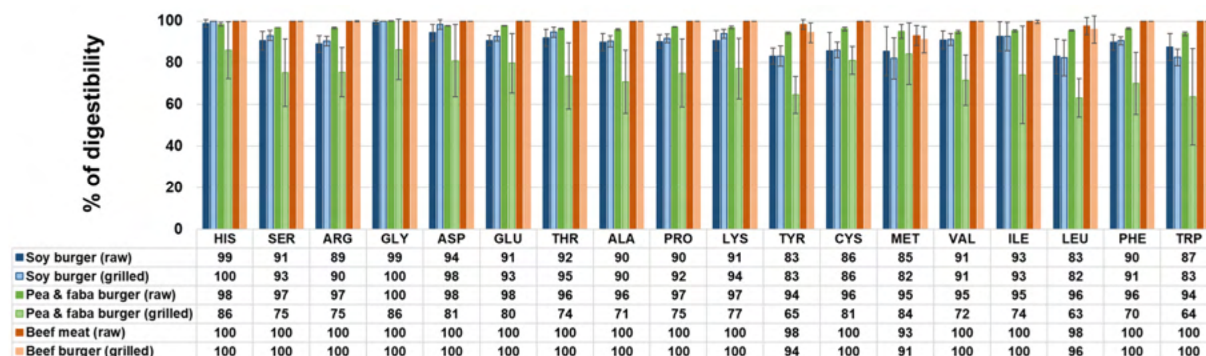


Figure 6. Grilling effect on individual amino acids digestibility. Comparison of digestibility of individual amino acids of the plant based burgers soy burger (blue bars) and pea-faba burger (green bars) with beef meat burger (orange bars) under raw and grilled conditions, respectively. Digestibilities were calculated as in (Sousa et al., submitted manuscript, Chapter 2). The error bars are the SD of triplicate analysis.

3.4. In vitro DIAAR values

The *in vitro* DIAAR values were calculated based on the amount of that AA in the food and the reference requirement values for that AA for preschool children (6 month to 3 years) given by FAO (FAO, 2013) and the digestibility of each individual indispensable amino acid accessed by HPLC.

In line with the data present in Figure 3, DIAAR values of the soy meat analog were higher than the DIAAR values of its ingredient showing a positive effect of the extrusion process.

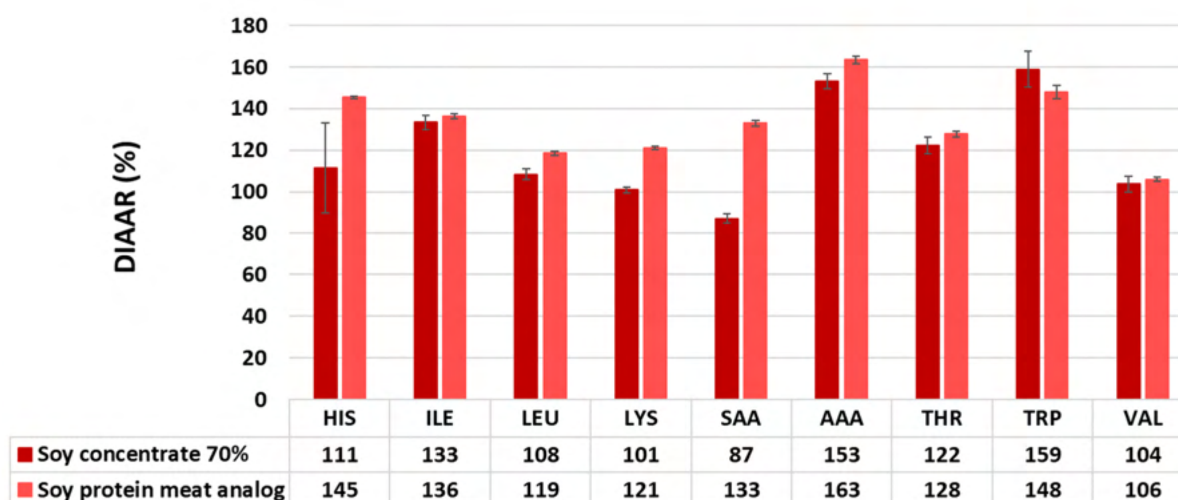
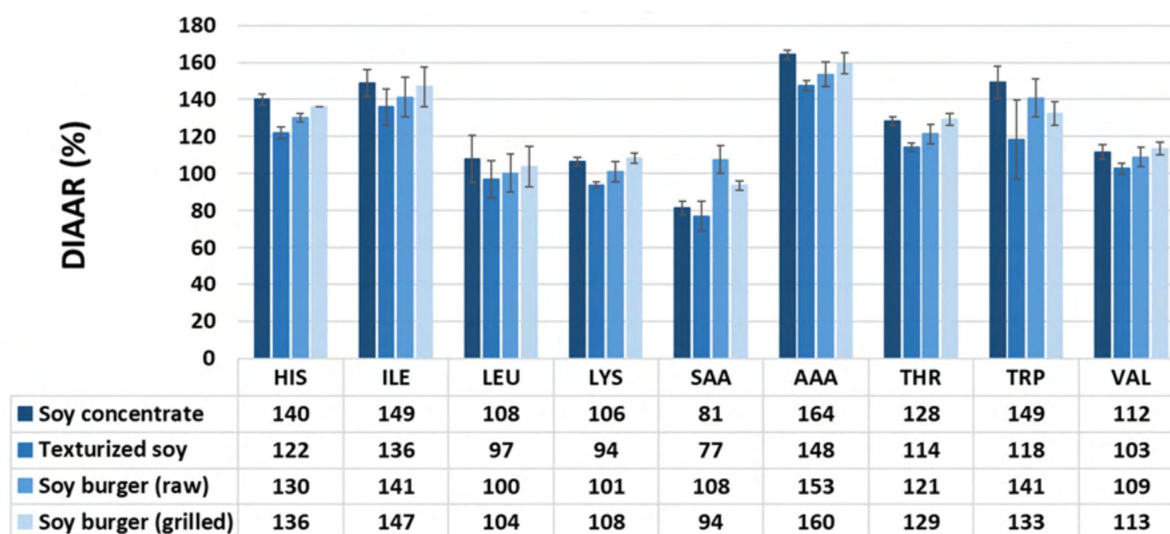


Figure 7. DIAAR values calculated for the ingredient soy concentrate 70 % (dark red) and for the final product soy protein based meat analog (light red). The soy concentrate was digested together with cookie. DIAAR values were based on total protein content (TN*6.25) and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). The error bars are the SD of three analysis.

Unlikely to what is shown in Figure 4, where the texturizing process seemed not to affect the digestibility of the individual amino acids, in Figure 8 the DIAAR values for texturized soy were lower than for the soy concentrate from which it was produced. When comparing soy burger before and after grilling, there was an increase of the DIAAR values (expect for sulfur-containing amino acids and tryptophan), indicating a positive effect of cooking on this parameter.



	HIS	ILE	LEU	LYS	SAA	AAA	THR	TRP	VAL
Soy concentrate	140	149	108	106	81	164	128	149	112
Texturized soy	122	136	97	94	77	148	114	118	103
Soy burger (raw)	130	141	100	101	108	153	121	141	109
Soy burger (grilled)	136	147	104	108	94	160	129	133	113

Figure 8. DIAAR values calculated for the ingredients soy concentrate (dark blue), texturized soy (blue), both plus cookie together with the finished product, soy burger raw (light blue) and grilled (very light blue), respectively. DIAAR values are based on total protein (TN*6.25) content and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). The error bars are the SD of three analysis.

When comparing the pea-faba burger (raw and grilled) with its ingredients, there was a clear drop in DIAAR values, except for sulfur-containing amino acids (Figure 9) indicating a negative effect of the food processing during production. Surprisingly, the grilling effect on DIAAR values was not as pronounced and clear as for the digestibility of the amino acids (Figure 5).

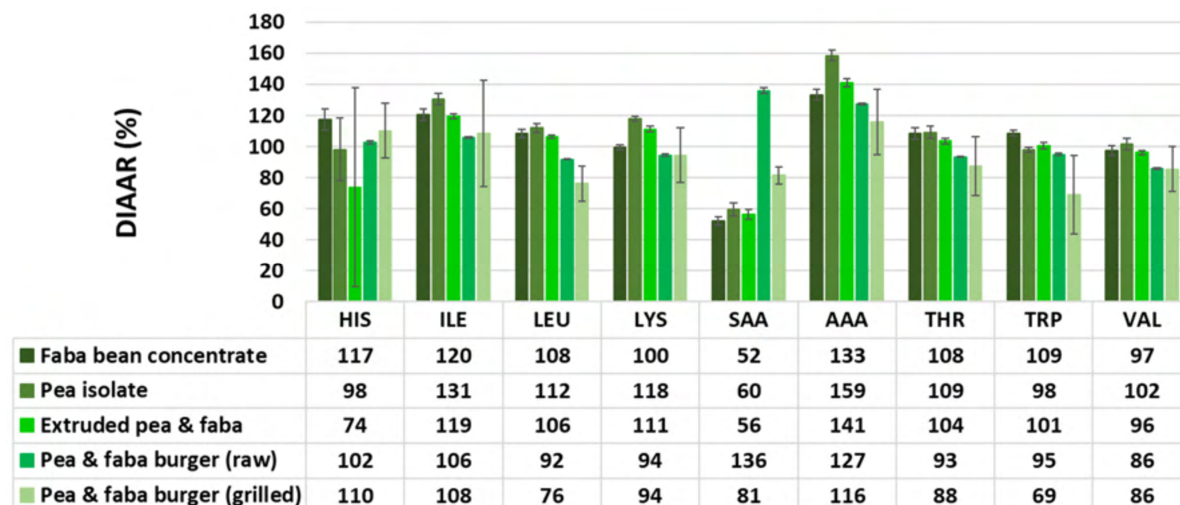
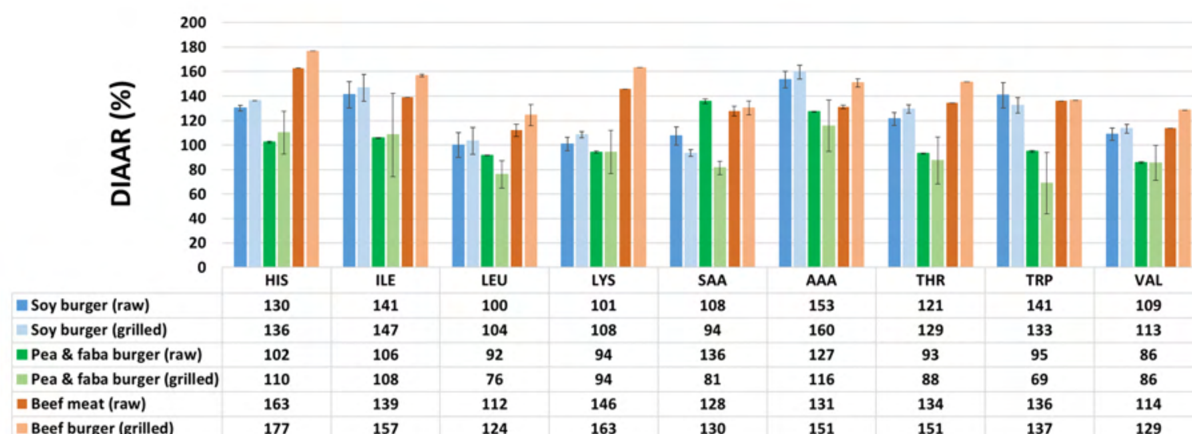


Figure 9. DIAAR values calculated for the ingredients faba bean concentrate (dark green), pea isolate (olive green), extruded pea-faba (neon green), all plus cookie together with the finished product, pea-faba burger raw (light green) and grilled (very light green), respectively. DIAAR values are based on total protein (TN*6.25) content and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). The error bars are the SD of three analysis.

Table 2. *In vitro* DIAAR values compared to *in vivo* DIAAR values determined in the growing pig by (Herreman et al., 2020).

Protein source	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
Fava bean *in vivo*	108 ± 4.1	106 ± 2.2	95 ± 5.4	95 ± 4.3	55 ± 5.1	119 ± 3.4	91 ± 6.2	68 ± 7.8	83 ± 2.2
Faba bean *in vitro*	117	120	108	100	52	133	108	109	97
Pea *in vivo*	99 ± 9.7	101 ± 13.1	87 ± 11.5	110 ± 10.8	70 ± 12.3	116 ± 16.3	94 ± 7.9	77 ± 7.1	83 ± 9.8
Pea *in vitro*	98	131	112	118	60	159	109	98	102
Soy *in vivo*	119 ± 9.4	124 ± 8.3	102 ± 6.1	96 ± 9.0	91 ± 11.5	147 ± 8.3	105 ± 6.0	132 ± 21.1	95 ± 7.3
Soy concent. 70% *in vitro*	111	133	108	101	87	153	128	122	104

In agreement with figures 2 and 6, beef burger presents the higher DIAAR values when comparing with soy and pea-faba burgers (Figure 10). A positive effect of grilling on DIAAR values was observed for beef burgers but was not clear or even decreased for the vegan burgers. Nonetheless, there is a slight increase in DIAAR values for the grilled soy burger and a slight decrease for pea-faba burger.



	HIS	ILE	LEU	LYS	SAA	AAA	THR	TRP	VAL
Soy burger (raw)	130	141	100	101	108	153	121	141	109
Soy burger (grilled)	136	147	104	108	94	160	129	133	113
Pea & faba burger (raw)	102	106	92	94	136	127	93	95	86
Pea & faba burger (grilled)	110	108	76	94	81	116	88	69	86
Beef meat (raw)	163	139	112	146	128	131	134	136	114
Beef burger (grilled)	177	157	124	163	130	151	151	137	129

Figure 10. DIAAR values calculated for the pea-faba burger (green), soy based burger (blue), and beef meat burger (orange) under raw (darker color) and grilled (lighter color) conditions, respectively. DIAAR values were based on total protein (TN*6.25) content and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). The error bars are the SD of at least three analysis.

4. Discussion

After the intestinal digestion and precipitation, three different analytical approaches were performed to access protein and individual amino acids digestibilities. All the ingredients of the plant-based burgers were digested together with 0.25 g of protein-free cookie to better simulate the macronutrient composition of a real meal (Sousa, et al., 2022, submitted), whereas the three burgers were digested alone.

The *in vitro* digestibility based on total protein TN gives higher values than the digestibility based on primary amines (OPA, R-NH2), and TAA, as shown in Fig. 1. For all the three methods, the digestibility was higher for beef burgers than for its vegan counterparts as expected. However, when not grilled, the tested plant-based protein sources (ingredients and final products) had good digestibilities with values around 85 % or higher.

Interestingly, grilling seemed not to have any impact on meat protein digestibility, however, for both vegan burgers, this cooking process had a negative impact on protein and amino acids digestibilities (figure 2 and 6). Pea-faba burgers seemeds to be the most affect by the grilling process, whereas this impact was less significant for the soy burger. The occurrence of Maillard reactions and consequent generation of Maillard reaction products are an undesirable effect of the protein-carbohydrate complexes presented on processed foods thtat are subjected to heat (Jaeger et al., 2010). Maillard reactions cause nutritional losses of amino acids and decreased amino acid digestibilities (Almeida et al., 2014; González-Vega et al.,

2011). As indicated in table 1, pea-faba burger has more than the double of carbohydrates content compared to the soy burger, therefore, the probability of occurrence of Maillard reactions on pea-faba burger is higher than for the soy burger. This can explain the decrease in digestibility observed for the grilled pea-faba burger but not for the grilled soy burger.

The *in vitro* DIAAR values for all the essential amino acids were calculated based on total protein (TN*6.25) content and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). Our results did not provide clear evidences that texturing process affected negatively the protein digestibility (figure 4 and 5). However, DIAAR values of the texturized soy were lower than the values of its precursor (Figure 8) indicating a negative impact of the texturizing process on this parameter, whereas this effect was not clear for the texturized pea-faba (figure 9).

Animal proteins are considered high-quality/complete proteins due to their high protein digestibility and amino acids profile. On the other hand, most of the plant proteins (except soy protein) are incomplete (lack one or more essential amino acids) and have lower digestibilities due to the presence of antinutritional factors (Mariotti, 2017). In agreement with this, our digestibility and DIAAR values for beef were higher than the values of the tested meat analogues. The difference in DIAAR values was bigger between beef and pea-faba burger than soy, which is in alignment with the concept of soy being a complete protein. Both plant based burger variants however can be considered as good (pea-faba burger) or even excellent (soy burger) protein sources according to FAO (FAO, 2013).

When compared to *in vivo* DIAAR values determined in growing pigs, our results for faba bean, pea, and soy concentrate 70 % show a good agreement (table 2), proving the robustness of the adapted INFOGEST *in vitro* digestion protocol.

5. Conclusion

In recent years, vegetable proteins have been widely used as ingredients in the food industry because of their relatively low cost, higher sustainability, reduced impact on the environment as well as reducing the ethical concerns when compared with animal proteins. Soy protein is the best known and widely used plant protein source since many years for a high variety of food products (e.g. tofu, soy-milk, yogurts, snacks and meat analogues). However, other legumes such as green peas, chickpeas, lentils, and faba beans are attracting the attention from the food industry. Therefore, it is important to understand and better know these new protein sources especially when it comes to digestibility, since it is well-known that plant proteins have in general a lower protein quality and as well digestibility than animal proteins. The alternative proteins sources tested in the present work proved to be good alternatives to meat due to their good protein digestibilities and amino acid profiles. Soy protein

seems to be more stable to temperature than pea and faba proteins and therefore, an improvement of the recipe and characteristics of the pea-faba burger would be recommended. The adapted INFOGEST *in vitro* digestion protocol proved to be suitable to access digestibility of highly processed and heated protein sources.

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Supplementary material

Supplemental table 1. Nonessential amino acids (NEAA) and the essential amino acids (EAA) ratios

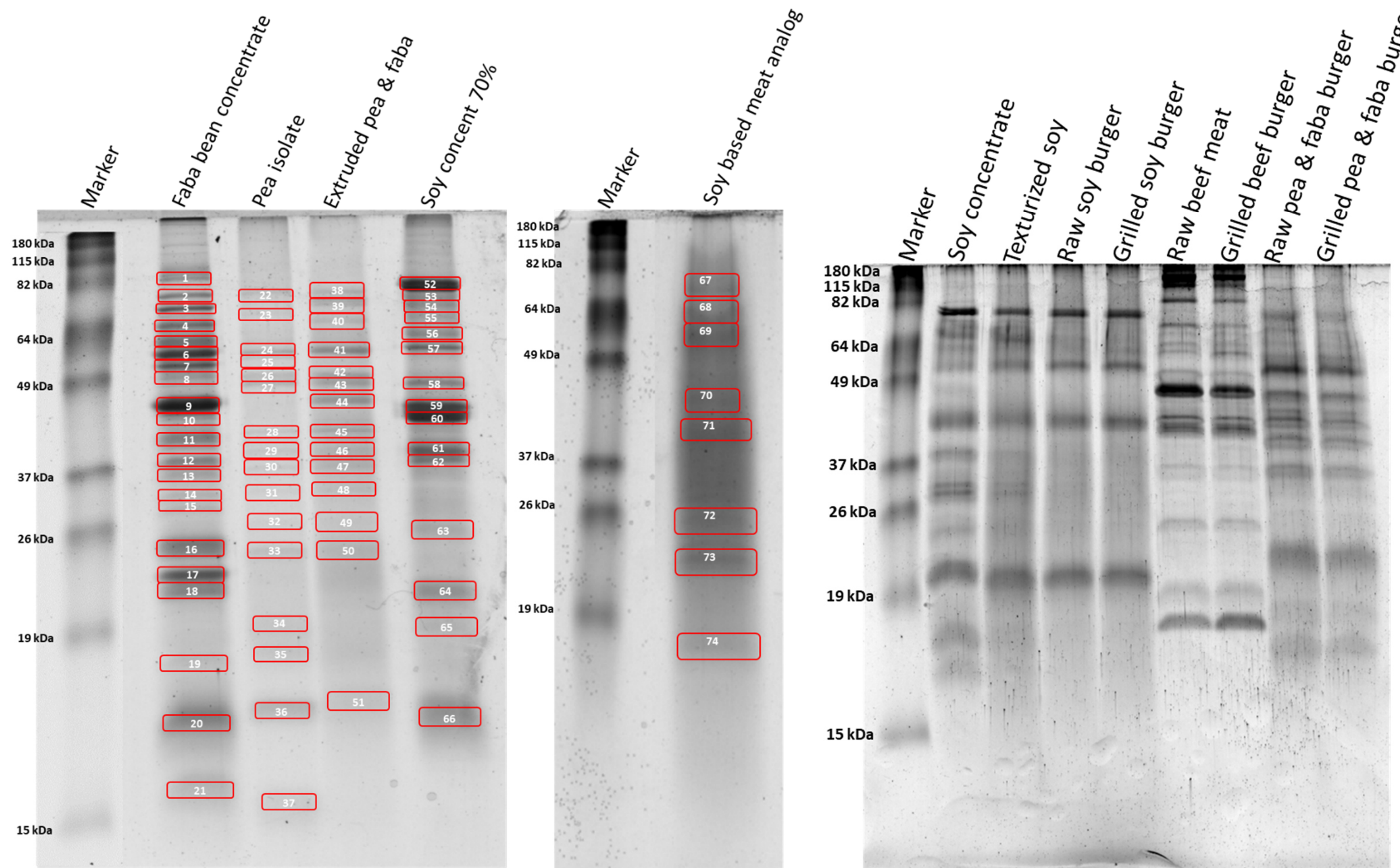
	Sum NEAA (g/kg product)	***Sum EAA (g/kg product)***	***EAA/NEAA***
Soy concentrate 70%	268.0	978.5	3.7
Soy protein based meat analog	110.0	347.5	3.2
Faba bean concentrate	218.0	771.1	3.5
Pea isolate	348.1	1166.5	3.4
Extruded pea & faba	124.7	421.3	3.4
Pea & faba burger	66.3	221.7	3.3
Soy protein concentrate	300.4	1091.9	3.6
Texturized soy	116.8	423.3	3.6
Soy based burger	38.6	142.1	3.7

Suppl. table 1. *Sum of the nonessential amino acids (NEAA) and the essential amino acids (EAA) and the EAA/NEAA ratio.*

Supplemental table 2. Identified proteins using peptide fingerprinting

Band nr.	Protein	Band nr.	Protein	Band nr.	Protein	Band nr.	Protein
1	LOX2_PEA; LOX3_PEA; LOXB_PHAVU	20	ALB1E_PEA	39	CVCA_PEA	58	GLYG5_SOYBN; GLYG4_SOYBN; GLYG1_SOYBN; GLCA1_SOYBN
2	HSP70_SOYBN	21	ALB1C_PEA	40	CVCA_PEA	59	GLYG4_SOYBN; GLYG2_SOYBN; GLYG1_SOYBN
3	CVCA_PEA	22	n.i	41	VCLC_PEA	60	GLYG2_SOYBN; GLYG1_SOYBN; GLYG4_SOYBN; GLYG3_SOYBN
4	CVCA_PEA; SBP65_PEA; LEGA2_PEA	23	n.i	42	LEGA2_PEA; LEGJ_PEA	61	GLYG4_SOYBN; LEC_SOYBN; GLYG2_SOYBN; GLYG1_SOYBN
5	CVCA_PEA	24	n.i	43	LEGA2_PEA; LEGJ_PEA	62	GLYG4_SOYBN; GLYG2_SOYBN; GLYG1_SOYBN
6	VCL_VICFA ; VCLC_PEA	25	VCLC_PEA	44	LEGB2_VICFA; VCLC_PEA; LEGA2_PEA	63	GLYG4_SOYBN
7	MATK_MEDSA	26	LEGA2_PEA	45	VCLB_PEA; VCL1_PEA	64	GLYG1_SOYBN; GLYG2_SOYBN; ITRA_SOYBN; GLYG5_SOYBN; GLYG4_SOYBN
8	ALF2_PEA; ADH1_PEA; CVCA_PEA; CVCB_PEA; LEG_CICAR	27	LEGA2_PEA; LEGJ_PEA	46	VCLB_PEA; VCLC_PEA	65	GLYG1_SOYBN; HSP12_SOYBN
9	LEGB4_VICFA; LEGB7_VICFA; LEG_CICAR	28	VCLB_PEA	47	VCLC_PEA; VCLA_PEA	66	2SS_SOYBN
10	VCL_VICFA; LEGB7_VICFA	29	n.i	48	ALB2_PEA; VCLC_PEA	67	GLYG4_SOYBN; PM1_SOYBN
11	VCL_VICFA; LEG_CICAR; LEGB2_VICFA; LEGB4_VICFA; VCLC_PEA	30	VCLC_PEA	49	LEGA2_PEA	68	GLYG4_SOYBN; GLYG2_SOYBN
12	VCL_VICFA; VCLC_PEA	31	VCLC_PEA	50	LEGA2_PEA; VCLB_PEA; LEGB_PEA	69	GLYG4_SOYBN; GLYG5_SOYBN; PM1_SOYBN
13	VCL_VICFA; VCLC_PEA	32	n.i	51	VCLB_PEA	70	GLYG4_SOYBN; GLYG2_SOYBN; GLYG5_SOYBN; GLYG1_SOYBN; GLYG3_SOYBN; SLE1_SOYBN
14	n.i	33	LEGA2_PEA	52	GLCAP_SOYBN; GLCA1_SOYBN	71	GLYG2_SOYBN; GLYG4_SOYBN
15	REHY_MEDTR	34	n.i	53	GLCA1_SOYBN; GLCAP_SOYBN	72	GLYG2_SOYBN; GLYG4_SOYBN; 2SS_SOYBN
16	LEGA2_PEA	35	n.i	54	GLCA1_SOYBN; GLCAP_SOYBN; SBP65_SOYBN	73	GLYG4_SOYBN; GLYG2_SOYBN; 2SS_SOYBN
17	LEGB2_VICFA; VCLB_PEA; LEGA2_PEA; VCL_VICFA	36	VCLB_PEA	55	GLCA1_SOYBN; GLCAP_SOYBN; GLYG2_SOYBN; SBP_SOYBN; GLYG1_SOYBN; GLYG4_SOYBN	74	GLYG4_SOYBN; GLCA1_SOYBN
18	LEGB_PEA; LEGB2_VICFA; LEC_VICFA; VCL_VICFA; LECB_LATOC; VCLC_PEA	37	ALB1A_PEA; ALB1C_PEA	56	GLCA1_SOYBN; GLCAP_SOYBN; GLYG1_SOYBN; GLYG4_SOYBN		
19	VCL1_PEA	38	CVCB_PEA	57	n.i		

Supplemental figure 2. SDS-PAGE gels of the different protein sources



Suppl. figure 2. SDS-PAGE gel from the different protein sources and position of the identified proteins.

Chapter 4

Assessment of the physiological relevance of the *in vitro* digestion protocol by evaluating the influence of digestion products on satiety using STC-1 cell lines

Manuscript 4

Stimulation of CCK and GLP-1 secretion and expression in STC-1 cells by *in vitro* gastrointestinal digests

Raquel Sousa[1,2], Reto Portmann[1], Lotti Egger[1], Marta Santos-Hernández[2], and Isidra Recio[2]

[1]Agroscope, Schwarzenburgstrasse, 161, 3003 Bern, Switzerland
[2]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain

Abstract

The present study evaluates gastrointestinal digests from seven protein sources: two isolated proteins (zein and collagen) and five foods (peanuts, sorghum flour, All-Bran® cereals, pigeon peas, and black beans) as inducers of cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) secretion and expression in STC-1 cells. All the seven protein sources were digested according to the *in vitro* digestion INFOGEST protocol and have been split into two fractions: soluble (bioavailable) fraction and solid (non-bioavailable) fraction. Secretion and gene expression of CCK and GLP-1 were evaluated in response to both gastric and intestinal soluble fractions. All digests showed dose-response effects for both, gastric and intestinal digests. In general, higher hormonal secretion was achieved in response to intestinal digests compared to gastric ones, with a significant difference for CCK release ($p < 0.023$) but not for GLP-1. The presence of small size peptides and free amino acids in the intestinal fractions induced a more potent CCK and GLP-1 secretion than gastric fractions. A positive correlation between protein and carbohydrate contents of the digests was observed for CCK and GLP-1, respectively. A dose-dependent behaviour was also observed for CCK and GLP-1 secretion. Both, GLP-1 and CCK secretion were maximal for intestinal fractions from black bean, sorghum, All-Bran® and zein. However, GLP-1 secretion was more intense than CCK secretion. Moreover, a marked increase in CCK mRNA levels for both gastric and intestinal fractions was observed. GLP- 1 mRNA levels were increased for zein, sorghum and black beans digests. Different peptides from zein and collagen digestion-resistant sequences were synthesized and tested for their ability to activate the enteroendocrine cell line STC-1. A clear sequence specificity was observed, leading to an increase in intracellular Ca^{2+} concentration. Our results demonstrate that the degree of protein hydrolysis during digestion plays an important role in CCK and GLP-1 secretion.

Keywords

Satiety, CCK, GLP-1, hormone secretion, *in vitro* digestion, ELISA, RT-qPCR, intracellular calcium

Abbreviations

CaSR, Calcium-sensing receptor; CCK, Cholecystokinin; cDNA, complementary DNA; DPP-IV, Dipeptidyl Peptidase IV; EECs, Enteroendocrine cells; ELISA, Enzyme-linked Immunosorbent Assay; GI, gastrointestinal tract; GLP-1, glucagon like peptide-1; HPLC-MS, High-Performance Liquid Chromatography- Mass Spectrometry; IVD, *in vitro* digestion; LC-MS, Liquid Chromatography -Mass Spectrometry; mRNA, messenger RNA; RNA, Ribonucleic acid; RT-PCR, *R*everse transcription polymerase chain reaction; RT-qPCR, Quantitative RT-PCR; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

1. Introduction

Dietary proteins have a wide range of nutritional and biological functions. In addition to their nutritional function as source of amino acids for protein synthesis, dietary proteins play a key role in regulation of food intake, glucose and lipid metabolism, blood pressure, bone metabolism and immune function. In the gastrointestinal (GI) tract, proteins and their digestion products induce several regulatory functions by interacting with receptors leading to a release of hormones, modulating stomach emptying, motility, appetite, and absorption (Gribble, 2018; Jahan-Mihan et al., 2011). Protein and GI tract interaction is source-dependent and based on protein characteristics, such as physico-chemical properties, amino acid composition and sequence, digestion kinetics, bioactive peptides and associated non-protein bioactive components (Jahan-Mihan et al., 2011).

Representing less than 1 % of the epithelial population, enteroendocrine cells (EECs) constitute the largest endocrine organ of the human body. More than 20 different types of EECs produce and secrete a variety of hormones. These cells are classified according to their shape and epithelial localization into two different groups: 'open-type cells' and the 'closed-type cells'. The open-type cells have microvilli that allow direct detection of the luminal content. This triggers the EECs to secrete hormones that will enter the blood vessels and activate extrinsic or intrinsic afferent nerves or other neighbouring cells. On the other hand, closed-type cells do not reach the epithelial surface and are only indirectly affected through neural pathways or via signals coming from the bloodstream (Janssen & Depoortere, 2013).

Among all macronutrients, protein has been reported as the most satiating macronutrient, relative to the caloric content (Soenen & Westerterp-Plantenga, 2008). Many factors are known to play a role in this satiating effect. After ingestion, proteins are partially hydrolysed by pepsin in the stomach. The protein hydrolysis is completed in the small intestine by several pancreatic proteases, of which trypsin is the most important. The generated peptides stimulate the secretion of anorexigenic hormones by the intestinal enterocytes, such as cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1). These hormones regulate the appetite and food intake, insulin secretion and energy expenditure (Anderson & Aziz, 2006; Janssen & Depoortere, 2013). CCK is manly secreted by I-cells located in the duodenum after the ingestion of fat or protein. Luminal peptides, resulting from protein gastrointestinal digestion stimulate CCK secretion via calcium-sensing receptor (CaSR) or GPR93 activation, causing an intracellular Ca^{2+} increase (Caron et al., 2017). CaSR is not only activated by ligands, such as aromatic amino acids, polyamines, and basic polypeptides (protamine, poly-lysine, and poly-arginine) but as well by extracellular calcium (Nakajima et al., 2012). CCK contributes to satiety by inhibiting gastric emptying, sustaining gastric distension, stimulating pancreatic secretion and gallbladder contraction (Dockray, 2009).

In vivo, CCK secretion is regulated via negative feedback in which active proteases, present in the pancreatic secretion, inhibit CCK secretion. Enteroendocrine cells secrete a luminal CCK releasing factor, which binds to receptors on the secreting cells of the gut and stimulates CCK secretion. In the presence of trypsin, the CCK releasing factor is degraded and inactivated. However, in the presence of dietary proteins, which are substrates for trypsin, binding competition occurs. This competition allows CCK releasing factor to remain active and interact with CCK secreting cells, leading to CCK secretion (Green & Lyman, 1972).

GLP-1 derives from post-translational modification of the larger precursor molecule proglucagon. In response to food intake, L-cells, distributed throughout the small and large intestines, synthesize proglucagon. GLP-1 elicits a range of biological functions, like stimulating glucose-dependent insulin secretion and insulin biosynthesis, reducing glucagon secretion, regulating acid secretion, gastric emptying, and gut motility, via the "ileal break" mechanism, and reduces food intake (Caron et al., 2016). After food intake, a nutrient-stimulated increase on GLP-1 levels occurs. However, native GLP-1 has a very short half-life mainly due to renal clearance and the GLP-1 N-terminal degradation by dipeptidyl peptidase IV (DPP-IV) (Jao et al., 2015). Peptides resulting from protein gastrointestinal digestion stimulate GLP-1 secretion via CaSR or GPRC6A activation (Caron et al., 2017).

Due to the ability to induce anorexigenic hormone production and satiety properties, dietary proteins contribute to the termination of the meal. Therefore, identification of specific proteins, protein hydrolysates or even specific peptides with optimized satiety hormone releasing properties can be an interesting target for developing functional food products for weight management purposes (Paddon-Jones et al., 2008; Westerterp-Plantenga et al., 2006). Many studies have been performed in order to assess the release of gut-derived hormones from isolated enteroendocrine cells in response to protein ingestion. Murine secretin tumour cells STC-1, have been commonly used in these kind of studies, since this cell line closely resembles of native intestinal enteroendocrine cells. They possess many similar features, such as the high endogenous expression levels of CCK and GLP-1 in response to physiological stimuli (Cordier-Bussat et al., 1998; Geraedts et al., 2011; Rindi et al., 1990).

In the present study, gastrointestinal digests from seven different protein sources were tested as inducers of CCK and GLP-1 secretion and receptor expression in the enteroendocrine cell line, STC-1. For this purpose, black beans, pigeon peas, All-Bran® cereals, sorghum flour, peanut, zein, and collagen were digested according to the harmonised INFOGEST *in vitro* digestion protocol. Protein, total and free fatty acids, and total and free glucose were determined in the digests. In addition to the protein content, free amino acids content was also determined. In order to establish a relationship between the observed effect and the digestion-derived peptide composition, a detailed peptidomic characterization of the gastrointestinal digests was performed by HPLC-mass spectrometry (HPLC-MS). CCK and

GLP-1 secretion and gene expression in STC-1 cells were evaluated after incubation with gastric and intestinal *in vitro* digests using ELISA and RT-qPCR, respectively.

2. Materials and methods

2.1. Sample preparation for in vitro digestion (IVD)

Seven protein sources, two isolated proteins (zein and collagen) and five foods (peanuts, sorghum flour, All-Bran® cereals, pigeon peas, and black beans) were *in vitro* digested according to the INFOGEST protocol (Brodkorb et al., 2019). Before digestion, black beans and pigeon peas were previously soaked and cooked. In brief, 40 g of each product was soaked in water for 18 h; after they were cooked in 200 mL of water and 288 mg of salt for 10 min (pigeon peas) or 20 min (black beans). Once cooked, the beans and peas were ground to simulate mastication. All-Bran® cereals and peanuts were ground as well with the same purpose. Sorghum flour, collagen, and zein powders were not subjected to any mechanical or heat treatment.

2.2. In vitro digestion with the INFOGEST static model

All substrates were digested according to the *in vitro* gastrointestinal INFOGEST protocol (Minekus et al., 2014). The enzyme activities and bile concentration were measured prior to the digestion experiment using the assays described in the harmonized protocol (Brodkorb et al., 2019). Briefly, for the digestion experiment, the amount of each substrate corresponding to 0.04 g of protein was dissolved in 1 mL of water and mixed with 1 mL of simulated salivary fluid (pH 7, 37 °C), containing amylase (300 U/mL of digesta), for 2 min. Then, 2 mL of simulated gastric juice (pH 3, 37 °C), containing pepsin (2000 U/mL of digesta), were added and incubated for 120 min. Subsequently, 4 mL of simulated intestinal juice (pH 7), containing pancreatin (100 U trypsin activity/mL of digesta) and bile (2.5 mmol/L of total digesta), were added and incubated for 120 min. Bile salts were added at a lower concentration than the one recommended in the original protocol (10 mmol/L of total digesta), due to cytotoxic effects on the cell line (Santos-Hernández et al., 2018). The whole digestion protocol was performed at 37 °C, under constant gentle mixing, on a rotating wheel. Digestion was stopped after 120 min of gastric digestion, by increasing the pH to 7 with NaOH (1 mol/L) and the intestinal phase was stopped by heating at 85 °C for 15 min. Immediately after stopping the digestion, all the samples were snap frozen in liquid nitrogen and kept at −20 °C until analysis. After de-freezing, the samples were split by centrifugation (13,000 × g at 4 °C for 15 min) into a soluble and insoluble fraction, by collecting the supernatant and the pellet, respectively.

2.3. *Gel electrophoresis*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described by Sanchón, et al., 2018 (Sanchón et al., 2018), with some adaptions: the undigested protein sources were dissolved at 0.5 mg of protein per mL in sample buffer, the soluble fractions at 2.5 mg/mL, and the insoluble fractions at 1.5 mg/mL. The gels were stained with Coomassie Blue (Instant Blue, Expedeon, Swavesey, UK) and images were taken with a Molecular Imager VersaDoc™ MP 5000 system (Bio-Rad, Hercules, CA, USA) and processed with Quantity One 1-D analysis software (Bio-Rad).

2.4. *Protein content, nitrogen and free amino acids determination*

The protein content of the undigested samples was determined by Kjeldahl according to ISO 8968-3:2007/IDF 20-3: 2007 (ISO 8968-3, 2007). Measurements were done in duplicate. Nitrogen distribution on the digests was determined using Dumas methodology (https://www.uam.es/uam/sidi/unidades-de-analisis/unidad-analisis-elemental/analisis-quimico-elemental) as a service provided by the Servicio Interdepartamental de Investigación de la Universidad Autónoma de Madrid. For free amino acids determination, freeze-dried samples were dissolved to a concentration of 4 mg of protein per mL in Milli-Q water. Then, proteins were precipitated with 5-sulfosalicylic acid at 4 °C for 1 h, and were centrifuged at 15,000 × g, at 4 °C for 15 min. The pH of the supernatant was adjusted to 2.2 with 0.3 mol/L NaOH and filtered through a 0.45 µm filter prior to analysis. The analysis was carried out in a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd, Cambridge, UK), equipped with a cation-exchange column. The post-column derivatization was achieved by mixing the eluent column with ninhydrin and by passing this mixture through a high temperature reaction coil. Finally, absorption was measured at 440 and 570 nm.

2.5. *Determination of total and free glucose*

The starch content of the undigested samples was measured using the Total Starch Assay Kit (AA/AMG) from Megazyme (Megazyme u.c., Wicklow, Ireland). Measurements were done in duplicate.

The free glucose present in the soluble fractions of the intestinal digests was determined with an enzymatic method where glucose is first phosphorylated through the action of hexokinase, yielding glucose-6-phosphate under the concomitant reduction of the present NAD+ into NADH, by the highly specific enzyme glucose-6-phosphate-dehydrogenase. The so generated NADH is equimolar to the glucose in the original sample and is analyzed spectrophotometrically at 340 nm.

2.6. Determination of total fat and free fatty acids

The fat content in the different substrates was determined according to ISO standard 1735:2004 (ISO 1735, 2004). The free fatty acids were extracted as described by Villasenor et al., 2014 (Villaseñor et al., 2014). Briefly, 6 mg of sample (lyophilized supernatants) were disolved with 30 µL milli-Q water and 175 µL of metanol and 175 µL of MTBE were added. Then, the samples were vortexed for 1 min and centrifuged at 3200 rpm at 15° C for 15 min. The supernatants were collected and evaporated with N_2 and dissolved with 100 µL of dichloromethane for injection by HPLC (Shimadzu Vp Series, Duisburg, F.R., Germany) coupled with an ELSD detector (SEDERE. SEDEX 85 model, Alfortville Cedex, France) (Rodríguez-Alcalá & Fontecha, 2010).

2.7. Analysis by HPLC-mass spectrometry (HPLC-MS)

Peptide identification in the digests was performed as described by (Egger et al., 2019) with minor modifications. Briefly, the digested samples were filtered through Amicon columns (Ultracel YM-30, Millipore, Zug, Switzerland), and the peptides were identified via HPLC (Rheos 2200, Flux Instruments), which was equipped with an XTerra MS C18 column (3.5 mm, 1.0 mm 3 150 mm, Waters) and coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland). The samples were measured in three overlapping narrow-mass windows for peptide fragmentation over a total range of 300-1300 m/z (i.e., 300-600, 600-900, and 900-1300 m/z). The obtained raw files were merged with Mascot Daemon, prior to the identification search with Mascot, using specific protein databases for zein and collagen. Peptides with a minimal length of 5 amino acids and an ion score cut-off of 20 were considered. Peptides were ordered according their abundance on the mass spec and their hydrophobicity for further selection.

2.8. Cell viability

STC-1 cells were seeded into 96-well plates (5×10^4 cells per well) and cultured at 37 °C for 24 h in a humidified atmosphere containing 5 % CO_2. The cells were exposed to the soluble fraction of the seven protein digests for 2.5 h, after which the medium was removed from the wells. Alamar Blue Cell Viability Reagent (ThermoFisher Scientific) (100 µL, diluted 1:10 v:v) was added to the cell plate and incubated at 37 °C for 1 h. Fluoresce was measured using an excitation wavelength of 570 nm and fluoresce emission at 600 nm in a FLUOstar OPTIMA microplate reader (BMG Labtech, Biogen Científica, S.L. Madrid, España). For the synthetic peptides, cell viability was tested following the same protocol. Th digests and the synthetic peptides were diluted in HEPES buffer (20 mmol/L HEPES 1 M, 10 mmol/L glucose, 140 mmol/L NaCl, 4.5 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, pH 7.4) and incubated for 2 h with the cells.

2.9. Secretion studies of CCK and GLP-1

STC-1 cells, provided by ATCC (ATCC® CRL3254), were cultured in Dulbecco's modified Eagle's medium (DMEM) (containing 4.5 g/L of glucose and 5 mmol/L L-glutamine) (Life Technologies, Paisley, UK), supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, 100 mg/L streptomycin and amphotericin, at 37 °C in a humidified atmosphere containing, 5 % CO_2, for 48 h, in 24-well plates (3×10^5 cells per well). Cells were washed twice with HEPES buffer (20 mmol/L HEPES 1 M, 10 mmol/L glucose, 140 mmol/L NaCl, 4.5 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, pH 7.4) and were incubated for 1 h in HEPES buffer prior to adding buffer (control) or buffer supplemented with the soluble fractions of the protein digests. After 2 h of incubation, supernatants (soluble fractions) were collected and stored at −80 °C with Halt Protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). After incubation, cells were collected to isolate RNA. The concentration of CCK and GLP-1 hormones was measured, using a commercial enzyme immunoassay CCK 26–33, non-sulphated EIA Kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and GlucagonLike Peptide-1 Active ELISA (EMD Millipore, Billerica, MA, USA), respectively. To detect cross-linked reactions, all food samples (zein, collahen, peanut, sorghum, black beans and pigeon peas) were directly tested at the highest assayed concentration using CCK and GLP-1 ELISA kits prior to assay cell supernatants. All experiments were conducted in triplicate using three biological replicates and measurements were performed in duplicate. For CCK, absorbance at 450 nm was measured using a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific, Madrid, España). For GLP-1, fluorescence was measured using an excitation wavelength of 570 nm and fluoresce emission at 600 nm in a FLUOstar OPTIMA microplate reader (BMG Labtech, Biogen Científica, S.L.).

2.10.RNA isolation and gene expression

Total RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel Gmbh & Co., Germany). The concentration and purity of each sample were evaluated on a Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific). cDNA was obtained by reverse transcription using a PrimeScript RT Reagent kit (RR037A, TaKaRa Bio Inc., Shiga, Japan). Quantitative RT-PCR amplification was carried out, using a real-time thermocycler (Viia 7 Real-Time PCR system; Applied Biosystems, Foster, CA, USA) in 384-well microplates (Axygen, Corning). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Amplification was initiated at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The following specific oligonucleotides were used: for CCK (accession no. NM_001284508.2) forward (F) 5′-CCAATTTTTCCTGCCCGCAT- 3′ and reverse (R) 5′-AGAAGGAGCAGTCAAGCCAAA- 3′; for GLP-1 (accession no. NM_008100.4) (F) 5′-AGAGACATGCTGAAGGGACC-3′ and (R) 5′-

CTTTCACCAGCCACGCAATG- 3′;14 and for reference gene β-actin (accession no. NM_007393.5): (F) 5′-AGCTGCGTTTTACACCCTTT-3′ and (R) 5′-AAGCCATGCCAATGTTGTCT-3′. The relative expression levels of the target gene were calculated using the comparative critical threshold method (ΔΔCt), by normalizing the data to the expression of β-actin. Experiments were performed at least three times in triplicate.

2.11.Intracellular calcium assay

Peptides derived from zein and collagen intestinal digests were selected based on their abundance in the digest, hydrophobicity, and the presence of aromatic amino acids and purchased from CSBio Ltd (Shanghai, China). Prior to the intracellular calcium assay, the effect of the synthetic peptides on the SCT-1 cell viability was tested as described for the gastrointestinal digests (section 2.8). Synthetic peptides were incubated at concentrations of 0.25 mmol/L and 1 mmol/L, diluted in HEPES buffer (20 mmol/L HEPES 1 mol/L, 10 mmol/L glucose, 140 mmol/L NaCl, 128 4.5 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, pH 7.4), for 2 h. After incubation, the supernatant was aspirated and Alamar Blue solution 1:10; v:v, (AlamarBlue™ Cell Viability Reagent, ThermoFisher Scientific) was incubated for 1 h prior to the fluorescence measurement at 590/530 excitation/emission wavelength. Intracellular calcium was measured using a Fluo-4 Direct™ Calcium Assay Kit (Invitrogen, Paisley, UK). STC-1 cells were seeded in a black 96-well plate (2×10^4 cells per well). After 24 h culture, the cell plate was washed with Hank's Balanced Salt Solution (HBSS, sigma-Aldrich) with 5.6 mmol/L glucose and 20 mmol/L Hepes (pH 7.4) and Fluo-4 Direct kit diluted 1:1 (v:v) with HBSS buffer and added to the cells and incubated for 1 h. Fluorescence was recorded every 5 sec for 3 min before and after sample addition at wavelengths of 490 nm excitation and 528 nm emission. A Synergy HTX Multi-Mode Reader (Biotek) was used for measurements. Synthetic peptides were tested at 0.5 mmol/L. The results were expressed as relative fluorescence, $(RFU_{max} – RFU_{min})/RFU_{min}$. RFU_{min} corresponds to the basal RFU value and RFU_{max} corresponds to the maximum value recorded after the sample addition.

2.12.Statistical analysis

ELISA, viability and intracellular calcium data were compared using one-way ANOVA with Tukey's post hoc test for pairwise comparisons. PCR results were compared using the Mann–Whitney test. The results were considered significant if $P < 0.05$. GraphPad Prism version 6.01 for Windows (La Jolla, CA, USA) was used for graphics and calculations.

3. Results

3.1. *CCK and GLP-1 secretion in SCT-1 cells*

Before the secretion assays, cell viability was assessed after incubation for 2.5 h with the samples at two different concentrations (1 and 2 mg protein/mL digest). All the soluble fractions from the different digests did not affect significantly cell viability at the concentration of 1 mg protein/mL digest (Figure 1). In addition, all samples were also assayed against the antibodies used for the quantitative determination of CCK and GLP-1 to discard those samples that show cross-reactivity in the immunoassays. No assayed samples were recognised by the CCK and GLP-1 antibodies.

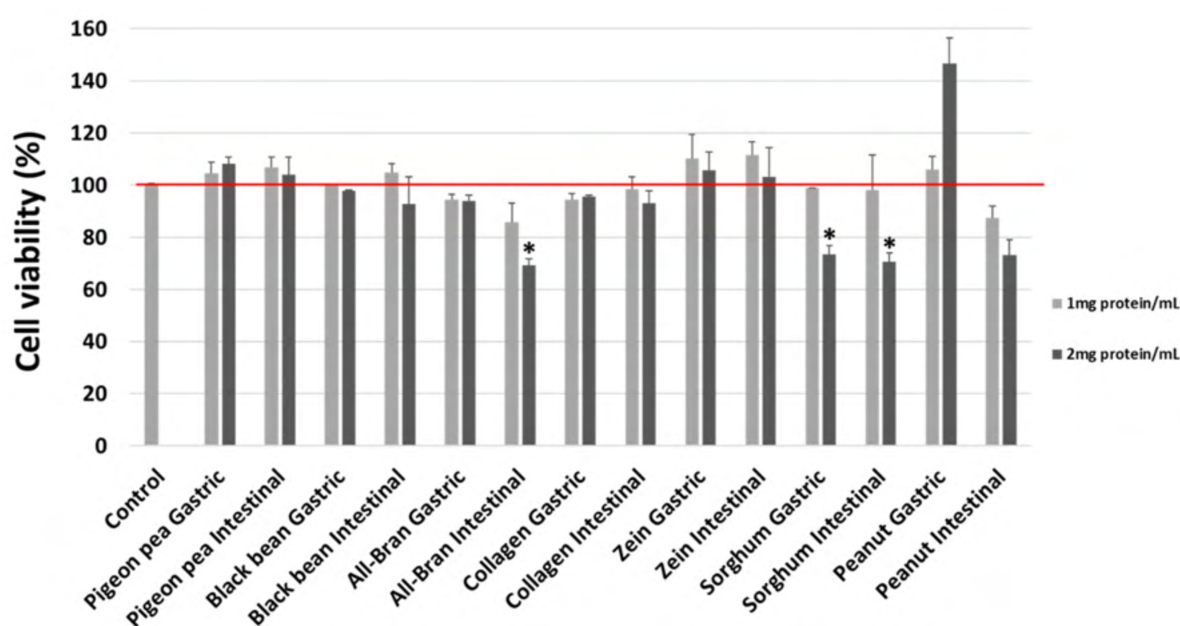


Figure 1. Cell viability in STC-1 cells (mean ± SEM, n=3) following 2.5 h of incubation with gastrointestinal digests, at 1 mg and 2 mg of protein per mL. Statistical significance compared with control (one-way ANOVA with Tukey's post hoc test) is indicated by *$P < 0.05$.

After incubation of gastric and intestinal soluble fractions with STC-1 cells for 2 h, the different digests induced significant secretion of GLP-1 and a moderate CCK secretion in a dose-dependent manner (Figure 2). This dose-response effect was clearer for the intestinal fraction than for the gastric fractions. Both, gastric and intestinal fractions induced a moderate CCK secretion (Figure 2a). CCK secretion was higher for black bean, sorghum, All-Bran® and zein intestinal digests. GLP-1 secretion was maximal in a dose-dependent manner for black bean, sorghum, All-Bran® and zein intestinal digests (Figure 2b). For gastric fractions, GLP-1 and CCK secretion was higher for pigeon peas, sorghum and All-Bran®.

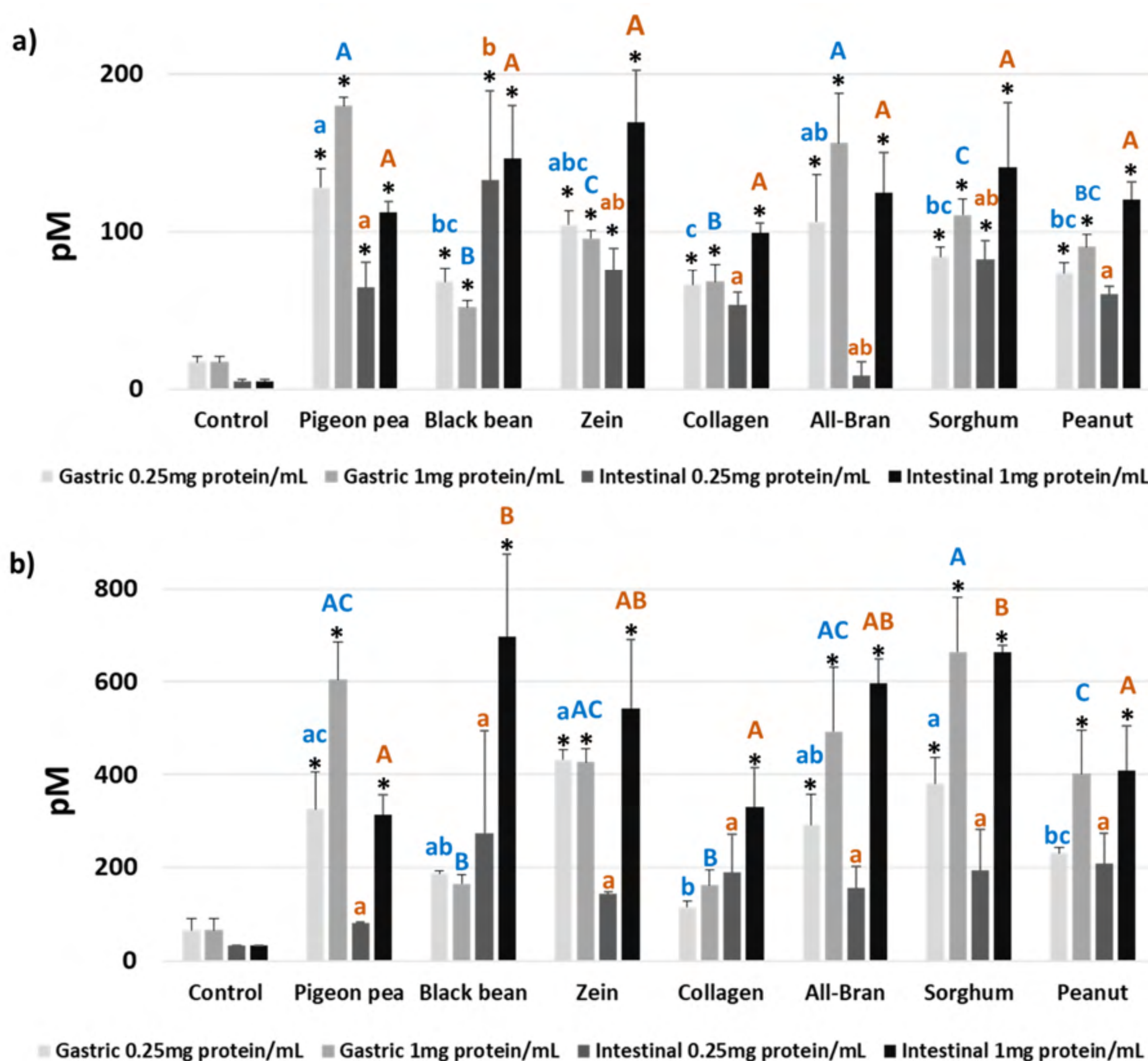


Figure 2. CCK (a) and GLP-1 (b) secretion after 2 h of incubation of STC-1 cells with the soluble fraction of the different digests resulting from *in vitro* digestion. All the samples were assayed at two different protein concentrations (1 and 0.25 mg mL^{-1}). Error bars indicate SEM (n = 3).). Statistical significance compared with the control (one-way ANOVA with Tukey's post hoc test) is indicated by **P* < 0.05. Digests were compared with their counterparts at the same concentration. Different lowercase letters with the same colour denote statistically significant differences (*P* < 0.05) between different substrates at the same type of digest, gastric digests in blue and intestinal digests in red, at 0.25 mg of protein/mL. Different uppercase letters with the same colour denote statistically significant differences (*P* < 0.05) between different substrates at the same type of digest, gastric digests in blue and intestinal digests in red, at 1 mg of protein/mL.

A dependence between the digestive phase and hormonal secretion evidenced in Figure 3, where a higher secretion is verified for intestinal fractions, with a significant difference for CCK release (*P* <0.023) (figure 3a) but not for GLP-1 (Figure 3b). This fact is because

hormone secretion is influenced by the size of the peptides. While gastric digests contain a mixture of peptides and non-digested or partly digested carbohydrates, the intestinal soluble fractions are rich in free amino acids, short peptides and free glucose which explains this different behaviour.

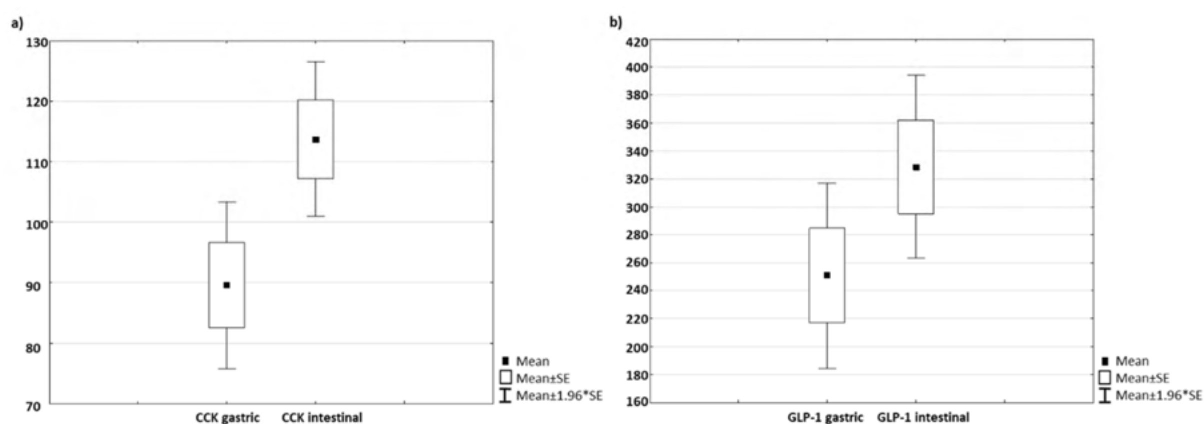


Figure 3. Phase digestion dependence on CCK and GLP-1 secretion by SCT-1 cells when incubated for 2 h with the soluble fraction of the different digests resulting from *in vitro* digestion. All the samples were assayed at two different protein concentrations (1 and 0.25 mg mL−1). Error bars indicate Mean ±1.96*SE (n = 3).

3.2. Correlation of protein, lipids and carbohydrates with hormone secretion

A significant correlation between the protein content of the digested fractions and the secretion of CCK is shown in Table 1. As expected, GLP-1 secretion is directly and significantly correlated with the carbohydrate content of the digested fractions.

Table 1. Correlation between protein, lipids and carbohydrates contents present on the digests with the CCK and GLP-1 secretion levels.

	CCK vs Macronutrients	CCK vs Protein	CCK vs Lipids	CCK vs Carbs
p-value	0.029451	0.001453	0.890027	0.191201
	GLP-1 vs Macronutrients	**GLP-1 vs Protein**	**GLP-1 vs Lipids**	**GLP-1 vs Carbs**
p-value	0.00047	0.124977	0.790464	0.002446

3.3. CCK and GLP-1 gene expression

Interestingly, in contrast to the results of hormone secretion, the mRNA levels are higher for CCK than for GLP-1 (figure 4) after incubation with gastric and intestinal fractions. Gastric fractions induced higher CCK mRNA levels than intestinal fractions (Figure 4a).

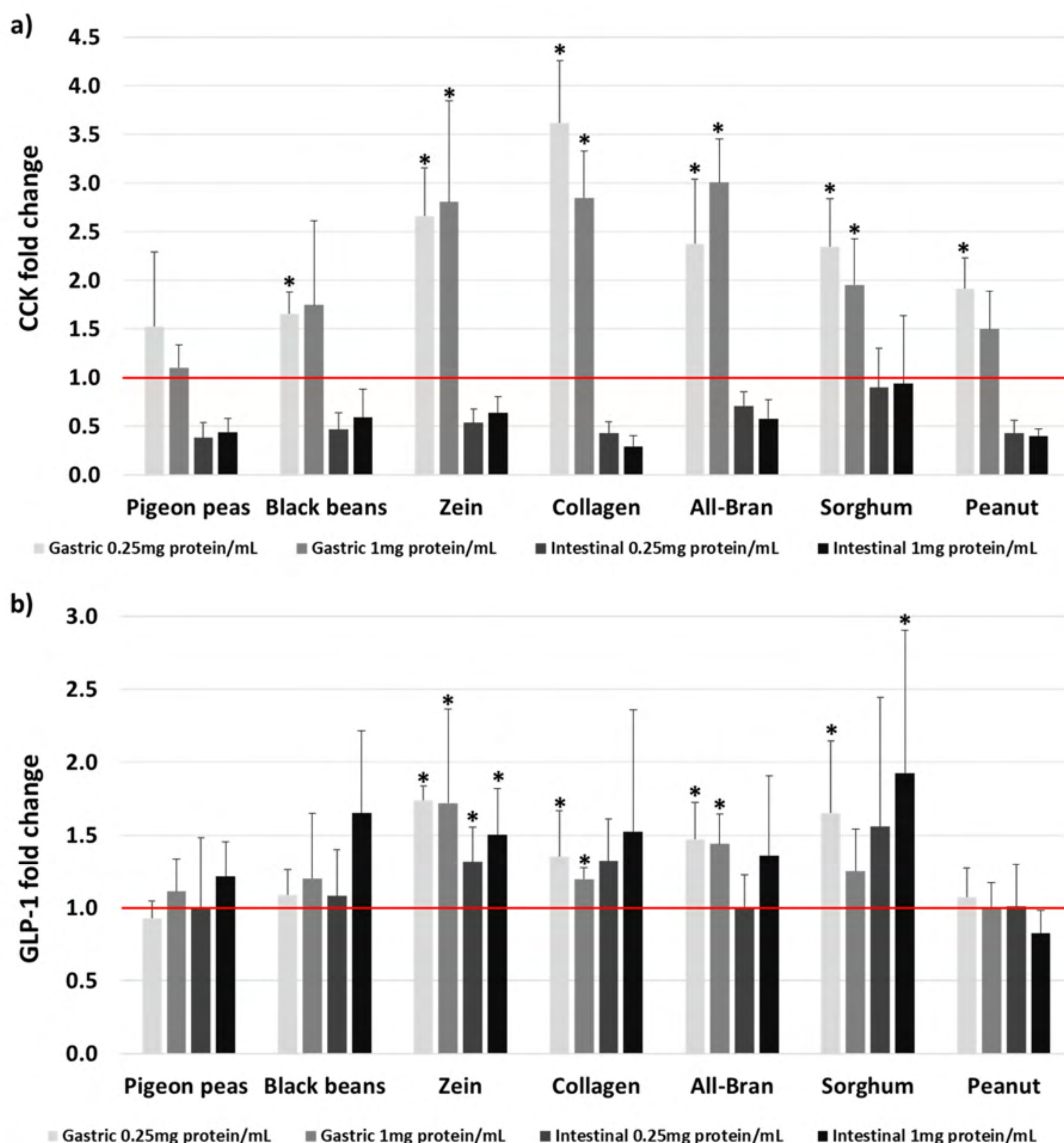


Figure 4. CCK (a) and GLP-1 (b) mRNA levels in STC-1 cells after 2 h of incubation with *in vitro* gastrointestinal digests. Data are normalized using β-actin as a reference gene and are expressed relative to the expression level of untreated cells (red line). Error bars correspond to SEM (n = 9). Results were compared using Mann-Whitney test after Shapiro-Wilk normality test. Statistical significance compared with the normalised control is indicated by, **P* <0.05.

No evidence of a dose-response effect on CCK mRNA levels for both fractions (gastric and intestinal). GLP-1 mRNA levels (except for peanuts digests), are increased for the intestinal fractions in a dose-response manner (Figure 4b). For gastric fractions, there is no evidence of dose-response effect on GLP-1 mRNA levels.

3.4. Selected peptides for synthesis

Peptides present at the end of the intestinal phase originiating from zein and collagen digests were identified by LC-MS and few of them were selected for synthesis based on their abundance on the digests, hydrophobicity and presence of aromatic amino acids. Structural characteristics of the selected peptides tested on the STC-1 cell line for their effect on the intracellular calcium concentration (section 2.11), such as length, hydrophobicity and isoelectric point (pI) are given in Table 2.

Prior to the cell assay, cell viability was tested at two different concentrations (0.25 mmol/L and 1 mmol/L). None of the synthetic peptides affected cell viability at the tested concentrations (Figure 5).

Table 2. Structural characteristics of selected peptides from zein and collagen gastrointestinal digests.

Code	Protein	Sequence	Start position	Length	Hydrophobicity	Theoretical pI
P1	CO1A2_BOVIN	S*RGDGGPPG	774	9	-13.6	5.46
P2	ZEAZW_MAIZE	ASNPLAL	117	7	5.3	4.92
P3	ZEAZW_MAIZE	FLPPV	38	5	7.6	4.64
P4	CO1A1_BOVIN	GPPGPMGPPG	994	10	-7.7	4.92
P5	ZEAZW_MAIZE	AVANPL	227	6	6.5	4.92
P6	E1BB91_BOVIN	QPQIVNLLK*SVQNGAPAP	1007	18	-3.3	8.27
P7	CO3A1_BOVIN	GSPGPAGPRGPVG	975	13	-7.7	10.01
P8	ZEAA_MAIZE	QQLLPFNQL	134	9	-1.4	4.54
P9	ZEAA_MAIZE	QQPIVGGAIF	258	10	8.4	4.27
P10	ZEAA_MAIZE	LPFSQL	216	6	4.5	4.9
P11	CO1A2_BOVIN	FGFDGDF	1108	7	0.6	2.78
P12	ZEAA_MAIZE	QQLLP	93	5	-1	4.36
P13	CO1A2_BOVIN	AGPTGPI	598	7	1.6	4.92
P14	CO1A1_BOVIN	GETGPAGPAGPIGPV	1066	15	-0.3	3.38
P15	CO1A2_BOVIN	PGPIGPA	475	7	0.7	5.35

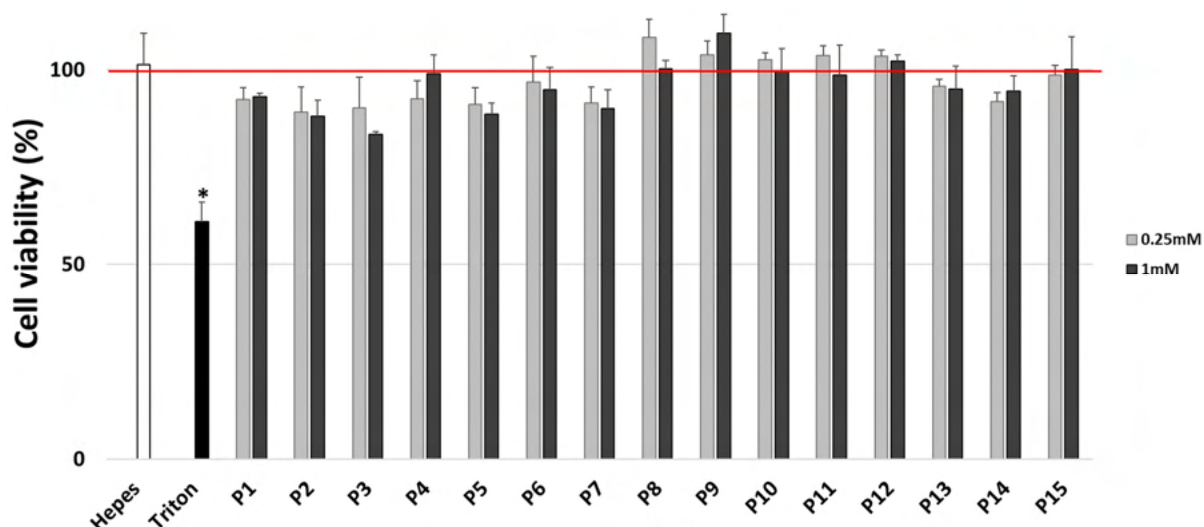


Figure 5. Cell viability in STC-1 cells (mean ± SEM, n=3) following 2 h of incubation with synthetic peptides, at two different concentrations (0.25 mmol/L and 1 mmol/L). There is no statistical significance between the synthetic peptides in comparison with control (Hepes) (one-way ANOVA with Tukey's post hoc test).

3.5. Intracellular Ca $^{2+}$ concentration induced by specific synthetic peptides

Exposure of synthetic peptides to STC-1 cells induced an increase in intracellular calcium levels at different extensions (Figure 6), for almost all peptides tested at a concentration of 0.5 mmol/L. The increase in Ca^{2+} was higher for the peptides *S*RGDGGPPG* (P1), *FLPPV* (P3), *and QQLLPFNQL* (P8), whereas the peptides *GPPGPMGPPG* (P4), *QPQIVNLLK*SVQNGAPAP* (P6), *QQPIVGGAIF* (P9), *and PGPIGPA* (P15) induced a moderate increase in intracellular calcium levels. The peptides *ASNPLAL* (P2), and *GSPGPAGPRGPVG* (P7) did not induce a significant intracellular calcium increase in comparison with the control, whereas the peptide *LPFSQL* (P10) did not induce any effect. The peptide *FGFDGDF* (P11) was not soluble at the assay conditions, and therefore was excluded from the assay. It has to be noted that HBSS with calcium and glucose was used as control and therefore, a slight increase of calcium levels (0.046 RFU) was observed.

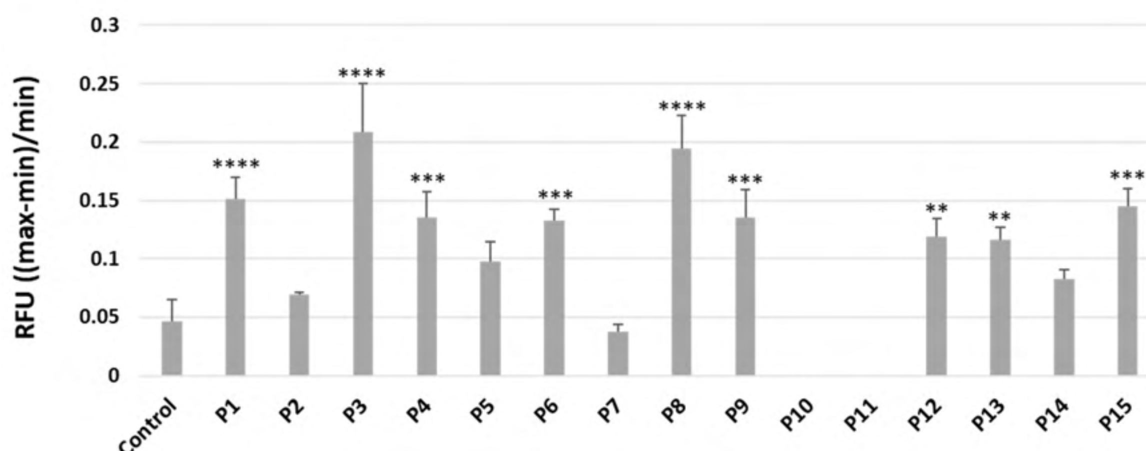


Figure 6. Changes in intracellular calcium concentration in enteroendocrine STC-1 cells exposed to synthetic peptides. Intracellular calcium was expressed in relative fluorescence units (RFU) as $(RFU_{max} – RFU_{min})/RFU_{min}$ after exposure of zein and collagen derived peptides at 0.5 mmol/L. Error bars indicate SEM (n=3). Statistical significance compared with the control is indicate **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ (one-way ANOVA with Tukey's post hoc test).

The selected peptides present at the end of the intestinal phase originating from zein and collagen digests were identified by LC-MS and were represented using Peptigram Bioware tool (figure 7). In the peptigrams, the location in the protein sequence is shown by the position of the green bar and the intensity of the green colour stands for signal intensity, in both lines. The first lane shows the abundance of the selected peptides in the soluble fraction of the intestinal digest (mass spec intensity); the increase in intracellular calcium concentration induced by the corresponding synthetic peptides is shown on the second line ($RFUx10^{8}$).

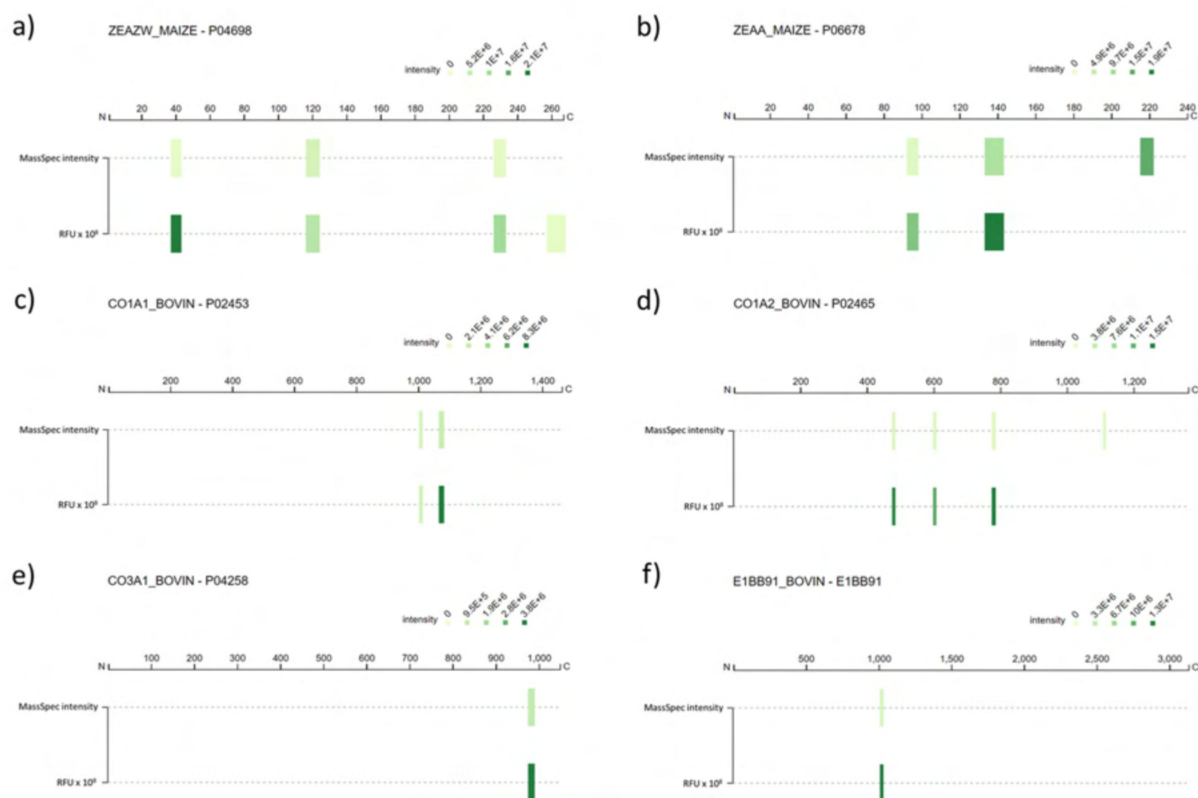


Figure 7. Peptides from 22 kDa alpha-zein 14 (a), Zein-alpha 19D1 (b), Collagen alpha-1(I) chain (c), Collagen alpha-2(I) chain (d), Collagen alpha-1(III) chain (e), Collagen type VI alpha 3 chain (f), resulting from zein and collagen *in vitro* gastrointestinal digestion, respectively, were represented using the Peptigram Bioware tool. The green bars represent the selected and tested peptides placed at their location on the respective proteins and the intensity of the green colour stands for signal intensity. The first line of the peptigrams corresponds to individual peptide intensity in the mass spec analysis, and the second line corresponds to the effect of these corresponding synthetic peptides on intracellular calcium concentration (RFU x 10^8) induced by the in SCT-1 cells.

4. Discussion

This work aimed to study the influence of *in vitro* digested protein sources with different digestibilities and macronutrient composition on CCK and GLP-1 secretion and expression in SCT-1 enteroendocrine cells.

The substrates were digested according to the harmonized INFOGEST 2.0 *in vitro* digestion protocol (Brodkorb et al., 2019). After digestion, the samples were separated into soluble (supernatant) and insoluble (pellet) fraction by centrifugation and SDS-PAGE gels (Supplemental Figure 1) showed the effectiveness of the protocol on protein digestion, where no intact proteins belonging to the substrates remained undigested at the end of the intestinal step. After incubation with the soluble fraction of the digests for 2 h, there was a significant

induction of GLP-1 secretion and a moderate CCK secretion. For gastric fractions, GLP-1 and CCK secretion was higher for pigeon peas, sorghum and All-Bran®. CCK and GLP-1 secretion was higher for black bean, sorghum, All-Bran® and zein intestinal digests (Figure 2a). It has to be noted that intestinal fractions contain as protein digestion products, free amino acids, peptides, in addition to free glucose and fatty acids as digestion products from carbohydrates and lipids, respectively. Protein is recognized as a strong stimulus for CCK release (Bowen et al., 2006), and fatty acids also significantly stimulate CCK secretion. In the case of zein, a protein isolate, the secretion of CCK is induced by peptides and amino acids, while for black bean, sorghum and All-Bran® have a moderate fat content and free fatty acids might contribute to the secretion of this hormone (Supplemental Table 1). As previously reported CCK is stimulated by peptides and free amino acids, while GLP-1 is stimulated by peptides (Santos-Hernández et al., 2020). GLP-1 secretion was higher for black bean, sorghum, All-Bran® and zein intestinal digests (Figure 2b). Carbohydrates are strong stimuli of GLP-1, consistent with its role as incretin, although free glucose has a more potent effect than complex carbohydrates (Elliot et al., 1993). Therefore, GLP-1 levels found in black bean, sorghum and All-Bran are caused by the contribution of digestion products from proteins and carbohydrates and especially free glucose, while for zein intestinal digests the secretion of this hormone is induced by peptides. GLP-1 is mainly induced by peptides with a MW > 500 Da (Santos-Hernández et al., 2020) or even intact proteins (Geraedts et al., 2011). Our results are in agreement with these previous studies, since GLP-1 secretion with the gastric digests, lacking free glucose and containing longer peptide fragments, was comparable with that obtained with intestinal digests.

Several authors suggested an important role of the aromatic amino acids (Phe + Tyr + Trp) in CCK secretion induction via CaSR (Caron et al., 2016; Colombel et al., 1988; Hira et al., 2008; Meyer et al., 1976). Zein, collagen, and peanut digests are the ones with higher concentration of free aromatic amino acids (Supplemental Figures 2 and 3). However, CCK secretion was maximized for black bean, sorghum, All-Bran® and zein, which did not corroborate this relation between aromatic amino acids content and CCK secretion.

Regarding the ability of synthetic peptides to activate the enteroendocrine cell line STC-1, it was observed a clear sequence specificity. Higher activation was achieved with the peptapeptide FLPPV (P3), followed by S*RGDGGPPG (P1), and QQLLPFNQL (P8). Peptides P3 and P8 have a marked hydrophobic character, while P1, containing a phosphorylated serine, is more hydrophilic. It remains to be studied the hormonal secretion induced by these peptides.

5. Conclusion

In conclusion, all digests showed dose-response effects for both, gastric and intestinal digests. In general, higher hormonal secretion was achieved in response to intestinal digests compared to gastric ones, with a significant difference for CCK release ($p<0.023$) but not for GLP-1. A positive correlation between protein and carbohydrate contents of the digests and GLP-1 levels was observed. However, our data show that CCK production was mainly dependent on protein content.

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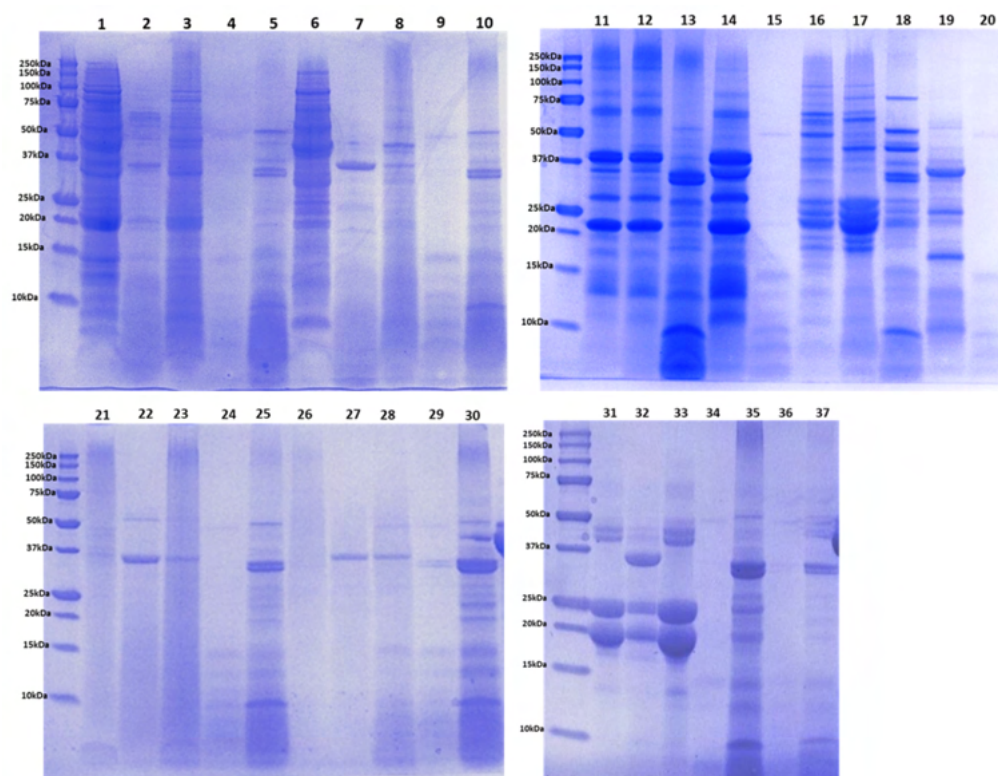
Supplemental material

Supplemental figure 1. SDS-PAGE gels of the undigested protein sources and gastrointestinal endpoints



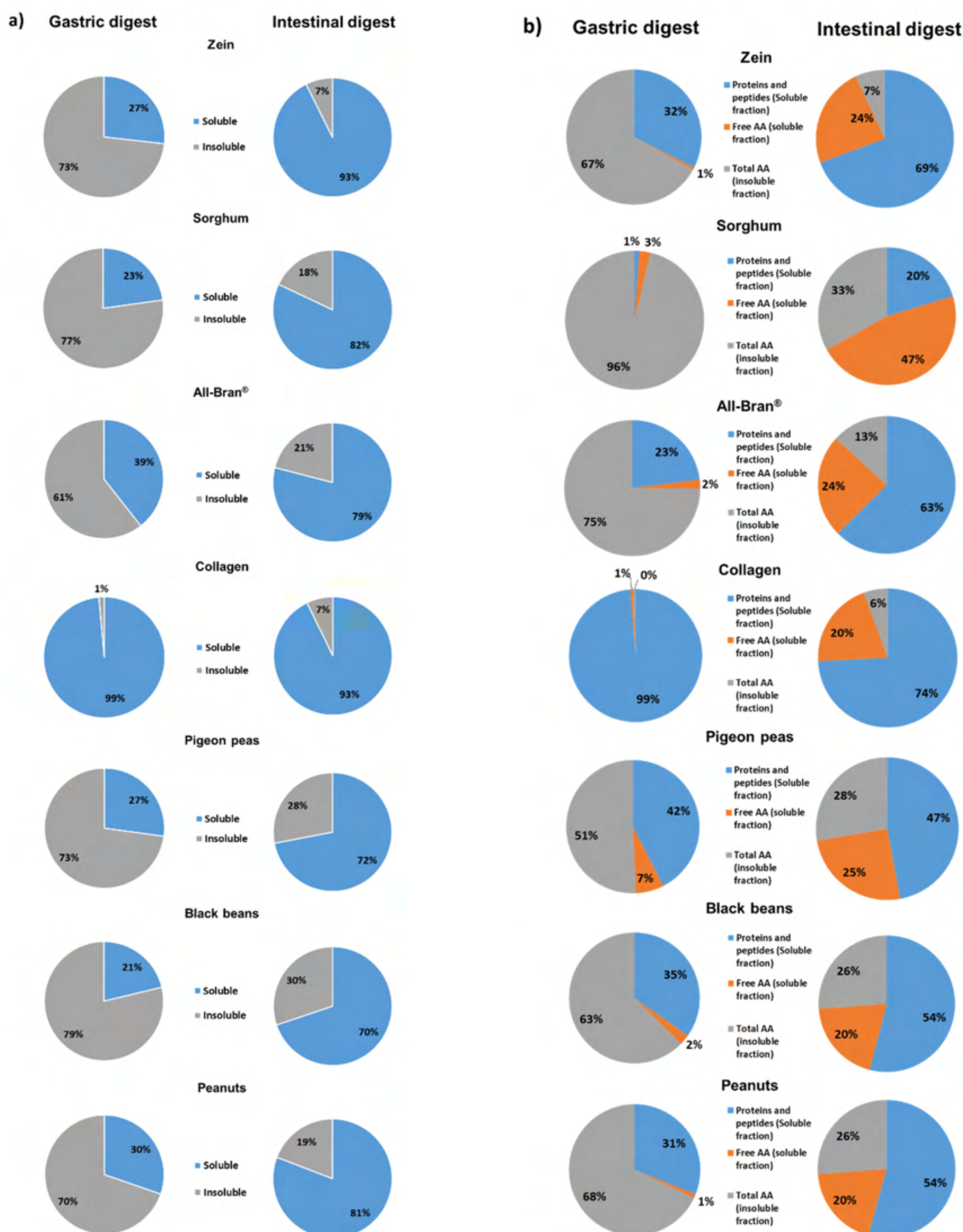
Suppl. figure 1. SDS-PAGE of the undigested samples and the soluble and insoluble fractions. Pepsin band appears at 35 kDa in the soluble gastric fractions. 1- Pigeon peas undigested; 2- Pigeon peas gastric soluble fraction; 3- Pigeon peas gastric insoluble fraction; 4- Pigeon peas intestinal soluble fraction; 5- Pigeon peas intestinal insoluble fraction; 6- Black beans undigested; 7- Black beans gastric soluble fraction; 8- Black beans gastric insoluble fraction; 9- Black beans intestinal soluble fraction; 10- Black beans intestinal insoluble fraction; 11- Peanut undigested; 12- Peanut gastric insoluble fraction; 13- Peanut intestinal insoluble fraction; 14- Peanut gastric soluble fraction; 15- Peanut intestinal soluble fraction; 16- Sorghum undigested; 17- Sorghum gastric insoluble fraction; 18- Sorghum intestinal insoluble fraction; 19- Sorghum gastric soluble fraction; 20- Sorghum intestinal soluble fraction; 21- All-Bran undigested; 22- All-Bran gastric soluble fraction; 23- All-Bran gastric insoluble fraction; 24- All-Bran intestinal soluble fraction; 25- All-Bran intestinal insoluble fraction; 26- Collagen undigested; 27- Collagen gastric soluble fraction; 28- Collagen gastric insoluble fraction; 29- Collagen intestinal soluble fraction; 30- Collagen intestinal insoluble fraction; 31- Zein undigested; 32- Zein gastric soluble fraction; 33- Zein gastric insoluble fraction; 34- Zein intestinal soluble fraction; 35- Zein intestinal insoluble fraction; 36- Intestinal water blank soluble fraction; 37- Intestinal water blank insoluble fraction.

Supplemental table 1

Sample	Protein (g/100g)	Total fat (g/100g)	Starch (g/100g)
Zein	92.86 ± 0.037	n.d	n.d
Sorghum	9.63 ± 0.055	4.43 ± 0.05	71.63 ± 0.68
All-Bran®	14.15 ± 0.003	5.49 ± 0.31	27.62 ± 0.33
Collagen	103.83 ± 0.007	n.d	n.d
Pigeon peas	26.11 ± 0.060	2.45 ± 0.16	n.d
Black beans	22.97 ± 0.092	2.29 ± 0.25	33.60 ± 0.89
Peanuts	31.98 ± 0.013	48.91 ± 0.49	3.4 ± 0.05

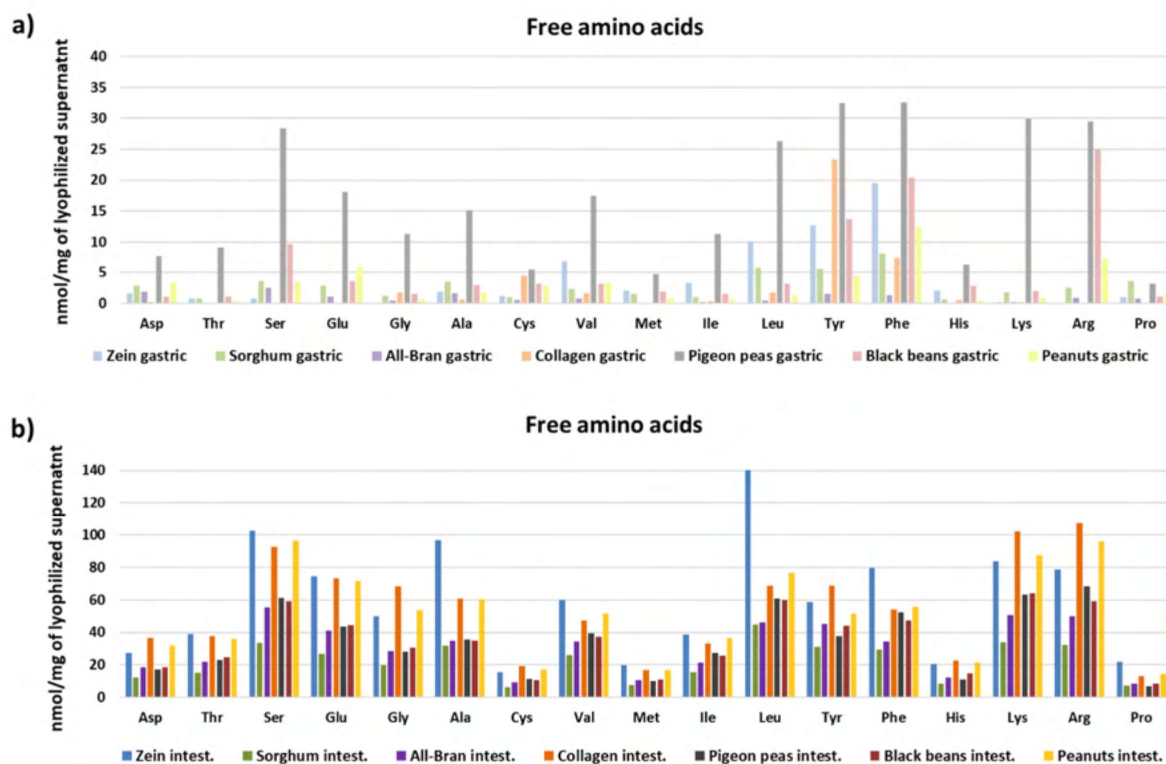
Suppl. table 1. Substrate composition in protein, fat and starch. The protein content was calculated based on a 6.25 conversion factor and Kjeldahl analysis; n.d = not determined. Starch, fat and TN were determined in duplicate.

Supplemental figure 2. Nitrogen distribution in the soluble and insoluble fraction from the gastric and intestinal endpoints



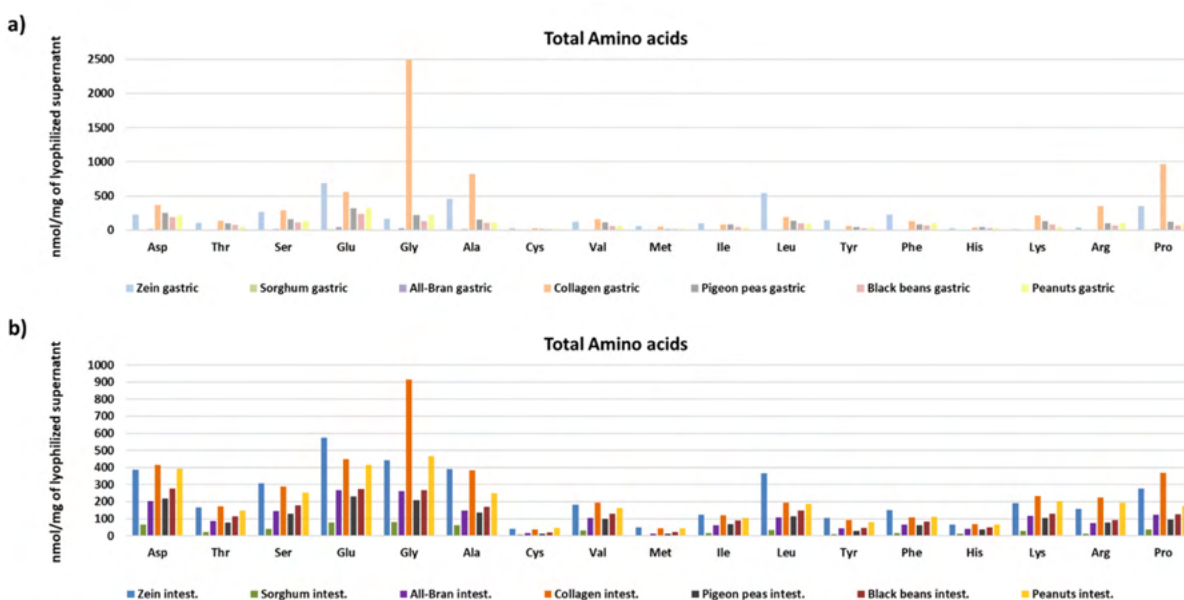
Suppl. figure 2. Distribution of the nitrogen content between soluble and insoluble fraction from the gastric and intestinal digests. Digests were centrifuged at 5000 *x* g for 20 min. Supernatant and pellet were freeze-dried and weighted. Nitrogen content in each fraction was determined by elemental analysis (a) and amino acid analysis (b). Total and free amino acids were separately determined in the soluble fraction.

Supplemental figure 3. Free amino acids content



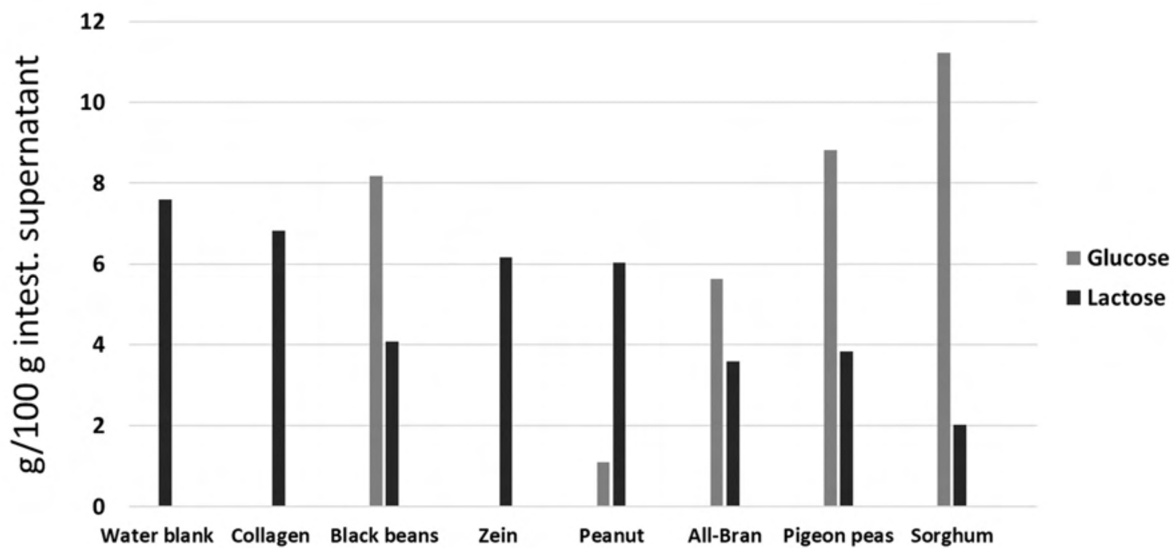
Suppl. figure 3. Free amino acid contents (nmol/mg of lyophilized supernatant) in (A) gastric and (B) intestinal digests.

Supplemental figure 4. Total amino acid content



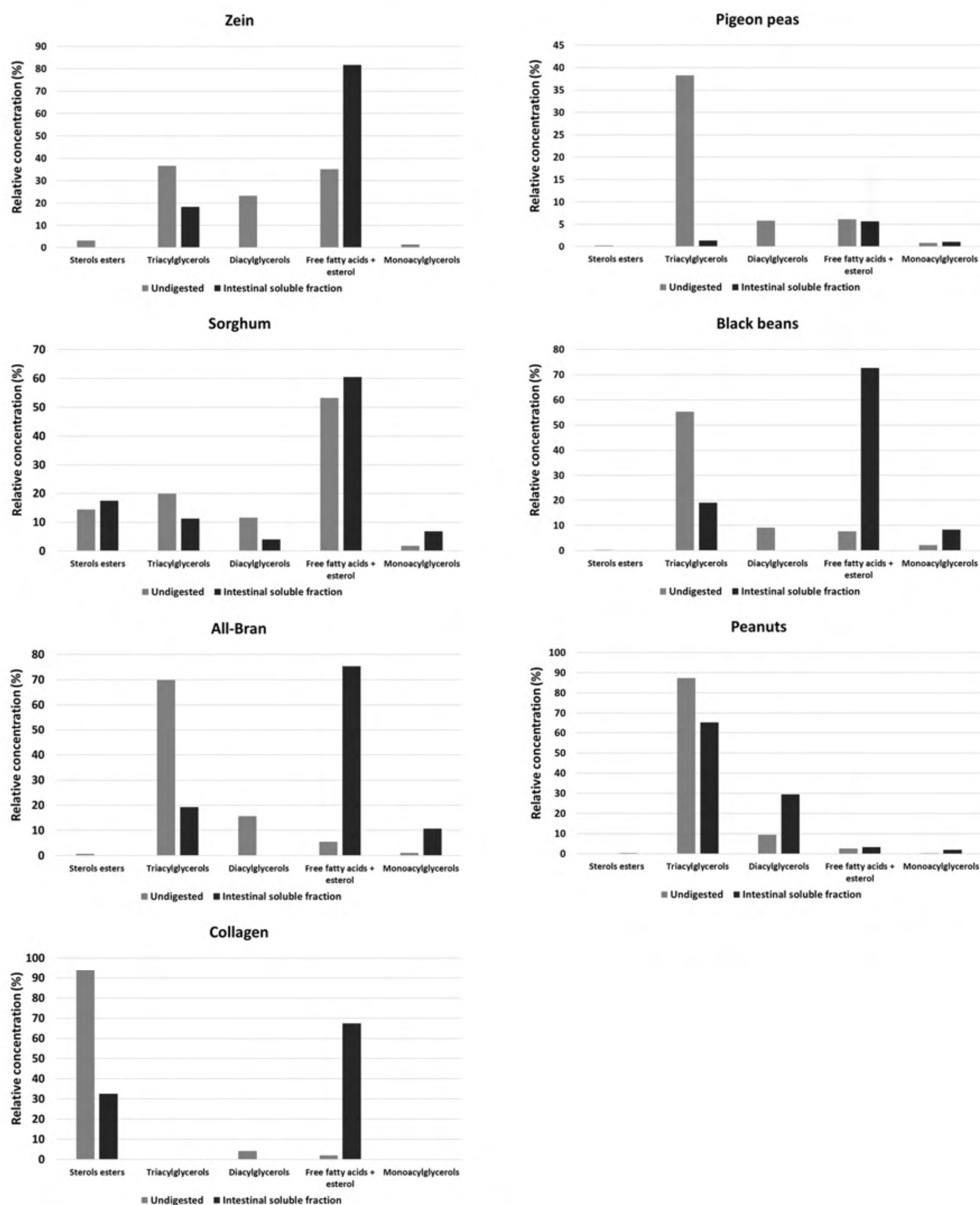
Suppl. figure 4. Total amino acid contents (nmol/mg of lyophilized supernatant) in (A) gastric and (B) intestinal digests.

Supplemental figure 5



Suppl. figure 5. Total glucose and lactose in intestinal soluble faction expressed in g/100g of intestinal soluble fraction.

Supplemental figure 6. Free fatty acids distribution in the undigested samples and intestinal soluble fractions



Suppl. figure 6. Free fatty acids distribution in the undigested samples and its corresponding intestinal soluble fractions. The results are expressed as relative concentration (%).

General discussion

Macronutrient composition is known to influence energy intake and metabolism. Protein is an indispensable constituent of the diet as it is a source of nitrogen and essential amino acids. Most of the proteins in our body have specific functions in the regulation of growth, repair, maintenance and replacement of the tissues and consequently, any loss in body proteins is a loss of cellular function. Thus, insufficient intake of dietary protein is incompatible with growth and life. Consequently, a control of the regulation of protein intake is critical for the organism. Contrary to lipids and carbohydrates, the human body does not have true reserves of protein and, therefore, low dietary protein intake is compensated by catabolizing some, but not all, proteins in our body's tissues. The protein pool irreversibly catabolized due to body metabolism is defined as the recommended daily intake of protein, and it is crucial that these recommended amounts are ingested in order to ensure the proper functioning of the body (Nadathur et al., 2017). Protein quality can be defined as the ability of a food protein to meet the body's metabolic demand for nitrogen and it can be determined by its essential amino acid profile, digestibility, and bioavailability of the individual amino acids (FAO, 2013).

For many years, bioassays, mainly using rats, were the chosen methods to assess the nutritional value of proteins. This value was expressed in parameters such as protein efficiency ratio, net protein utilization and biological value. In 1991, Food and Agricultural Organization of the United Nations/World Health Organizations (FAO/WHO) proposed the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), and they made PDCAAS the official standard. The method expresses the content of the first limiting essential amino acid of the test protein as a percentage of the content of the same amino acid in a reference pattern of essential amino acids. This reference pattern derives from the essential amino acid requirements of the preschool-age child. This percentage is then corrected for the true fecal digestibility of the test protein, as measured in a rat assay. PDCAAS improved significantly the standardization of the results and it has been widely used to determine protein quality for more than 25 years, however it has some disadvantages. One of the biggest criticisms of this method is the use of true fecal nitrogen digestibility for estimating the bioavailability of the amino acids, which can lead to over- or underestimation of the protein quality. To address the limitations of the PDCAAS, in 2013, FAO proposed the Digestible Indispensable Amino Acid Score (DIAAS), based on the true ileal digestibility of each (indispensable) amino acid. This method is based on the individual concentration of amino acids and their digestibility at the end of the small intestine. The true ileal digestibility should preferably be determined in humans (naso-ileal intubation or ileostomized patients). Since this goes beyond the practical and ethical limits for routine studies, it is recommended to use (ileum-fistulated) growing pigs, as the preferred model of choice, or growing rats (Huang et al., 2018). At the moment, DIAAS cannot currently be fully implemented by industry as there is limited data for the true ileal digestibility of amino acids in foods (Hodgkinson et al., 2020). However, taking into account that each food

undergoes six different analyses to calculate coefficients of ileal digestibility for indispensable amino acids, the use of ileal-cannulated pigs as recommended by FAO to determine digestibility coefficients for hundreds, if not thousands, of food items is not practical. In addition, and according to the 3Rs principle, consumers, governments and industry aim to reduce and replace animal experiments wherever possible, and therefore the development, validation and implementation of *in vitro* methods as an alternative for these bioassays is urgently required. However, to date only two studies determining the DIAAS using *in vitro* methods have been published, and both of them lack the validation of their data (Ariëns et al., 2021; Havenaar et al., 2016). In 2014, the international COST Infogest network proposed a standardised static *in vitro* digestion method (Minekus et al., 2014) based on relevant physiological conditions, which was revised in 2019 (Brodkorb et al., 2019). Since its first publication, this protocol has been used numerous times for a wide variety of studies, for example to investigate the release of carotenoids and phenolic compounds, the bioaccessibility of vitamins, and the digestion of proteins, lipids, and starch. The studies presented in this doctoral thesis reveal the potential of the INFOGEST static *in vitro* digestion model to estimate the *in vivo* total digestibility, digestibility of individual amino acids and DIAAS.

i. Protein sources characterization

In the frame of the PROTEOS project, eight different protein sources, three isolated proteins (whey protein isolate, collagen, and zein), and five foods (sorghum, peanuts, pigeon peas, black beans, and All-Bran®), were selected. The international PROTEOS project, led by Distinguished Professor Paul Moughan (Riddet Institute, New Zealand), aims to develop a database of values for true ileal amino acid digestibility of individual amino acids, established in ileal cannulated pigs and human studies, for a large number of commonly consumed foods. Each protein source used in this experiment was distinct, however, they were selected to reflect protein sources used in diets around the globe. In our *in vitro* project, at first, all the substrates were fully characterized for their individual protein profiles, and amino acid composition. After subjecting them to the INFOGEST static protocol, protein hydrolysis was followed by the OPA method and SDS-PAGE. The peptide patterns from the different digests at the end of the gastric and intestinal step were identified via HPLC in order to follow the peptide formation as a consequence of the protein digestion, as well to investigate the existence of digestion-resistant peptides. In the course of the experiments towards quantification of protein hydrolysis, we observed that the water blank digest, widely used in *in vitro* digestion studies, was not the best option to subtract as enzymatic background due to the high degree of auto-digestion of the digestive enzymes in the absence of substrate. Therefore, a protein-free cookie (only containing carbs and fat) digestion was used as alternative. This

mimics the approach used *in vivo* studies, where a protein-free diet was administrated to the animals in order to measure the endogenous protein losses.

ii. Adaptation of the INFOGEST static protocol to assess *in vitro* DIAAS

After an extensive characterization of the protein sources and a better understanding of their behaviour during and after digestion, the next step was to develop an algorithm able of calculating the DIAAS of the different tested proteins using the INFOGEST static *in vitro* protocol. For that, all the substrates were again digested according to the INFOGEST static protocol with minor adaptations. In the original protocol (Brodkorb et al., 2019), it is suggested to mix the food with simulated salivary fluid (SSF) at a 1:1 ratio, for example, 1 g of food 1 mL of SSF. However, foods and substrates are very different in protein content and, by adding the same amount of food different amounts of protein would be in the system, which would make it impossible to compare the protein digestibility between different protein sources. The INFOGEST static *in vitro* protocol was validated by inter-laboratory studies using skim milk powder (SMP) (Egger et al., 2016). For this inter-laboratory study 5 mL of a 1/10 dilution of SMP in H_2O (w:v), which corresponded to 0.04 g of protein per digest. The protocol also proved its physiological relevance by comparing *in vivo* data with *in vitro* results where protein hydrolysis obtained by the *in vitro* digestion was similar to *in vivo* protein hydrolysis in pigs at the gastric and intestinal endpoints (Egger et al., 2017). Therefore, we decided to normalize the protein input for the *in vitro* digestions in this project to 0.04 g protein. However, to confirm this decision, different amounts of protein were *in vitro* digested (0.04 g, 0.06 g and 0.08 g) and it could be observed that a higher the protein input, led to a decrease in protein digestibility, which was more important in substrates with lower digestibility. These results have shown that for concentrations of 0.06 g and 0.08 g of protein, the *in vitro* system was overloaded and the amount of enzymes present is not enough to guarantee the enzymatic reactions to their full extent. In addition, it was also observed that the standard deviation of the results increased with the increase in protein input. Thus, these results supported the decision of normalizing the *in vitro* digestion system to 0.04 g of protein input. Moreover, when comparing the digestibility of the three isolated proteins (zein, whey protein isolate, and collagen), no differences in digestibilities was observed, and they were all very well digested (≈ 100 % for all the three sources). However, according to the *in vivo* data available from the PROTEOS project (Hodgkinson et al., 2022) a very low digestibility for zein (≈ 60 %) was reported, which is a highly hydrophobic protein, and around 80 % for collagen. In the *in vivo* assays, they were feeding the isolated proteins as part of a meal, however, we were feeding the *in vitro* system with the isolated proteins alone. Therefore, we decided to mimic the *in vivo* conditions and fed the *in vitro* system with the isolated proteins plus protein-free cookie. Several digestions were performed with different amounts of cookie added to the isolated proteins (0.1 g, 0.2 g, and

0.25 g of protein-free cookie added to 0.04 g of protein). Analysing the data from these digestions it was clear that by digesting the isolated proteins as part of a “meal” the digestibility values were changing. When comparing the three different tested conditions (0.1 g, 0.2 g, and 0.25 g of protein-free cookie) we could conclude that the condition that most closely resembled the *in vivo* results was 0.25 g of protein-free cookie. Therefore, we decided to do all the following digestions of isolated proteins with 0.25 g protein-free cookie.

The INFOGEST nature protocol (Brodkorb et al., 2019) identified the enzyme activity as a critical point. It is know that the activity indicated by the manufacturer is not corresponding to the real measured enzyme activity. Moreover, the real measured activity can vary significantly between lots. Therefore, it is recommended to measure the enzyme activity before use and at the same time use the same lot of enzyme during the whole set of experiments to improve experimental repeatability. The protocol recommends the addition of the amount of pancreatin that would correspond to 100 U/mL of trypsin activity. However, this corresponds to a considerable amount of pancreatin, especially for lots with lower trypsin activity, increasing the ratio of substrate to background. In addition, it was found that pancreatin formed a suspension with undissolved particles, resulting in non-reproducible measurements due to difficulties or inconsistences in the pipetting of this suspension, leading to differences in pellet size after the precipitation step. Therefore, different procedures were tested to improve the homogeneity of the pancreatin suspension without reduction of trypsin activity. For that, it was first dissolved in simulated intestinal fluid (SIF) at the concentration indicated in the protocol (Brodkorb et al., 2019), then an ultrasonication step for better solubilisation was added, and finally the non-solubilized material was removed by centrifugation. The subsequently measured activities of the different pancreatic enzymes in the supernatant showed, that, despite, the slight decrease in activity, no significant differences we found (trypsin p-value = 0.60; pancreatic amylase p-value = 0.35; lipase p-value = 0.08) and the reproducibility of the results were highly improved by only using the supernatant of pancreatin suspension.

The next challenge faced was the determination of the bioavailable fraction. It is know that in the small intestine not only dietary proteins are absorbed as free amino acids and di- and tripeptides but also as bigger peptides. Absorption of insulin (MW 5700 Da; (Laskowski et al., 1958), ribonuclease (MW 13700 Da; (Alpers & Isselbacher, 1967), ferritin, horseradish peroxidase (Warshaw et al., 1971), and whey protein oligopeptides (MW 1633 Da; (Ozorio et al., 2020)) have previously been demonstrated. Based on the fact that the INFOGEST model lacks the brush border enzymes, the bioavailable fraction was determined consisting of free amino acids and peptides up to 10 amino acids. In the *in vivo* situation these peptides would be further degraded by the brush border enzymes. Thus, to obtain the so called bioavailable fraction, different precipitation agents and conditions (MeOH, TCA, perchloric acid, tanic acid, acetone, and 3 K filter) were tested in order to split the whole digest into a bioavailable

(supernatant) fraction and non- bioavailable (pellet) fraction. Among all the tested conditions, MeOH 80 % showed to be the most reproducible condition since there were no additional peaks given by the precipitation agent and the recovery from both fractions was good. Therefore, both fractions (bioavailable and non-bioavailable fractions) from all the digests were precipitated with MeOH 80 %. In order to decrease the number of repetitions, several aliquots from one whole digest were precipitated with MeOH, in order to do the further analysis based on one single digestion experiment. However, this procedure led to non-reproducible results, due to a variability in pellet size and weight between replicates of the same food. Therefore, it had to be concluded that aliquots did not represent the whole digest, due to the heterogeneity of the particles in the digest and the precipitation needed to be performed on the whole digest. As a next step, both fractions (bioavailable and non-bioavailable) were characterized for their peptide size distribution using size exclusion chromatography, which was previously calibrated with compounds of know molecular weight, in order to evaluate the molecular weight of the molecules present in both fractions. Analysing the chromatograms, it could be observed that the majority of the molecules with molecular weight > 1000 Da were retained in the pellet (non-bioabsorbable fraction), and most of the molecules < 1000 Da were retained in the supernatant fraction. Thus, we concluded that the MeOH 80% precipitation was a good condition to obtain the bioavailable fraction out of the whole digest.

Once the bioavailable fraction was defined, both fractions (bioavailable and non-bioavailable) were analyzed for their total nitrogen content (Kjeldahl), primary amines (OPA) and total amino acids (HPLC). Even though Kjeldahl being a very well implemented routine method some inconsistencies were faced which were caused by the low Nitrogen contents in the supernatants after precipitation with MeOH 80 %, and amount of moisture in the pellet, which was variable. It was therefore absolutely indispensable to dry the pellet and concentrate the supernatant to have reproducible Kjeldahl measurements. For HPLC and OPA measurements, the pellet was also dried and the supernatant fully evaporated and re-dissolved in water in order to reduce interferences in the analytics due to the high concentration of MeOH and guarantee the reproducibility of the results.

iii. Application of the adapted INFOGEST protocol to highly transformed foods

Adequate dietary protein intake is very important for humans during all stages of life, and in particular it is critical to meet the requirements for indispensable amino acids (Reeds, 2000). In case the requirements for individual indispensable amino acids are not met, several physical and biochemical problems can occur (Reeds & Hutchens, 1994). Animal protein, such as meat, is a very important element in the human diet, it is known to be well digested and rich in essential amino acids (McAfee et al., 2010). In contrast, plant proteins are not as well digested, and usually have larger amounts of non-essential amino acids, and are deficient or

lack some essential amino acids (Joye, 2019). Despite its traditional consumption, there is growing pressure from activists and government agencies to reduce meat production due to ethical and environmental concerns. This is due to evidence that animal protein production causes a far greater environmental impact than the same yield of protein from other sources than meat, such as grains, beans and seeds (Nijdam et al., 2012). In the last few years, the number of people adopting a vegetarian or vegan diet increased significantly. As matter of fact, according to Google Trends, the interest in plant-based diets increased seven-fold between 2014 and 2019. However, it is important to consider and review the nutritional content of the plant-based food, making up a well-planned vegetarian or vegan diet when compared with the food products from more traditional meat-consuming diet. A critical factor is the bioavailability of the amino acids after digestion. Several factors have been identified which could affect the bioavailability of amino acids from plant-based proteins, and impair their digestion. These factors are related to their low content in sulphur-containing amino acids, the compact proteolysis-resistant structure of the native seed proteins, the structure and conformation of the proteins, the presence of non-protein compounds (dietary fiber, tannins, phytates) and anti-nutritional factors (protease inhibitors, lectins). Thus, a lack of bioavailability can represent a serious problem for the full exploitation of legumes, and nuts as an alternative sources of proteins (Neacsu et al., 2017).

Two plant-based burgers (soy protein, and pea plus faba proteins) and a meat analogue, together with their ingredients (faba bean concentrate, pea isolate, extruded pea & faba, soy concentrate 70 %, soy concentrate, and texturized soy) were digested according to the INFOGEST static protocol. In parallel, a beef burger was also digested for comparison purposes. For all of them, protein digestibility was evaluated and DIAAR values calculated using the developed method mentioned above, and the impact of grilling and food processing on protein quality and digestibility was also evaluated. When raw, the tested plant-based protein sources (ingredients and final products) had a good digestibility, with values around 85 % or higher.

An increase in amino acid digestibility was observed, when comparing the results obtained for soy concentrate 70 % (ingredient) with the values obtained for the final product (soy meat analog). This indicates a positive effect of the extrusion process on amino acid digestibility. The positive effect of extrusion in protein digestibility was previously reported by several authors (Dahlin & Lorenz, 1993; Fapojuwo et al., 2006; Omosebi et al., 2018; Patil et al., 2016). However, no effect of the texturizing process on amino acid digestibility was found when comparing the results for soy concentrate and texturized soy, and faba bean concentrate, pea isolate and extruded pea & faba. More examples need to be explored in the future, allowing a conclusion on the impact of the extrusion process on protein digestibility.

Grilling did not seem to affect meat protein digestibility, however, the veggie burgers, were negatively affected in their protein digestibility. This decrease in protein digestibility was greater for the pea-faba burger then for the soy burger. The occurrence of Maillard reactions and consequent generation of Maillard reaction products are an undesirable effect of the protein-carbohydrate complexes presented in processed foods that are subjected to heat (Jaeger et al., 2010). Maillard reactions cause nutritional losses of amino acids and decreased amino acids digestibilities (Almeida et al., 2014; González-Vega et al., 2011). Pea-faba burger has more than the double of carbohydrates when compared with soy burger, therefore, the probability of occurrence of Maillard reactions is higher for pea-faba burger than for soy burger. This can explain the decrease of protein digestibility of the pea-faba burger, which was not observed for soy burger, after grilling.

The *in vitro* DIAAR values for all of the essential amino acids were calculated based on TN content and the reference requirement values for preschool children (6 month to 3 years) given by the FAO (FAO, 2013). As expected, beef burger was better digested, yielding higher DIAAR values than the veggie burgers. The difference in DIAAR values was more pronounced between beef and pea-faba burger compared soy, which is in alignment with the concept that soy is a complete protein. DIAAR values for faba bean concentrate, pea isolate, and soy concentrate calculated by means of the *in vitro* method were compared with *in vivo* DIAAR values assessed in growing pigs and were found to be in good agreement.

iv. Physiological relevance of the *in vitro* digestion protocol

It is well accepted that protein is more satiating than the isoenergetic ingestion of carbohydrate or fat in animals and humans, and diets high in protein are associated with weight loss and improved body composition. These observations support the idea that in addition to total energy intake, nutrient-specific mechanisms are also involved to induce satiety and control food intake. Various mechanisms such as secretion of gut satiety hormones, an increase in energy expenditure or an increase in plasma amino acids are possible candidates for protein-induced satiety. It has been postulated that protein-induced satiety could be due to changes in gut neuropeptides secretion with an increased secretion of the gut anorexigenic hormones GLP-1, and CCK, among others (Potier et al., 2009). This has led to a growing scientific interest in recent years in the study of the products of protein digestion in the release of satiety-related hormones in enteroendocrine cells.

The experimental work included in this thesis reveals new data on the secretion of anorexigenic hormones CCK and GLP-1 in the enteroendocrine cell line STC-1 induced by products derived from the digestion of different protein sources. The secretion and gene expression of cholecystokinin (CCK) and glucagon like peptide 1 (GLP-1) were evaluated in

SCT-1 cells in response to *in vitro* gastric and intestinal digests from zein, collagen, sorghum, black beans, pigeon peas and peanuts. After incubation with the soluble fraction of the *in vitro* gastrointestinal digests for 2 h, there was significantly high secretion of GLP-1 and a moderate secretion of CCK, in a dose-dependent manner.

It is worth noting that intestinal fractions contain as protein digestion products, free amino acids, peptides, in addition to free glucose and fatty acids as digestion products from carbohydrates and lipids, respectively. A dependence between the digestion stage and the hormone secretion was evident, although not significant for GLP-1. Intestinal fractions from black bean, sorghum, All-Bran® and zein induced a higher increase in CCK and GLP-1 secretion. *In vivo*, CCK is released in response to protein and fat in the duodenum (Rehfeld, 2021), while GLP-1 is mainly induced by carbohydrates and lipids (Layer et al., 1995). In agreement with these, a significant correlation between the protein content of the digested fractions and the secretion of CCK was found, while GLP-1 secretion was directly and significantly correlated with the carbohydrates content of the digested fractions. However, protein and amino acid stimulation of GLP-1 secretion has been also demonstrated in humans (Chen & Reimer, 2009). In the case of zein, a protein isolate, the secretion of CCK was induced by peptides and amino acids, while for black bean, sorghum and All-Bran® fat and free fatty acids might contribute to the secretion of this hormone. GLP-1 secretion induced by black bean, sorghum and All-Bran is due to the digestion products from proteins and carbohydrates and especially free glucose, while for zein intestinal digests the secretion of this hormone is induced by peptides. GLP-1 secretion is mainly associated to peptides with a MW > 500 Da (Santos-Hernández et al., 2020) or even intact proteins (Geraedts et al., 2011). Our results are in agreement with these previous studies, as the GLP-1 secretion levels for gastric digests, without free glucose and containing longer peptide fragments, were comparable to those obtained with intestinal digests.

Different peptides from zein and collagen digestion-resistant sequences were synthesized and tested for their ability to activate the enteroendocrine cell line STC-1. A clear sequence specificity in the response to the STC-1 cells to these peptides was observed, by evaluating the intracellular Ca^{2+} concentration. However, it remains to be studied the hormonal secretion induced by these peptides in order to better understand the role of these resistant sequences and their characteristics on hormone secretion.

Currently, protein is the only macronutrient requesting *in vivo* studies to evaluate its quality. Thus, it is of extreme importance to develop *in vitro* methods that can be used for this purposes. The high correlation between the *in vitro* DIAAR values calculated using the newly developed protocol and the DIAAR values obtained *in vivo* by human and pig trials state its physiological relevance. This *in vitro* method proved to be a powerful tool that can be used by the food industry and producers as a first approach to determine protein quality of their products. This will be of big help for industry and producers, once they can evaluate their products for protein digestibility and quality in a fast, cheap, robust and reproducible way, and without ethical challenges associated to *in vivo* trials. The method was so far only validated with *in vivo* data for seven protein sources and, in the future, it needs to be further validated with a wide range of foods.

In addition, this method has proven its usefulness in satiety studies. This demonstrates the versatility of this method, making it not only suitable to evaluate protein quality but also to study the satiety effect, by means of hormone secretion, of different foods.

Furthermore, allergenicity studies can also be performed using this method. By evaluating the digestion-resistant proteins/peptides present at the end of intestinal endpoint it is possible to identify possible proteins/peptides responsible for allergies. More and more new alternative proteins are entering the food market and little is known about their behaviour on the human digestive system. Therefore, it is of extreme importance to study and understand the risks of allergenicity of these novel protein sources.

Conclusions/Conclusiones

Conclusions

1. A method to evaluate protein nutritional quality and digestibility was developed based on the INFOGEST static digestion protocol. Among others, the addition of the pancreatic enzymes and the separation of the bioavailable and non-bioavailable fractions was optimized and the use of MeOH 80% was proved to be an efficient precipitation solvent. Protein-free cookie proved to be a good enzymatic background since it avoids the auto-digestion of the enzymes observed in the water blank digest.

2. Total nitrogen by Kjeldahl, primary amines by OPA and sum to the total amino acids by HPLC give similar results for protein digestibility and can be considered equivalent to assess this parameter.

3. The developed *in vitro* method to assess protein quality and DIAAR calculation proved to be a powerful tool to predict *in vivo* data in different types of foods (bias between the two systems is 0.1 % for DIAAR calculation and 1.2 % for protein digestibility). However, the values obtained for isolated proteins were slightly overestimated, especially for low digestible proteins such as zein.

4. The developed method can be used to assess protein quality in highly processed foods such as plant-based burgers and meat analogues. Texturizing process does not to affect significantly protein digestibility in the tested products, while extrusion improves protein digestibility for soy protein, and grilling seems to have a negative impact on DIAAR values of pea and/or faba protein but not for soy or animal protein.

5. Despite well digestible and the high essential/nonessential amino acids ratio, the plant-based products have lower DIAAR values than the beef burger. However, both sources can be considered as good (faba-pea burger) or even excellent (soy burger) protein sources.

6. Gastric and gastrointestinal digests of all studied substrates induced anorexigenic hormone secretion and expression in STC-1 cells, although differences were found with the substrate and the degree of hydrolysis. CCK and GLP-1 secretion was maximized by black bean, sorghum, All-Bran® and zein intestinal digests.

7. A significant positive correlation between the protein content of the digested fractions and the secretion of CCK was found. However, GLP-1 secretion was directly and significantly correlated with the carbohydrate content of the digested fractions.

Conclusiones

1. Se ha optimizado un método basado en el protocolo de digestión estático de INFOGEST para evaluar la digestibilidad y la calidad nutricional de las proteínas alimentarias. Entre otros, se optimizó la incorporación de las enzimas pancreáticas y la separación de la fracción biodisponible y no disponible para lo que la precipitación con MeOH 80 % ha demostrado ser un método efficiente. Se requiere la incorporación de una matriz libre de proteína para calcular el nitrógeno aportado por las enzimas, evitando la excesiva autolisis de estas como ocurre en un blanco con agua.

2. La evaluación de la digestibilidad proteica mediante las determinaciones de nitrógeno total por Kjeldahl, aminas primarias por OPA o la suma de aminoácidos totales cuantificados mediante HPLC arrojan resultados similares y por tanto, cualquiera de estos métodos pueden emplearse en el cálculo de la digestibiidad.

3. El protocolo *in vitro* desarrollado para calcular la calidad nutricional de las proteínas de la dieta y DIAAR ha demostrado ser una herramienta potente para predecir los datos *in vivo* en distintos tipos de alimentos. Sin embargo, los valores obtenidos para aislados proteicos estaban ligeramente sobreestimados, especialmente para proteínas de baja digestibilidad como la zeína.

4. El protocolo desarrollado puede ser empleado para evaluar la calidad nutricional de las proteínas en alimentos altamente procesados, tales como hamburguesas de origen vegetal y análogos cárnicos. El proceso de texturización no afecta significativamente a la digestibilidad proteica en los productos ensayados, mientras que la extrusión mejora la digestibidad de las proteínas de soja. El cocinado tiene un impacto negativo sobre los valores de DIAAR de guisante y/o habas pero no en el caso de la proteína de soja o proteína de origen animal.

5. A pesar de la elevada digestibilidad y el elevado ratio de aminoácidos esenciales/no esenciales determinados en los productos de origen vegetal, estos presentaron valores de DIAAR inferiores a los de la hamburguesa de carne bovina.

6. Los digeridos gástricos y gastrointestinales de todos los sustratos estudiados inducen la secreción y la expresión de hormonas anorexigénicas, aunque se encontraron diferencias en función del sustrato y el grado de hidrólisis. La secreción de CCK y GLP-1 fue máxima en los digeridos intestinales de alubia negra, sorgo, All-Bran® y zeína.

7. Se encontró una correlación positiva significativa entre el contenido en proteína de los digeridos y la secreción de CCK. Sin embargo, la secreción de GLP-1 está directamente relacionada con el contenido en carbohidratos de los digeridos.

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