Molecular composition of lipid and protein fraction of almond, beef and lesser mealworm after *in vitro* simulated gastrointestinal digestion and correlation with the hormone-stimulating properties of the digesta

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Abstract
The current production of meat presents many disadvantages for the environment and much research focuses on alternative protein sources. Insects are novel protein sources highly valued for their nutritional and sustainable potential. However, many aspects concerning biological and nutritional proprieties of the insects after digestion, in comparison with other protein sources, are still overlooked. In this work, a comparative study on three different protein sources, namely almond, lean beef and insect *Alphitobius diaperinus* (lesser mealworm), was performed after *in vitro* simulated gastrointestinal digestion. An in-depth characterization of the chemical composition of the solubilized protein and lipid fractions of the digesta was performed by applying different analytical techniques, including chromatographic methods coupled to mass spectrometry and ^{1}H NMR spectroscopy. Beef and insect were proven to be very similar in amino acid composition and protein solubilization after digestion, when considering the proper corrections for the chitin content. Lipid fraction from insects was solubilized during digestion as the one of almonds, but with a fastest kinetics. Thus, lesser mealworms are a good source of both lipids and highly nutritional proteins. Then, the amino acid composition of raw and digested protein fraction from the three sources was related to the ~~biological activity of the digesta was investigated focusing on the enterohormone secretion of GLP-1 by *in vitro* cell tests. Moreover, the possible correlations of~~ PYY, ghrelin, GLP-1 and CCK release and rats' food intake~~ with the molecular composition of the digesta were also examined~~. ~~Results indicated a comparable level of solubilized protein from lesser mealworm larvae and lean beef digestion, together with a good lipolysis rate. Insects proved to be good meat equivalent, in terms of solubilized proteins and amino acid profile. Almond gave the least solubilized protein, while it presented a high lipolysis rate similar to the one measured in lesser mealworm at the end of the digestion~~. ~~A positive correlation was observed between the biological activity and the amino acid composition of the solubilized protein fraction~~. ~~The increase of GLP-1 hormone secretion was mostly related to His, HPro, Lys, Leu, Met, Cys amino acids when present in the digestate mixture.~~ The composition of amino acids in insect digesta ~~seemed to~~was found to be related to ~~have~~ specific effects on enterohormone release~~ of CCK, total ghrelin in the human colon~~, and the modulation of food intake in rats.

KEYWORDS: gastrointestinal digestion; *in vitro* digestibility; hormone release; food intake; almond; beef; insect

Abbreviations A, almond; B, beef; CCK, cholecystokinin; DG, diacylglycerols; DH, degree of hydrolysis; FAMEs, fatty acid methyl esters; FFA, free fatty acids; I, insects; MG, monoacylglycerols; OG, oral-gastric; OGD, oral-gastric-duodenal; PYY, peptide YY; TFA, total fatty acids; TG, triacylglycerols; tGLP-1, total glucagon-like peptide 1; TTG, degree of transformation of triacylglycerols;

1. Introduction

Nowadays, there is a continuous growth in the global protein demand, driven by the increase of the global population and the socio-economic shifting of dietary preferences (Henchion et al., 2017). Indeed, many researchers are focusing their attention on the use of new, sustainable and high-value protein sources. Among them, edible insects are considered good candidates to be new protein sources for feed and food applications. Insects are appreciated for their nutritional value, quite similar to the traditional animal product, having a good amount and a good nutritional quality of their main constituents, namely proteins (Food and Agriculture Organisation (FAO), 2010; G. Leni et al., 2020; Sánchez-Muros et al., 2016) and fat (Aguilar, 2021; Caligiani et al., 2019; Womeni et al., 2009).

The nutritional quality of a given food is influenced by its composition in essential nutrients, including its ability, once ingested, to be properly absorbed by the organism to satisfy the body's requirements.

Nutrient-gut interactions define the effects, other than the nutritive ones, that food can have in our organism. To study the bioactivity of foods is necessary to understand the underlying mechanisms of these nutrient-gut interactions. Ex vivo and in vitro techniques are essential to pursue this purpose before translating the results to the clinic (Huang et al., 2020).

Current methods of static or dynamic *in vitro* gastrointestinal digestion can simulate the complex digestive process of food. Therefore, *in vitro* digestion from the chemical and biochemical point of view becomes a valuable tool to determine the bioaccessibility (Dima et al., 2020) of food matrices in terms of enzymatic hydrolysis and solubilization of their components. Indeed, the availability of nutrients after digestion can be evaluated on the soluble fraction of the digestate. The soluble fraction can be studied through various analytical techniques and the value of the soluble fraction is calculated based on the total amount of the nutrient. (Etcheverry et al., 2012) Working with digested samples is fundamental in secretory studies with intestinal models, as in physiological conditions the food always reaches this tissue already digested.

An example of one of the most employed *in vitro* static methods is represented by the harmonized INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014), used in this work to obtained digested protein and lipid fractions of selected foods and insects.

From a nutritional point of view, the value of a protein depends mainly on its amino acids composition and digestibility (Meade et al., 2005). Dietary proteins can be found in animal (e.g., eggs, dairy products, meat, fish, insects) and vegetable (e.g. cereals, legumes) sources. Animal proteins are considered to be high-quality among other food sources since they have more balanced proportions of essential amino acids than plant-based food. Furthermore, the animal-derived proteins are appreciated for their higher digestibility, about 95 % more than the 80-85 % exhibited by those from vegetable sources (Wu, 2016).

Most dietary lipids consist of triacylglycerols (TG), which are composed of glycerol esterified to three fatty acid chains of varying length, degree of unsaturation and location within the TG molecule (Ratnayake & Galli, 2009).

The nutritional value of dietary lipids, in terms of energy source and their role on human health, is strongly affected by their fatty acid profile (Masson, 1981). Saturated fatty acids (SFAs), such as palmitic and stearic acids, are mainly supplied by animal fat (i.e., meat, seafood, milk, cheese, eggs, etc.) but also by some plant oils (i.e., coconut and palm oils). Meat content in SFAs depends on animal species, growth, and environmental conditions (Bordoni et al., 2021). On the other hand, lipids derived from plants (e.g., olive oil) or marine algae generally contain higher levels of unsaturated fatty acids (such as oleic, linoleic and linolenic acids), while fish oils are most notable for containing the omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DPA) (Ye et al., 2019).

The availability for absorption of fatty acids into the human digestive system largely depends on the extent of lipolysis after GD digestion as well as their stereospecific distribution in the TG, as, for example, long-chain SFAs, located at the outer positions sn-1 and sn-3, have limited absorption (Umberto Bracco, 1994).
The information on the molecular composition of gastrointestinal digesta both at the protein and lipid level is still very scarce for insects, in comparison with other common protein sources. Therefore, the study of the changes of the chemical and biological properties after gastrointestinal digestion has become a challenge.
In our previous published work (Miguéns-Gómez et al., 2020), on the same protein sources, the interaction between different proteins sources and the gastrointestinal tract was evaluated, concerning the enterhormone secretion in human and pig intestine membrane (i.e., *ex vivo*) and food intake regulation in rats (i.e., *in vivo*). The data obtained in our previous investigation underline the different hormone secretory profiles of all the digested protein sources tested as well as the different modulation of food intake in rats, although no information on the molecular composition of the digesta was obtained.
In this study, lesser mealworm larvae (*Alphitobius diaperinus*), almond (*Prunus dulcis*) and lean beef (*Bos taurus*) were evaluated for their raw composition, and the percentage of released lipids and proteins was measured after having performed *in vitro* simulated gastrointestinal digestion. Degree of hydrolysis of both lipid and protein fractions was also measured and related to the solubilisation after the digestion process. Then, the previously observed biological properties were related to the compounds present in the digesta, to better understand the relation between the food digestate composition and hormones released. ~~In addition, the ability of the digesta to affect the secretion of GLP-1 enterohormone was evaluated and related to its specific amino acid composition.~~

2. Materials and methods

2.1. Solvent and reagent

20x XT MES running buffer, 20x XT reducing agent, 4x XT sample buffer, Coomassie brilliant blu protein stain powder R-250, Criterion TMXT Bis-Tris Precast Gel (12% Bis-Tris, 13.3 x 8.7 x 0.1 cm), and Precision Plus Protein Standards from BIO-RAD (Hercules, CA, USA). Quant-iT™ Protein Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA). Kjeldahl defoamer was purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, Kjeldahl tablets catalyst (3.5 g/tablet), sulfuric acid (96 %), Ultrapure water obtained with Milli-Q® system, The ELISA kit for total GLP-1 were purchased from Merck Millipore (Burlington, MA, USA). Aspartic acid, bile salts, boric acid, Cysteine, formic acid (>95 %), Glutamine, hydrochloric acid (37 % HCl), L-Isoleucine, Methionine sulfone, NAC (N-acetyl-cysteine), DL-norleucine, OPA (o-phthaldialdehyde), pancreatin (containing enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease) from porcine pancreas, porcine α-amylase, porcine pepsin from gastric mucosa, SDS (Sodium dodecyl sulfate), Tryptophan and the amino acids standards for biological assay were purchased from Sigma Aldrich (St. Louis, MO, USA). Amino Acid Standard Mixture (2.5 mM) was purchased from Thermo Scientific (Waltham, MA, USA). ~~Kjtabs VS defoamer was purchased from VELP Scientifica (Usmate Velate, Italy).~~ Acetonitrile (ACN) and Copper (II) oxide were purchased from VWR Chemicals (Radnor, PA, USA). AccQ•Fluor Reagent Kit for Amino Acid Analysis was purchased from Waters (Milford, MA, USA).

2.2. Sample selection

Raw insect larvae belonging to lesser mealworm species (*Alphitobius diaperinus* powder) were provided by Protifarm (Protifarm NV, Ermelo, the Netherlands). Beef (*Bos taurus*), a lean portion, was purchased at a local market (Mercat Central, Tarragona, Spain) and almond (*Prunus dulcis*) flour was provided by Borges Agricultural & Industrial Nuts

(BAIN). All the samples were stored in the dark at −20ºC for optimal conservation. We chose to compare insects as a new protein source with beef, as one of the traditional protein sources and almond as a vegetal protein source.

2.3. *In vitro* digestion

Foods were digested according to the INFOGEST harmonized protocol with some modifications (Brodkorb et al., 2019~~Egger et al., 2016~~), first published in 2014 (Minekus et al., 2014), which consists of the simulation of the three main stages of the *in vivo* digestion: the oral, gastric, and duodenal stages. In our study, we used samples digested up to the gastric phase (Oral-Gastric digestion) and gastrointestinal digested samples (Oral-Gastric-Duodenal digestion). The two types of digestion occurred in parallel. Food quantity was adjusted by protein content to achieve the same ratio of protein per volume for all the samples (0.12 g protein/mL simulated saliva).

Oral-Gastric digestion. The different foods were minced with the simulated saliva using a mincer (Ultra-Turrax T25; IKA Werke, Staufen, Germany) for 2–5 min. Amylase was added after this step (75 U mL−1) and mixed with the minced food for 2 min. Then, the simulated gastric juice containing pepsin (2000 U mL−1), which not included the rabbit gastric lipase, was added to a final ratio of 50:50 (v/v) and mixed for 120 min at 37ºC. The Oral-Gastric digested samples were stopped at this point, and the Oral-Gastric-Duodenal samples went on to the next step.

Oral-Gastric-Duodenal digestion. The Oral-Gastric digested samples were added with the same volume of simulated intestinal juice containing pancreatin (having a trypsin activity of 100 U mL−1) and bile salts (10 mM) to obtain a final ratio of 50:50 (v/v) and mixed for 120 min at 37ºC.

We also applied the same procedures to all three food samples but without enzymes as a negative control for digestion, and as an enzyme control, we followed the procedure using only the enzymes and simulated fluids without food. Finally, we placed all the digestions and the controls in a 90 °C bath for 20 min to stop the enzymatic reactions. After that, we minced the samples to avoid the protein clotting formed after the heating. Then we centrifuged all the samples (3220*g*, 5 min, 4 °C) to discard the undigested fractions: a pellet for all samples and an upper layer of fat for the almond and insect digestions. The samples were frozen at -80 °C and freeze-dried. After that there were stored at -20 ºC.

2.4. Determination of residual moisture

The determination of the relative moisture was performed on each lyophilized sample. 0.5 g of each sample, in duplicate, was dried at 105 °C until reaching a constant weight.

2.5. Lipid analysis

2.5.1. Determination of lipids content

The determination of fat content on lyophilized raw, gastric and gastrointestinal digested samples using Soxhlet extraction was performed. The fat content was extracted by Soxhlet apparatus (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) using 1 g of each lyophilized sample and 70 mL of diethyl ether. The following method was used for the beef and almond samples: 30 minutes of extraction, 60 minutes of washing and 15 minutes of recovery. For the insect, the method used included: 60 minutes of immersion, 30 minutes of washing and 15 minutes for recovery.

2.5.2. Determination of fatty acids profile

Soxhlet lipid extracts obtained from samples before simulated gastrointestinal digestion (A RAW and I RAW) were subjected in duplicate to acid-catalys~~z~~ed transmethylation according to the ISO 12966-2:2017 protocol (*ISO 12966-2:2017 Animal and Vegetable Fats and Oils — Gas Chromatography of Fatty Acid Methyl Esters — Part 2: Preparation of Methyl Esters of Fatty Acids*, 2017), slightly adapted. Briefly, 100 mg of fat were added to 0.5 mL of

H_2SO_4/MeOH (1:15 v/v) and heated at 100 °C for 3 hours. After cooling, fatty acid methyl esters (FAMEs) were recovered with 5 mL of n-hexane containing 0.2 mg of internal standard tetracosane. This solution was diluted to match the linearity range of the GC–MS instrument, by taking 100 µL and diluting with 800 µL of n-hexane. Then, samples were split-injected (1 µL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a SUPELCOWAX® 10 capillary column (30 m x 0.25 mm x 0.25 µm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The concentration of each detected fatty acid was calculated in relation to the concentration of the internal standard, after calculating the response factors using the Supelco 37 Component FAME Mix (Sigma Aldrich, Saint Louis, MO, ®USA). Finally, results were expressed as relative percentage of total FAMEs.

2.5.3. ^{1}H NMR analysis of lipid classes before and after simulated gastrointestinal digestion

^{1}H NMR analysis was performed on the same samples analyzed for fatty acid profiles and the Soxhlet lipid extracts of samples before (A RAW and I RAW) and after simulated oral-gastric phase (A OG, I OG), simulated oral-gastric-duodenal phase (A OGD, I OGD) and, as control, the simulated gastrointestinal fluid (blank). For NMR analysis, about 100 mg of fat were dissolved in 0.8 mL of deuterated chloroform (CDCL3) in a 5mm glass tube. ^{1}H NMR spectra were registered on a Bruker Avance III 400 MHz NMR. Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic eld-strength of 9.4 T. Spectra fi were acquired at 298 K, with 32 K complex points, using a 90° pulse length and 3 s of relaxation delay (d1). 128 scans were acquired with a spectral width of 9595.8 Hz and an acquisition time of 1.707 s. The relaxation delay and acquisition time allow the complete relaxation of the protons, allowing their integrals for quantitative purposes. The whole zone ranging from 0.87 to 2.90 ppm plus signal centered at 5.35 ppm were used as determinant indicative of total fatty acid moles, both free and bound. For the glycerol esters, the specific signals (listed in Table 5) were integrated. Integrals were normalized for the number of hydrogens contributing to the specific signal. In the case of fatty acid integral, the mean number of hydrogens in fatty acids was inferred from the mean fatty acid composition obtained by GC analysis, and it was found as 33.27 for almond and 32.83 for insects. The normalized areas obtained were converted as relative molar percentages.

Quantification of the lipolytic products

To determine the molar percentage (mol %) of free fatty acids (FFA), the total fatty acid molar percentage was subtracted from the contribution of fatty acids bound to triacylglycerols (TG), diacylglycerols (DG) and monoacylglycerols (MG), according to the simple relation previously reported (Nieva-Echevarría et al., 2014) :

$$[FFA] = [TFA] - (3[TG] + 2[DG] + 1[MG])$$

The extent of lipid digestion

The extent of lipid in vitro digestion has been estimated as the degree of transformation of TG (TTG_%), which considers the hydrolysis that occurred in TG, according to the following equation reported (Nieva-Echevarría et al., 2016):

$$TTG_\% = 100\ [(mol_\%\ TGi - mol_\%\ TG)/\ mol_\%\ TGi]$$

where mol_% TGi is the molar percentage of TG initially present in the sample.

2.6. Protein analysis

2.6.1. Protein content

The protein content was evaluated for raw and digested samples using the official Kjeldahl method (DKL heating digester and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) according to European Regulation EC 152/2009, the nitrogen was quantified and subsequently multiplied by the conversion factors respectively: almond 5.18 (Food and Agriculture Organisation (FAO) and World Health Organization (WHO), 1973), beef 5.57 (Mariotti et al., 2019) and insect 6.25.

2.6.2. Electrophoretic profile

The protein pattern of raw and digested samples was evaluated using SDS-PAGE analysis. 0.01 g of each lyophilized sample in 10 mL of water/acetonitrile (50:50 ratio) solution was dissolved. Each sample was mixed with an overhead shaker for 1 h at room temperature and centrifuged for 15 min at 3220*g* and 4 °C. A rate of all the samples corresponding to 30 μg of protein (quantified by Quant-iT™ Protein Assay) was dried under nitrogen flux. Each sample was added with 25 μL of a solution consisting of sample buffer xT 4X, reducing agent 20x and ultrapure water, subsequently loaded into the electrophoretic gel. The electrophoretic run was performed at a constant voltage of 150 V using a Mini-Protean II electrophoresis chamber (Bio-Rad, Hercules, CA, USA). The main proteins were visualized on the gel by staining through Coomassie Blue.

2.6.3. Evaluation of the degree of hydrolysis in digested samples

The degree of hydrolysis (DH) is the percentage of severed peptide bonds compared to the number of total peptide bonds. The DH % was calculated using the OPA method as previously described (Bustamante et al., 2021; Spellman et al., 2003) to obtain a solution that absorbs at a wavelength of 340 nm, using a UV-VIS spectrophotometer (B530 JASCO, Oklahoma City, OK, USA). The calibration curve was prepared using L-isoleucine from 0 to 2 mg / mL. The OPA assay was carried out using 3 μL of the digested sample (or digestion blank), 17 μL of milli-Q water and 2.4 mL of OPA/NAC reagent. During digestion proteins were hydrolyzed, a high number of free amino groups determines a greater protein digestibility. The percentage of the degree of hydrolysis was calculated as reported below:

$$DH\% = \left(\frac{N_{free}}{N_{total}}\right) x\ 100$$

N_{free} was calculated from OPA and N_{total} corresponded to the total nitrogen moles present in the solution before hydrolysis, calculated by the Kjeldahl method.

2.7. Total amino acid

For evaluation of total amino acid profile, 0.1 g of lyophilized sample (raw and digested) were added with 6 mL of 6 M HCl, mixed and hydrolyzed for 23 hours at 110 °C. Subsequently, the samples at room temperature were added with 7.5 mL of 5 mM norleucine water solution, used as internal standard. In conclusion, in a volumetric flask (250 mL) the samples were filtered up to the mark. 10 μL of the final solution obtained, from total amino acid procedures, were derivatized using AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and analyzed by UPLC-MS as previously reported (Buhler et al., 2019).

In addition, another protocol for the determination of the sulfur-containing amino acids (methionine and cysteine) was used in the present study. 0.05 g of each lyophilized sample was added with 0.2 mL of fresh performic acid (previously mixing 95 % formic acid and hydrogen peroxide in a 90:10 ratio and incubating 1 hour at room temperature and 1 hour at 4 °C). The samples were incubated in an ice bath at 0 °C for 16 hours. Subsequently, 0.03 mL of pure hydrobromic

acid (48 wt % in water) was added, mixed and dried under nitrogen flux. The dry matter was resuspended by the addition of 0.6 mL of 6 M HCl, mixed for 1 min under nitrogen flux and incubated at 110 °C for 23 hours. Finally, the samples at room temperature were added with internal standard (0.75 mL of 5 mM norleucine), filtered and made up to the mark in a 25 mL volumetric flask with demineralized water. The samples were derivatized using AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and analysszed by UPLC-MS. Tryptophan was determined as previously described (Prandi, Faccini, et al., 2019) reducing the amount of sample to 0.1 g.

2.8. Protein and peptides identification by high-resolution mass spectrometry

Each lyophilized digested sample was analyzed in high-resolution mass spectrometry using tandem mass spectrometry analysis to identify the main protein and peptide release after oral-gastric and oral-gastric-duodenal digestion. µHPLC (Dionex Ultimate 3000, Sunnyvale, CA, USA) coupled to Orbitrap LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used in the present study.

The condition parameters were previously reported (Prandi, Varani, et al., 2019) except for the loading flow: 30 µL/min, 98% eluent A and 2% eluent B and for protein identification the precursor ion tolerance: 10 ppm.

2.9. Correlation with biological properties of the digested mixtures

A heat map was created to visualize the total amino acid content associated with every source (insect, almond, beef) based on the Wilcoxon rank-sum test, logistic regression, and random forest classification. The different samples as well as the amino acids were reordered by hierarchical clustering. On top of the heatmap a dendrogram of the experiments is given, that is a tree showing the order in which the samples are merged. Samples with similar outcomes are grouped next to each other. The dendrogram on the left of the heatmap gives the similarity between the amino acid percentage in the samples.

Unsupervised principal component analysis (PCA) was performed with XLSTAT 2021.2.1 software (Addinsoft, New York, NY, USA) to assess relationships between the enterohormone secretions obtained from intestinal tissue explants, previously published (Miguéns-Gómez et al., 2020), and the amino acid composition of the digested samples and between the food intake from rats and the amino acid composition of the raw food administered. Principal components (PC) were considered significant if they contributed >50 % to the total variance.

2.10. Cell culture

GLUTag cells were kindly donated by Prof. Staels (Institut Pasteur de Lille, Lille, France) with permission of Prof. Drucker (Lunenfeld-Tanenbaum Research Insitute, Toronto, Canada). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 1 g L−1 glucose, supplemented with 10 % fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 100 U mL−1 penicillin, and 100 mg L−1 streptomycin (Lonza, O Porriño, Spain) and incubated under a 5 % CO_2-humidified atmosphere at 37 °C.

2.11. GLP-1 secretion studies

GLUTag cells were plated onto 24-well plates precoated with matrigel (Lonza, O Porriño, Spain) at a density of 200.000 cells/ml 24 h before the secretion study. Cells were then washed twice with HEPES buffer and treated for 2h with the mix of the amino acids more abundant in the OGD digested beef (His, HPro, Lys, Leu, Met, Cys with a concentration of 0.34, 0.11, 0.42, 0.88, 0.22, 0.34, 0.26 mg/ml respectively) or vehicle (HEPES buffer). After the treatment the medium was collected and stored at -80ºC until the determination of total GLP-1 by ELISA kit. Cells were lysed with RIPA buffer and lysates were analyzedanalysed for total protein content (to control for an equal number of

cells). At least three replicates using cells with different cell passage numbers were performed for each experiment, including at least three wells for each condition in every replicate. Cells with passage numbers below 30 were used.

2.12. Statistical analysis

Data analysis was performed using SPSS version 26.0 software (SPSS Inc., Chicago, IL., USA). The data were subjected to one-way analysis of variance (ANOVA), Tukey's-b test for the homogeneous variance of sample to define significant differences between samples ($p < 0.05$). For GLP-1 secretion analysis Students' unpaired t-test was used and only differences with P-values <0.05 were considered significant.

3. Results and discussion

3.1. Composition of the food matrices: lipids, protein and amino acid profile

3.1.1. Lipids and protein content

Lipids were analysed in all samples by the Soxhlet method, to evaluate the total amount of lipid fraction before digestion. Furthermore, also the nitrogen content was determined in raw samples by the Kjeldahl method in all samples and converted to protein content by multiplying for specific conversion factors. The reported conversion factors for almond and beef were used (Food and Agriculture Organisation (FAO) and World Health Organization (WHO), 1973; Mariotti et al., 2019) (5.18 and 5.57 respectively). On the other side, the commonly used 6.25 conversion factor was used for insects, due to its extensive use by insect-producing companies. It is any way to be reminded that previous papers already demonstrated that this conversion factor is not the most accurate to determine the protein content of insects (Janssen et al., 2017; G. Leni et al., 2020), due to the specific amino acid composition of insect, and mostly to their content of chitin (nitrogen-containing polysaccharide): both features lead to an overestimation of the actual protein value when the 6.25 factor is used.

The amount of lipids and proteins found in raw samples, on dry weight, is reported in Table 1. Lipid and protein content for lesser mealworm (*Alphitobius diaperinus*) samples were in agreement with previous literature data (Adámková et al., 2016).

<<Insert Table 1>>

For all raw samples statistically differences were observed. Lipids in almonds showed the highest value, followed by insects, whereas their amount was found to be very low in raw beef. On the contrary, the protein content on the dry matter was higher in beef than in insects, with almonds showing the lowest value.

3.1.2. Fatty acid profile

The almond and insect raw lipids were characterized for their fatty acid profiles by GC-MS. Results are reported in Table 2. The analysis was not performed in the case of beef considering the negligible amount of lipids contained in the sample.

<<Insert Table 2>>

In both cases the values found agreed with previous literature data (Hernandez, 2015). The fatty acid composition of almond oil consisted primarily of oleic acid (C18:1 cis-9) at about 60 %, followed by linoleic acid (C18:2n-6) at about 30 % and only trace amounts of α -linolenic acid (C18:3n-3; ALA). The remaining 10 % of the fatty acid profile is constituted by saturated fatty acids (SFA), such as lauric (C12:0), myristic (C14:0), C15:0, palmitic (C16:0) (the most abundant at 7 %) and stearic (C18:0) acids. Regarding insect samples, the main fatty acids were represented by oleic and linoleic acids at about 30 % each and palmitic acid (C16:0). Minor amounts of α-linolenic acid (C18:3n-3; ALA) were also detected. This composition is in line with what has already been reported for *Alphitobius diaperinus* (Adámková et al., 2016; Tzompa-sosa et al., 2014), performing the same extraction method, except for the above reported minor amount of α-linolenic acid detected.

3.1.3. Total amino acid profile

Total amino acid content is shown in Table 3 with the results expressed as g of amino acids / 100 g of food matrix (on dry weight). From the total amino acids, it was possible to calculate the true protein content of every matrix, by calculating the total mass of the residual amino acid moieties, i.e. the mass of the amino acid after subtraction of a water molecule (since the amino acids are bound in a condensed form in proteins). This latter method for protein content gives indeed more realistic results than the one based on nitrogen determination through Kjeldahl analysis and subsequent multiplication for a predefined factor to obtain the protein mass. In particular, the use of conversion factors not perfectly tailored to the proteins under analysis, and the presence of non-proteic sources of nitrogen, are definite sources of error in the Kjeldahl analysis. Obviously, this source of error can be avoided if we deduce the protein content only considering the amounts of total amino acids, which are the protein constituents.

<<Insert Table 3>>

Thus, the data obtained on total protein content evaluated by amino acids composition was compared to the protein amount calculated by the Kjeldahl method. For almonds, no significant differences were observed, the protein content was in perfect agreement between the two methods. For beef and insect significant differences were observed between the two methods. Indeed, for beef, the values obtained by Kjeldahl were slightly overestimated compared with total protein calculated by total amino acid (6 % more with Kjeldahl), very likely due to the presence of nitrogen-containing compounds naturally present in meat such as carnosine, anserine and creatine (non-proteinogenic origin) (Crush, 1970; Rao & Gault, 1989; Wu, 2020). In stark contrast, the lesser mealworm protein content obtained by Kjeldahl was highly overestimated (30 % more with Kjeldahl): this can be ascribed, as already anticipated above, to the 6.25 factor used to convert the amount of nitrogen in protein (not the correct one given the amino acid composition), and to the fact that chitin is also contained in the insect biomass, yielding a consistent amount of non-proteic nitrogen wrongly included in the Kjeldahl analysis.

3.2. Characterization of solubilized lipids and proteins after digestion

Samples underwent simulated gastrointestinal digestion according to the procedures detailed in the experimental section (2.3) and already reported in the previous paper (Miguéns-Gómez et al., 2020). Lipids and proteins were quantified after digestion in the soluble part of the digested samples. It can be assumed that the substance solubilized during the digestion phases is closely related to the substance available for absorption after digestion, thus the percentage of protein and lipids solubilized can be related to protein and lipid digestibility in the various matrices (Rieder et al., 2021).

3.2.1. Solubilized lipids after digestion

The lipid content (% dry weight) after oral-gastric (OG) and oral-gastric-duodenal (OGD) digestion of insect and almond were obtained by the Soxhlet method. Beef samples were not analys~~z~~ed, as giving the very low content of lipids in the original raw samples, would have led to unreliable data. Results are reported in Table 4 as percentage of solubilized lipids relative to the total amount of lipids originally present in the sample.

<<Insert Table 4>>

~~Given that the OG digestion contains no lipases, the solubility data obtained after OG is likely to be the same as the original raw sample, possibly increased by the triglyceride hydrolysis induced by the acidic environment. Anyway, even after the duodenal phase, which contained lipases, the increase in solubilized lipids was somehow limited, implying a low activity of the lipase (possibly due to poor accessibility of the enzyme) or to an intrinsic poor solubility of lipids also in form of free fatty acids.~~

^{1}H NMR analysis was performed to evaluate the lipid class distribution in almond and larvae before and after OG and OGD digestion. To obtain quantitative data on the relative amount of each lipid class, a simple procedure based on ^{1}H NMR spectroscopy was used, adapted for the identification and quantitation of different lipid species in the analys~~z~~ed oils, including triglycerides (TG), diglycerides (DG), monoglycerides (MG) and free fatty acids (FFA), as described above (Nieva-Echevarría et al., 2014). The quantitative molar proportions of the different acyl groups present in the lipid extracts of the analys~~z~~ed samples are reported in Table 5.

<<Insert Table 5>>

From the above data, it clearly emerges that the TG fraction after the OG digestion, and even more after OGD digestion, ~~markedly decreased~~markedly decreased as compared to the raw samples, and FFA became the predominant lipid class in both the digested matrices. Given that the OG digestion phase contains no lipases, the increase in FFA in that phase is likely due to the TG hydrolysis induced by the acidic environment. The high free fatty acid content determined in raw lesser mealworm lipids may be probably ascribed to the activity of endogenous lipases, due to killing method and storage, as previously reported for other insects (Caligiani et al., 2019).

Anyway, the marked increase of FFA and decrease of TG, even after the OGD phase, did not result in a strong increase in lipid solubility, implying an intrinsic poor solubility of lipids also in form of free fatty acids.

~~while FFA (at 76.5 % in digested larvae and 39.4 % in almond oils), followed by total MG (as the sum of 1-MG and 2-MG, at 17.6 % in digested insect and 20.8 % in almond oils) became the predominant lipid classes in both the digested matrices.~~

~~However, by Before the digestion experiment, comparing the distribution of the lipid classes of the raw materials already showed a difference in the relative abundance of the FFA among the two matrices can be observed, which were resulted to be higher in insect lipids than in almond lipids, both before (22.8 % vs and 0.0 %, respectively), and after digestion (76.5% and 39.4%, respectively). Indeed, as previously reported, the natural content of FFA in vegetable oils is generally limited (< 10 %) and increases with increasing storage time, temperature, and humidity (Ouzir et al., 2021). On the other hand, the high free fatty acid content determined in raw lesser mealworm lipids is may be probably due to the high concentration of lipase enzyme, consequently its strong activity depending on killing method and storage, as previously reported for other insects (Caligiani et al., 2019). Anyway, this higher amount of FFA in insects both before and after digestion did not affect lipid solubility, as seen above, which resulted in quite similar for both matrices.~~

It is to be noted a consistent increase in free fatty acids, in both matrices (but in a more consistent way in insects) also after gastric digestion, which, as said above, given the absence of any lipase in that phase, can only be due to the hydrolysis induced by the acidic environment.

3.2.2. Protein profile after digestion

The protein pattern of undigested and digested samples, including negative digestion control (same digestion mixtures, but without enzymes), is shown in Figure 1. All raw undigested samples showed clearly separated protein bands. The most abundant proteins found in almond seed were Prunin 1 (61 kDa) and Prunin 2 (63 kDa) which under reducing conditions yielded the acidic and the basic subunits at 42 kDa and 20 kDa, as previously described (De Angelis et al., 2018). The most intense bands of the proteins found in raw beef were shown at ~250 kDa and over 37 kDa corresponding to Myosin (220 kDa) and Actin (41-42 kDa). However, other lighter bands were visible from 15 kDa to 150 kDa region. For raw lesser mealworm, two main bands were visible at 25 kDa and over 37 kDa, but the incomplete insect database hampered a clear-cut attribution of proteins.

For almond and beef, after oral-gastric (OG) digestion only weak protein bands were observed, with intense smearing in the low molecular weight region, indicating the presence of peptides coming from digested proteins. For insect samples, after oral-gastric (OG) digestion the main bands originally present in the raw sample did not weaken, suggesting pepsin resistance. Anyway, in all samples the protein bands completely disappeared after oral-gastric-duodenal (OGD) digestion. Negative digestion control showed intact protein bands, confirming that their disappearance was due to the action of proteolytic enzymes.

<<Insert Figure 1>>

3.2.3. Protein solubilization and hydrolysis degree after digestion

To characterize protein digestibility, the solubilized fractions after digestion was determined by the Kjeldahl method, total and free amino acid and degree of protein hydrolysis, the results are reported in Figure 2. The nitrogen content obtained by Kjeldahl was converted to protein content for all samples, by using the same conversion factors reported in section 3.1.1, and compared with the protein amount originally present before digestion, by calculating the percentage of solubilized proteins, analogously to lipids. Protein hydrolysis degree was also calculated by using the OPA method (details in the experimental section 2.6.3.) and was associated with the solubilized protein content. The amount of solubilized proteins via total amino acids measurement was obtained by relating the amount of solubilized total amino acids (calculated in the digested mixtures as reported before for raw samples) related to the starting value of total amino acids.

For almond and insect samples, protein solubilization increased when comparing the oral-gastric (OG) phase to the oral-gastric-duodenal (OGD) phase, and also the degree of hydrolysis followed the same trend, indicating that such solubilization is strictly linked to their susceptibility to being cleaved during the digestive process. For the beef sample, no statistically significant differences between oral-gastric and oral-gastric-duodenal digesta were detected, possibly due to the action of proteolytic enzymes involved during the *in vitro* simulated gastric phase.

Both the amount of the solubilized proteins and the degree of hydrolysis indicated a higher digestibility of beef, followed by almond and lesser mealworm more or less at the same level. Free amino acids were also determined, but their amount turned out to be very low and scarcely significant in all samples, being about 1 % of the dry mass in beef and almond digesta, and about 10 % of the dry mass in insects digesta.

<<Insert Figure 2>>

The data obtained for almond and beef samples showed that the Kjeldahl method lead to a slight overestimation of the actual protein content, as compared to the protein calculated from the sum of amino acids (11-12 % higher values with Kjeldahl), indicating the presence of small amounts of non-proteinogenic nitrogen in the digestive mixtures.

In stark contrast, for the lesser mealworm samples the degree of protein solubilization was found to be much higher when calculated on the total amino acid amount (37 % higher than the value obtained by Kjeldahl). Again, this is likely due to the presence of chitin: when calculating the solubilization degree by using Kjeldahl, the total initial protein figures also include chitin, whereas solubilized proteins do not (chitin is not digested and therefore does not solubilize). In this way, based on Kjeldahl data, the protein solubilization after digestion is grossly underestimated. This bias completely disappears when using total amino acid as a way to calculate proteins, which allows the correct calculation of both initial proteins and solubilized proteins. Thus, after this re-evaluation of protein solubilization, the bioavailability of insect proteins reached a level comparable to the one of beef.

Also, the total amino acid analysis of the digested mixtures allowed to compare the percentage of solubilization for every single amino acid, to outline if any amino acid had some positive or negative preference in solubilization during digestion, as compared to all the others. Anyway, for all samples, there was no selection effect for amino acids in the digestion phases and the amino acid composition of the digested mixtures mirrored the one found in the raw materials (data not shown), meaning that all amino acids were solubilized in the same way, yielding a total amino acid distribution in solution (hence a nutritional ~~score~~value) very similar to that originally present. This also means that there is no particular bias in the protein digestion, and all the proteins present are digested in the same way, confirming what was already seen in the SDS-PAGE analysis which showed the complete disappearance of all proteins after digestion. Accordingly, analysis of peptide composition of the digested mixtures allowed to determine the main proteins present in the samples, confirming what already seen in SDS-PAGE analysis, i.e. seed storage proteins for almond (prunin and vicilin), and muscle proteins in beef (myoglobin, myosin, tropomyosin, actin) and insects (actin and cuticular proteins).

~~**3.2.4. Protein and peptides identification by high-resolution mass spectrometry**~~

~~To complete the molecular characterization of solubilized protein fraction the identification of the main peptides present in the soluble fraction of the digested samples was performed by high-resolution mass spectrometry on LTQ-Orbitrap, both for oral-gastric and oral-gastric-duodenal digested samples (details in the experimental section). The reason to perform such an analysis was to identify, through the peptides detected, the main proteins undergoing digestion. This serves a twofold purpose: first, identifying the main proteins present in the food matrices, and also, by comparison with literature data, assess if those proteins undergo digestion (hence we observe peptides coming from them) or they are somehow resistant (hence no peptides from those proteins are generated).~~

~~All detected peptides are reported in the Supplementary Material (S1). The protein databases for Almond, Bovine and Insect were used for the identification of the peptides and to identify the proteins which originated them. The main identified proteins, according to the number of identified peptides and their intensity, are reported in Table 6. The scarce presence of protein sequences in the insect databases limited the attribution of proteins and peptides for insect samples.~~

~~<<Insert Table 6>>~~

~~(Giulia Leni et al., 2020; Li et al., 2016; G. J. Wang et al., 2018). This means that the solubilized fraction is composed mostly of peptides coming from the most abundant proteins, which, generate most of the soluble nitrogen fraction after digestion clearly indicating that there is not a particular resistance to digestion in the main proteins from the mixture,~~

~~somehow confirming the data obtained by gel. This also indicates that the lower solubilization observed for almond proteins is due to a generic resistance of all the protein fractions, possibly induced by the diverse matrix or the different amino acid composition, and it is not due to specific resistance to the digestion of specific proteins in the fraction.~~

~~Based on the identified peptides after in vitro digestion, bioactive dipeptides (e.g. RF or GF) can be identified inside the sequences of larger peptides prunin, vicilin, myosin and actin. These dipeptides have been found to control food intake, in particular relating to the stimulation of CCK secretion, as previously reported by Tulipano (Tulipano, 2020) for dairy proteins. Also, peptides derived from muscle proteins showed bioactivity as antihypertensives or ACE inhibitors in previous work (Ryan et al., 2011).~~

3.3 Correlation between amino acid composition and biological properties of the digested mixtures

In a previous paper (Miguéns-Gómez et al., 2020), the ability of digested mixtures from the same biomass matrices to affect hormone secretion at the intestinal level was measured. Here, a further analysis was performed to identify a possible relationship between the amino acid composition and the biological effects previously reported. The data used for the correlation was the total amino acid content of the digesta, which mostly means the amino acid contained in the solubilized peptides and proteins, and not only to free amino acids, whose content in the digesta, as stated above, was nearly irrelevant. ~~and the biological effects previously reported.~~

A heat map was initially constructed to analyze features and sample clustering simultaneously. The differences in amino acid composition for almond, beef and insect, samples both raw and submitted to *in vitro* digestions were analyzed (Figure 3).

<<Insert Figure 3>>

Figure 3 shows that in all the conditions (raw or digested) insect and beef are clustered together, indicating that there are more similarities in the amino acid composition between these protein sources and that they are more different compared with almond amino acid composition.

To ~~understand whether there was a~~outline the relationships between the amino acid composition and the biological effects previously observed, ~~we performed a~~ several PCA analys~~i~~es were performed. The PCA results for all the variables analyzed are shown in Table S2 in the supplementary material.

First, a PCA analysis including ~~The projections in the plane of~~ the amino acid composition of the OG digestions and the enterohormone secretion (ghrelin, CCK and PYY) data obtained from the treatment of pig duodenum explants with the different protein digestions ~~are shown in~~was performed (Figure 4). ~~The distribution of the protein sources in the space based on the different variables is represented in the corresponding right panel. Axis 1 was responsible for explaining 63.48% of the total variance, while axis 2 explained 35.52%.~~ The first principal component (PC1) clearly discriminated between insect and almond gastric digestions. Overall, PC1 was characterized, on ~~the~~ one hand, by Gly, Arg, Glu, Asp, grouped on the left side of the graphic together with PYY, ~~which were grouped on the left side of the graphic~~. Those amino acids were most abundant in the protein fraction coming from digested almonds On the other hand, ~~we observed that~~ Ser, Val, Trp, Ala, Tyr, Pro, Ile, Thr, Phe and CCK were grouped on the right side of the PC1. ~~These groupings indicate a positive association between CCK and the amino acids located on this side of the panel,~~These latter amino acids ~~which~~ are most abundant in the protein fraction coming from digested lesser mealworm. By contrast, CCK was negatively associated with Arg, Glu and Asp, more abundant in the protein fraction coming from digested almonds. ~~PYY was positively associated with Gly, Arg, Glu and Asp, all abundant in the protein fraction coming from digested almonds, and negatively with Ala, Trp, Ser and Val. The contribution of PC2 explains the~~

~~differences between insect or almond, and beef.~~ PC2 ~~i~~was mainly described by HPro, Lys, Leu, His, Cys, Met, and Thr, located in the upper part of the graphic, and active ghrelin and Phe, located in the lower part of the graphic. The~~se~~ former amino acids are the most abundant in the digested protein fraction coming from digested beef, showing that ~~. The distribution of the variables in PC2, clearly showed that~~ active ghrelin was negatively associated with those amino acids.

<<Insert Figure 4>>

Next, a PCA analysis with the data from the amino acid composition of the OGD digestions and the enterohormone secretion data (obtained after the treatment of human colon explants with these OGD digestions) was run (Figure 5). ~~In that case, axis 1 and 2 were responsible for explaining around 50% of the total variance.~~ Like in gastric digestions, the PC1 discriminates between insect and almond digestions and it~~. PC1~~ showed that PYY was associated with ~~Asp, Glu, Cys, Arg and Gly and negatively with Val, Ala and Ser. This amino acid profile corresponds to~~ the amino acid composition of the protein fraction coming from OGD digestion in almonds. Total ghrelin was positively associated with Gly, Arg and Cys and negatively associated with Tyr, Pro and Phe, the latter among ~~some of~~ the most abundant amino acids in the protein fraction from OGD digested lesser mealworm. In PC2, ~~still explains the different compositions observed for almonds and insects as compared to beef. Here~~ total GLP-1 ~~i~~was positively associated with Leu, Lys, Met, His, HPro, Ile, Thr, Trp and Cys, the amino acids more abundant in the protein fraction from OGD digested beef. Even if the effect is likely mostly due to bound amino acids, ~~S~~since GLUTag cells produce GLP1, this possible correlation was tested experimentally. GLUTag cells were treated with the most abundant amino acids present in the protein fraction of OGD digested beef, to see if they could exert the same result as the digested protein. His, HPro, Lys, Leu, Met, Cys were used, with an equivalent concentration as the one present in the protein fraction from OGD digested beef. Indeed, these amino acids induced a 1.7-fold increase in total GLP-1 secretion as compared to control (82.86 ± 10.63 ~~and~~against 49.1 ± 5.69 pM ~~for beef amino acids and vehicle respectively~~; $p<0.05$), indicating that the more abundant amino acids in the OGD digested beef might participate in the stimulation of the total GLP-1 secretion previously published in human colon (Miguéns-Gómez et al., 2020).

<<Insert Figure 5>>

Finally, a PCA was run with the amino acid composition of the raw samples and the effects on food intake observed after the administration of these samples to rats. ~~Axis 1 explained 54,47% of the total variance, and similar to what occurred with the PCA of the OG and OGD digestions, it contributed to the differences between~~PC1 clearly discriminated between almond and lesser mealworm amino acid composition. Figure 6 shows that food intake, measured either at 3 or 20 hours after food replacement (beginning of the dark cycle), was directly associated with Ser, Ala, Tyr, Val and Pro (the most abundant amino acids in raw insects) and negatively associated with Gly, Arg and Glu, the mo~~re~~st abundant amino acids in ~~the protein fraction of raw insect and~~ almond ~~respectively (Figure 3)~~.

<<Insert Figure 6>>

Overall, the present data on total amino acid content indicates that *Alphitobius diaperinus* and beef are more similar as compared to almonds, as described before (Orkusz, 2021). In our previous study (Miguéns-Gómez et al., 2020), the secretome of intestinal segments (both pig duodenum or human colon) yielded different results with different protein

sources, albeit at the same concentration, which suggested that the total amount of protein was not the only factor responsible for the secretion of these enterohormones. In addition, no relation was observed between enterohormone release and the amount of energy present in each digested sample, suggesting that the amino acid composition or the specific peptides present were the primary factors responsible for the observed results. ~~With t~~The present ~~complete~~correlation found between the amino acid composition of the different digesta and the enterohormone secretion confirms our previous results, and indicate in the released peptide fraction (different for every source, since different is the amino acid composition of the protein fraction) a possible reason for the observed differences~~characterization of the protein samples, together with the PCA analysis, we can now better explain our previous results~~. Anyway, it must be kept in mind that these observed correlations might be spurious, and further experiments trying to isolate specific peptides released during digestion and able to modify enterhormone secretion will be needed in order to confirm the observed correlations.

The positive and negative associations here described between the amino acids and CCK secretion mostly agree~~d~~ with what reported in the literature~~the high or low content of the respective amino acids in the protein fraction from OG digested lesser mealworm~~. The aromatic amino acids ~~L-Phenylalanine and Tryptophan~~Phe and Trp, are considered potent stimuli for CCK secretion (Liddle, 1997). ~~Daly et. al, observed an increase in CCK secretion after stimulation with L-Phe ex vivo with proximal intestinal tissue in mice and in vitro with STC-1 cells (Daly et al., 2013). The same effect was observed when working with isolated intestinal cells from mice (Y. Wang et al., 2011). On the other hand,~~ Ile also stimulated CCK secretion *ex vivo* in pigs (Tian et al., 2019), while Val~~ine~~ has been reported to not stimulate CCK secretion in vivo in men (Elovaris et al., 2019) or ex vivo in pigs (Tian et al., 2019)~~, but in this last experiment the authors also reported CCK secretion after treatment with Isoleucine~~.~~ No information has been found for similar experiments about Ser, Ala, Tyr and Pro amino acids and their CCK secretory capacity~~. ~~Then, these results suggest that CCK secretion in pig duodenum by OG digested insects could be mainly triggered by Phe, Trp and Ile.~~ Thus, the results of the reported correlation with the amino acids found in the digesta, together with literature, suggest that CCK secretion in pig duodenum by OG digested insects could be mainly triggered by the presence of Phe, Trp and Ile. As indicated above, that does not necessarily involve that they exert their effects as free amino acids after they have been released during the digestion, but this effect can also be due to their presence in peptides having a specific sequence. (Tulipano, 2020; Caron et al., 2017). Thus, a further study on the peptides released after digestion and their relationship with CCK secretion, as said above, is certainly needed in order to shed more light into it.

For total GLP-1 secretion measured in the human colon, our results showed that it could be explained mostly for the amino acid composition of the OGD beef, This partially agrees with Rigamonti et al. which found a positive correlation between Leu, Lys, Met and Ile plasmatic levels in humans and GLP-1 (Rigamonti et al., 2020).

~~When the same tissue was treated with OG digested beef, an increase in the CCK secretion was also observed, but this increase may be due to the presence of other components, as the amino acid profile here is not positively associated with increased secretion of this hormone. A clear positive association was also observed between the amino acid composition of the protein fraction of the digested almond and the PYY secretion.~~ Finally, active ghrelin seemed to be negatively related to the main amino acids present in the protein fraction of OG digested beef (HPro, Lys, Leu, His, Cys, Met, and Thr). In humans, Leucine and Cysteine have shown a reducing effect in ghrelin plasmatic levels (Mcgavigan et al., 2015; Steinert et al., 2017). ~~No information in similar models has been found regarding the rest of the amino acids negatively associated with ghrelin. Nevertheless, this observation confirms our previous results indicating that beef was the most active source at reducing the active ghrelin secretion in pig duodenum and that it could be caused at least by Leu and Cys. On the other hand, the positive association between ghrelin and Phe was surprising, as none of the samples stimulated ghrelin secretion. The commonly described effect is a reduction of active ghrelin by~~

Phenylalanine, *in vivo* in rats (Alamshah et al., 2017), and ex vivo with mouse gastric mucosa (Nunez-Salces et al., 2021).

Total GLP-1 secretion measured in the human colon can be explained mostly for the amino acid composition of the OGD beef, being Leu, Lys, Met, His, HPro, Ile, Thr, and Cys the more abundant ones in this sample. We checked this in vitro treating GLUTag cells with a mix of some of the most abundant amino acids in OGD beef, obtaining a substantial increase in secretion compared to the vehicle. Rigamonti *et al.* found a positive correlation between Leu, Lys, Met and Ile plasmatic levels in humans and GLP-1 (Rigamonti et al., 2020). Then, with these results and our findings, we can say that the OGD beef amino acid composition is probably responsible for the GLP-1 secretion observed in the human colon *ex vivo*, even though Leucine was shown to not elicit any increase in GLP-1 after an intragastric infusion in lean participants (Meyer-Gerspach et al., 2016).

On the other hand, the amino acid composition of the OGD digested lesser mealworm could be responsible for the secretion of the total ghrelin in the human colon. Phenylalanine is described to exert a reduction of active ghrelin secretion in vivo in rats (Alamshah et al., 2017), and *ex vivo* with mouse gastric mucosa (Nunez-Salces et al., 2021). No other comparable studies have been found for other amino acids (Flynn et al., 2020).

Regarding the effect of these protein sources in the modulation of food intake in rats, we previously observed that the lesser mealworm increased it, almond reduced it and beef did not modify it after an acute administration of 300 mg protein/kg body weight (Miguéns-Gómez et al., 2020). It should be mentioned that rat food intake at 3 and 20 hours was directly associated with the amino acids mostly present in the lesser mealworm and inversely associated with those mostly present in almonds as shown in Figure 4 and Figure 7. These results agree with other authors that have demonstrated that Arg and Glu (in our case mostly abundant in almond) are two of the most anorectic amino acids in rats (Jordi et al., 2013).

A positive association between food intake and the amino acids Val, Ser and Ala and a negative association between the secretion of the anorexigenic enterohormone PYY and these amino acids was found. In humans, an increased food intake in children consuming more Val has been suggested (Lu et al., 2020). On the other hand, Val was one of the 8 amino acids implied in the appetite-suppressant effects of whey proteins through GLP-1 stimulatory effects (Rigamonti et al., 2020). In piglets under a low protein diet, Leu and Val balance markedly increased the feed intake (Yin et al., 2020). In mice fed a high-fat diet supplemented with Val, it did not increase food intake (Bishop et al., 2020). These discrepancies strengthen that the effects of the amino acid Val on food intake depend on the experimental model and design. In this sense, in mice, Ser supplementation led to reduced food intake (Holm et al., 2018; Zhou et al., 2018), but this was observed only after chronic supplementation and linked to hypothalamic effects, while our results showed a positive association of Ser and food intake refer to measures at 3 and 20 hours after an acute protein source administration. Also, a negative association with food intake and a positive for PYY was found with Arg, Gly and Glu. In agreement, acute administration of Arg to rodents led to reduced food intake and increased PYY secretion (Alamshah et al., 2016; Jordi et al., 2013), but. Also in healthy human volunteers Arg in combination with a meal can significantly elevate PYY, although no reductiondid not reduce in food intake was found (Amin et al., 2018). Other authors have often reported food intake modulation in vivo without correlations with intestinal hormone release (Caron et al., 2017). In the same study, they found no effects of Gly on PYY secretion.

ThusIn conclusion, the present results show that the previously observed effects of different protein sources on food intake arecan be asscoiated mostly associated with the amino acid profile of these sources. As already indicated above, in order to validate the results obtained by these correlations more experiments with actual peptides released during digestion will be needed., being a higher % of Val, Ser and Ala and lower % of Arg, Glu and Gly related to an increased food intake in rats and inversely associated with the PYY intestinal release.

4. Conclusion

Beef and insect were proven to have high similarities in amino acid composition and protein solubilization after digestion (considering the proper corrections for the chitin content)~~ and peptide composition of the digested mixture proved all major proteins being accessible for the digestive enzymes~~. Lipid fraction from insects appeared to be as accessible as the one of almonds, but with a fastest kinetics, likely due to the starting higher content of free fatty acids in insects. Thus, lesser mealworms are a good source of both lipids and highly nutritional proteins.

Looking beyond pure nutrition, the secretion of the different enterohormones ~~CCK in pig duodenum was positively~~ was found to be correlated to the amino acid~~s~~ composition of the digesta coming from the different sources ~~digestions mostly present in the protein fraction of gastric digested insects, while PYY secretion was positively associated with the amino acid composition of the protein fraction coming from gastric digested almond~~. ~~A major positive correlation with the amino acid composition of the digested protein fractions was found between GLP-1 secretion and beef, and total ghrelin in the human colon and insect.~~ The modulation of food intake in rats, which was differently modified by the three protein sources, was also associated with their different amino acid profile. Thus, ~~B~~besides the nutritional value, insect proteins seem to have specific effects on enterohormone release and food intake, which might even increase their value as a new source of food and feed. The actual amino acids/peptides present after digestion and exerting these effects will have to be validated in the future through dedicated experiments

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References

Adámková, A., Kourimská, L., Borkovcová, M., Kulma, M., & Mlček, J. (2016). Nutritional values of edible Coleoptera (Tenebrio molitor, Zophobas morio and Alphitobius diaperinus) reared in the Czech Republic. *Potravinarstvo*, *10*(1), 663–671. https://doi.org/10.5219/609

Aguilar, J. G. dos S. (2021). An overview of lipids from insects. *Biocatalysis and Agricultural Biotechnology*, *33*(December 2020). https://doi.org/10.1016/j.bcab.2021.101967

Alamshah, A., Mcgavigan, A. K., Spreckley, E., Kinsey-Jones, J. S., Amin, A., Tough, I. R., O'Hara, H. C., Moolla, A., Banks, K., France, R., Hyberg, G., Norton, M., Cheong, W., Lehmann, A., Bloom, S. R., Cox, H. M., & Murphy, K. G. (2016). L-arginine promotes gut hormone release and reduces food intake in rodents. *Diabetes, Obesity and Metabolism*, *18*(5), 508–518. https://doi.org/10.1111/dom.12644

~~Alamshah, A., Spreckley, E., Norton, M., Kinsey-Jones, J. S., Amin, A., Ramgulam, A., Cao, Y., Johnson, R., Saleh, K., Akalestou, E., Malik, Z., Gonzalez-Abuin, N., Jomard, A., Amarsi, R., Moolla, A., Sargent, P. R., Gray, G. W., Bloom, S. R., & Murphy, K. G. (2017). L-phenylalanine modulates gut hormone release and glucose tolerance, and suppresses food intake through the calcium-sensing receptor in rodents. *International Journal of Obesity*, *41*(11), 1693–1701. https://doi.org/10.1038/ijo.2017.164~~

Amin, A., Neophytou, C., Thein, S., Martin, N. M., Alamshah, A., Spreckley, E., Bloom, S. R., & Murphy, K. G. (2018). L-Arginine Increases Postprandial Circulating GLP-1 and PYY Levels in Humans. *Obesity*, *26*(11), 1721–1726. https://doi.org/10.1002/oby.22323

Bishop, C. A., Schulze, M. B., Klaus, S., & Weitkunat, K. (2020). The branched-chain amino acids valine and leucine have differential effects on hepatic lipid metabolism. *FASEB Journal*, *34*(7), 9727–9739. https://doi.org/10.1096/fj.202000195R

Bordoni, L., Petracci, I., Zhao, F., Min, W., Pierella, E., Assmann, T. S., Martinez, J. A., & Gabbianelli, R. (2021). Nutrigenomics of dietary lipids. *Antioxidants*, *10*(7). https://doi.org/10.3390/antiox10070994

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Buhler, S., Solari, F., Gasparini, A., Montanari, R., Sforza, S., & Tedeschi, T. (2019). *LWT - Food Science and Technology UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey*. *105*(July 2018), 127–134. https://doi.org/10.1016/j.lwt.2019.01.051

Bustamante, S. Z., González, J. G., Sforza, S., & Tedeschi, T. (2021). Bioactivity and peptide profile of whey protein hydrolysates obtained from Colombian double-cream cheese production and their products after gastrointestinal digestion. *Lwt*, *145*(November 2020). https://doi.org/10.1016/j.lwt.2021.111334

Caligiani, A., Marseglia, A., Sorci, A., Bonzanini, F., Lolli, V., Maistrello, L., & Sforza, S. (2019). Influence of the killing method of the black soldier fly on its lipid composition. *Food Research International*, *116*(January 2018), 276–282. https://doi.org/10.1016/j.foodres.2018.08.033

Caron, J., Domenger, D., Dhulster, P., Ravallec, R., & Cudennec, B. (2017). Protein Digestion-Derived Peptides and the Peripheral Regulation of Food Intake. *Frontiers in Endocrinology*, *8*(APR). https://doi.org/10.3389/FENDO.2017.00085

Crush, K. G. (1970). Carnosine and related substances in animal tissues. *Comparative Biochemistry And Physiology*, *34*(1), 3–30. https://doi.org/10.1016/0010-406X(70)90049-6

~~Daly, K., Al-Rammahi, M., Moran, A., Marcello, M., Ninomiya, Y., & Shirazi-Beechey, S. P. (2013). Sensing of amino~~

acids by the gut-expressed taste receptor T1R1-T1R3 stimulates CCK secretion. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *304*(3). https://doi.org/10.1152/ajpgi.00074.2012

De Angelis, E., Bavaro, S. L., Forte, G., Pilolli, R., & Monaci, L. (2018). Heat and pressure treatments on almond protein stability and change in immunoreactivity after simulated human digestion. *Nutrients*, *10*(11), 1–20. https://doi.org/10.3390/nu10111679

Dima, C., Assadpour, E., Dima, S., & Jafari, S. M. (2020). Bioavailability and bioaccessibility of food bioactive compounds; overview and assessment by in vitro methods. *Comprehensive Reviews in Food Science and Food Safety*, *19*(6), 2862–2884. https://doi.org/10.1111/1541-4337.12623

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M. J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., … Portmann, R. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International*, *88*, 217–225. https://doi.org/10.1016/j.foodres.2015.12.006

Elovaris, R. A., Fitzgerald, P. C. E., Bitarafan, V., Ullrich, S. S., Horowitz, M., & Feinle-Bisset, C. (2019). Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men. *Nutrients*, *11*(1). https://doi.org/10.3390/nu11010099

Etcheverry, P., Grusak, M. A., & Fleige, L. E. (2012). Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B 6, B 12, D, and E. *Frontiers in Physiology*, *3 AUG*(August), 1–22. https://doi.org/10.3389/fphys.2012.00317

Flynn, N. E., Shaw, M. H., & Becker, J. T. (2020). Amino acids in health and endocrine function. *Advances in Experimental Medicine and Biology*, *1265*, 97–109. https://doi.org/10.1007/978-3-030-45328-2_6

FAO. (2013). Dietary protein quality evaluation in human nutrition. In Food and Agriculrure Organization of the United Nations. https://www.fao.org/documents/card/en/c/ab5c9fca-dd15-58e0-93a8-d71e028c8282/

Food and Agriculture Organisation (FAO). (2010). Forest insects as food: humans bite back. In *Rap* …. http://ultimatepreparednesslibrary.com/wp-content/uploads/2012/03/EdibleInsects-humansbiteback.pdf https://www.fao.org/3/i1380e/i1380e00.pdf

Food and Agriculture Organisation (FAO) and World Health Organization (WHO). (1973). *Energy and protein requirements*. https://apps.who.int/iris/bitstream/handle/10665/41042/WHO_TRS_522_eng.pdf

Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., & Tiwari, B. (2017). Future protein supply and demand: Strategies and factors influencing a sustainable equilibrium. *Foods*, *6*(7), 1–21. https://doi.org/10.3390/foods6070053

Hernandez, E. M. (2015). Specialty Oils: Functional and Nutraceutical Properties. Functional and Nutraceutical Properties. In *Functional Dietary Lipids: Food Formulation, Consumer Issues and Innovation for Health*. Elsevier Ltd. https://doi.org/10.1016/B978-1-78242-247-1.00004-1

Holm, L. J., Haupt-Jorgensen, M., Larsen, J., Giacobini, J. D., Bilgin, M., & Buschard, K. (2018). L-serine supplementation lowers diabetes incidence and improves blood glucose homeostasis in NOD mice. *PLoS ONE*, *13*(3), 1–11. https://doi.org/10.1371/journal.pone.0194414

Huang, W. K., Xie, C., Young, R. L., Zhao, J. B., Ebendorff-Heidepriem, H., Jones, K. L., Rayner, C. K., & Wu, T. Z. (2020). Development of innovative tools for investigation of nutrient-gut interaction. *World Journal of Gastroenterology*, *26*(25), 3562–3576. https://doi.org/10.3748/wjg.v26.i25.3562

ISO 12966-2:2017 Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 2: Preparation of methyl esters of fatty acids. (2017). https://www.iso.org/standard/72142.html

Janssen, R. H., Vincken, J. P., Van Den Broek, L. A. M., Fogliano, V., & Lakemond, C. M. M. (2017). Nitrogen-to-Protein Conversion Factors for Three Edible Insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens. *Journal of Agricultural and Food Chemistry*, *65*(11), 2275–2278. https://doi.org/10.1021/acs.jafc.7b00471

Jordi, J., Herzog, B., Camargo, S. M. R., Boyle, C. N., Lutz, T. A., & Verrey, F. (2013). Specific amino acids inhibit food intake via the area postrema or vagal afferents. *Journal of Physiology*, *591*(22), 5611–5621. https://doi.org/10.1113/jphysiol.2013.258947

Leni, G., Soetemans, L., Jacobs, J., Depraetere, S., Gianotten, N., Bastiaens, L., Caligiani, A., & Sforza, S. (2020). Protein hydrolysates from alphitobius diaperinus and hermetia illucens larvae treated with commercial proteases. *Journal of Insects as Food and Feed*, *6*(4), 393–404. https://doi.org/10.3920/JIFF2019.0037

Leni, Giulia, Prandi, B., Varani, M., Faccini, A., Caligiani, A., & Sforza, S. (2020). Peptide fingerprinting of Hermetia illucens and Alphitobius diaperinus: Identification of insect species-specific marker peptides for authentication in food and feed. *Food Chemistry*, *320*(November 2019), 126681. https://doi.org/10.1016/j.foodchem.2020.126681

~~Li, S., Geng, F., Wang, P., Lu, J., & Ma, M. (2016). Proteome analysis of the almond kernel (Prunus dulcis). *Journal of the Science of Food and Agriculture*, *96*(10), 3351–3357. https://doi.org/10.1002/jsfa.7514~~

Liddle, R. A. (1997). Cholecystokinin cells. *Annual Review of Physiology*, *59*(2), 221–242. https://doi.org/10.1146/annurev.physiol.59.1.221

Lu, J., Gu, Y., Liu, H., Wang, L., Li, W., Li, W., Leng, J., Zhang, S., Qi, L., Yang, X., & Hu, G. (2020). Daily Branched-Chain Amino Acid Intake and Risks of Obesity and Insulin Resistance in Children: A Cross-Sectional Study. *Obesity*, *28*(7), 1310–1316. https://doi.org/10.1002/oby.22834

Mariotti, F., Tomé, D., & Mirand, P. (2019). Converting Nitrogen into Protein – Beyond 6 . 25 and Jones ' Factors To cite this version : HAL Id : hal-02105858. *Critical Reviews in Food Science and Nutrition*, 1–21.

Masson, L. (1981). Relative nutritional value of various dietary fats and oils. *Journal of the American Oil Chemists' Society*, *58*(3), 249–255. https://doi.org/10.1007/BF02582351

Mcgavigan, A. K., O'hara, H. C., Amin, A., Kinsey-Jones, J., Spreckley, E., Alamshah, A., Agahi, A., Banks, K., France, R., Hyberg, G., Wong, C., Bewick, G. A., Gardiner, J. V., Lehmann, A., Martin, N. M., Ghatei, M. A., Bloom, S. R., & Murphy, K. G. (2015). L-cysteine suppresses ghrelin and reduces appetite in rodents and humans. *International Journal of Obesity*, *39*(3), 447–455. https://doi.org/10.1038/ijo.2014.172

Meade, S. J., Reid, E. A., & Gerrard, J. A. (2005). The impact of processing on the nutritional quality of food proteins. *Journal of AOAC International*, *88*(3), 904–922. https://doi.org/10.1093/jaoac/88.3.904

Meyer-Gerspach, A. C., Häfliger, S., Meili, J., Doody, A., Rehfeld, J. F., Drewe, J., Beglinger, C., & Wölnerhanssen, B. (2016). Effect of L-Tryptophan and L-Leucine on gut hormone secretion, appetite feelings and gastric emptying rates in lean and non-diabetic obese participants: A randomized, double-blind, parallel-group trial. *PLoS ONE*, *11*(11), 1–15. https://doi.org/10.1371/journal.pone.0166758

Miguéns-Gómez, A., Grau-Bové, C., Sierra-Cruz, M., Jorba-Martín, R., Caro, A., Rodríguez-Gallego, E., Beltrán-Debón, R., Blay, M. T., Terra, X., Ardévol, A., & Pinent, M. (2020). Gastrointestinally digested protein from the insect alphitobius diaperinus stimulates a different intestinal secretome than beef or almond, producing a differential response in food intake in rats. *Nutrients*, *12*(8), 1–15. https://doi.org/10.3390/nu12082366

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., MacIerzanka, A., MacKie, A., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). A method based on 1H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures. *Food Research International*, *66*, 379–387. https://doi.org/10.1016/j.foodres.2014.09.031

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). A study by 1H NMR on the influence of some factors affecting lipid in vitro digestion. *Food Chemistry*, *211*, 17–26. https://doi.org/10.1016/j.foodchem.2016.05.021

~~Nunez-Salces, M., Li, H., Young, R. L., & Page, A. J. (2021). The secretion of total and acyl ghrelin from the mouse gastric mucosa: Role of nutrients and the lipid chemosensors FFAR4 and CD36. *Peptides*, *146*(October), 170673. https://doi.org/10.1016/j.peptides.2021.170673~~

Orkusz, A. (2021). Edible insects versus meat—nutritional comparison: Knowledge of their composition is the key to good health. *Nutrients*, *13*(4). https://doi.org/10.3390/nu13041207

Ouzir, M., Bernoussi, S. El, Tabyaoui, M., & Taghzouti, K. (2021). Almond oil: A comprehensive review of chemical composition, extraction methods, preservation conditions, potential health benefits, and safety. *Comprehensive Reviews in Food Science and Food Safety*. https://doi.org/10.1111/1541-4337.12752

Prandi, B., Faccini, A., Lambertini, F., Bencivenni, M., Jorba, M., Droogenbroek, B. Van, Bruggeman, G., Schöber, J., Petrusan, J., Elst, K., & Sforza, S. (2019). Food wastes from agrifood industry as possible sources of proteins : A detailed molecular view on the composition of the nitrogen fraction , amino acid pro fi le and racemisation degree of 39 food waste streams. *Food Chemistry*, *286*(December 2018), 567–575. https://doi.org/10.1016/j.foodchem.2019.01.166

~~Prandi, B., Varani, M., Faccini, A., Lambertini, F., Suman, M., Leporati, A., Tedeschi, T., & Sforza, S. (2019). Species specific marker peptides for meat authenticity assessment: A multispecies quantitative approach applied to Bolognese sauce. *Food Control*, *97*(July 2018), 15–24. https://doi.org/10.1016/j.foodcont.2018.10.016~~

Rao, M. V., & Gault, N. F. S. (1989). The influence of fibre-type composition and associated biochemical characteristics on the acid buffering capacities of several beef muscles. *Meat Science*, *26*(1), 5–18. https://doi.org/10.1016/0309-1740(89)90053-3

Ratnayake, W. M. N., & Galli, C. (2009). Fat and fatty acid terminology, methods of analysis and fat digestion and metabolism: A background review paper. *Annals of Nutrition and Metabolism*, *55*(1–3), 8–43. https://doi.org/10.1159/000228994

Rieder, A., Afseth, N. K., Böcker, U., Knutsen, S. H., Kirkhus, B., Mæhre, H. K., Ballance, S., & Wubshet, S. G. (2021). Improved estimation of in vitro protein digestibility of different foods using size exclusion chromatography. *Food Chemistry*, *358*(November 2020). https://doi.org/10.1016/j.foodchem.2021.129830

Rigamonti, A. E., Leoncini, R., De Col, A., Tamini, S., Cicolini, S., Abbruzzese, L., Cella, S. G., & Sartorio, A. (2020). The appetite−suppressant and GLP-1-stimulating effects of whey proteins in obese subjects are associated with increased circulating levels of specific amino acids. *Nutrients*, *12*(3), 775. https://doi.org/10.3390/nu12030775

~~Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: Meat and fish. *Nutrients*, *3*(9), 765–791. https://doi.org/10.3390/nu3090765~~

Sánchez-Muros, M. J., de Haro, C., Sanz, A., Trenzado, C. E., Villareces, S., & Barroso, F. G. (2016). Nutritional evaluation of Tenebrio molitor meal as fishmeal substitute for tilapia (Oreochromis niloticus) diet. *Aquaculture Nutrition*, *22*(5), 943–955. https://doi.org/10.1111/anu.12313

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*. https://doi.org/10.1016/S0958-6946(03)00053-0

Steinert, R. E., Feinle-Bisset, C., Asarian, L., Horowitz, M., Beglinger, C., & Geary, N. (2017). Ghrelin, CCK, GLP-1, and PYY(3-36): Secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. *Physiological Reviews*, *97*(1), 411–463. https://doi.org/10.1152/physrev.00031.2014

Tian, M., Heng, J., Song, H., Zhang, Y., Chen, F., Guan, W., & Zhang, S. (2019). Branched chain amino acids stimulate gut satiety hormone cholecystokinin secretion through activation of the umami taste receptor T1R1/T1R3 using an: In vitro porcine jejunum model. *Food and Function*, *10*(6), 3356–3367. https://doi.org/10.1039/c9fo00228f

Tulipano, G. (2020). Role of bioactive peptide sequences in the potential impact of dairy protein intake on metabolic health. *International Journal of Molecular Sciences*, *21*(22), 1–27. https://doi.org/10.3390/ijms21228881

Tzompa-sosa, D. A., Yi, L., Valenberg, H. J. F. Van, Boekel, M. A. J. S. Van, & Lakemond, C. M. M. (2014). Insect lipid pro fi le : aqueous versus organic solvent-based extraction methods. *FRIN*, *62*, 1087–1094. https://doi.org/10.1016/j.foodres.2014.05.052

Umberto Bracco. (1994). Effect structure on fat absorption. *American Journal of Clinical Nutrition*, *February*.

~~Wang, G. J., Zhou, G. Y., Ren, H. W., Xu, Y., Yang, Y., Guo, L. H., & Liu, N. (2018). Peptide biomarkers identified by LC–MS in processed meats of five animal species. *Journal of Food Composition and Analysis*, *73*(April 2017), 47–54. https://doi.org/10.1016/j.jfca.2018.07.004~~

~~Wang, Y., Chandra, R., Samsa, L. A., Gooch, B., Fee, B. E., Michael Cook, J., Vigna, S. R., Grant, A. O., & Liddle, R. A. (2011). Amino acids stimulate cholecystokinin release through the Ca2+-sensing receptor. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *300*(4), 528–537. https://doi.org/10.1152/ajpgi.00387.2010~~

Womeni, H. M., Linder, M., Tiencheu, B., Mbiapo, F. T., Villeneuve, P., Fanni, J., & Parmentier, M. (2009). Oils of insects and larvae consumed in Africa: Potential sources of polyunsaturated fatty acids. *OCL - Oleagineux Corps Gras Lipides*, *16*(4), 230–235. https://doi.org/10.1684/ocl.2009.0279

Wu, G. (2016). Dietary protein intake and human health. *Food and Function*, *7*(3), 1251–1265. https://doi.org/10.1039/c5fo01530h

Wu, G. (2020). Important roles of dietary taurine, creatine, carnosine, anserine and 4-hydroxyproline in human nutrition and health. In *Amino Acids* (Vol. 52, Issue 3). Springer Vienna. https://doi.org/10.1007/s00726-020-02823-6

Ye, Z., Li, R., Cao, C., Xu, Y. J., Cao, P., Li, Q., & Liu, Y. (2019). Fatty acid profiles of typical dietary lipids after gastrointestinal digestion and absorbtion: A combination study between in-vitro and in-vivo. *Food Chemistry*, *280*(November 2018), 34–44. https://doi.org/10.1016/j.foodchem.2018.12.032

Yin, J., Ma, J., Li, Y., Ma, X., Chen, J., Zhang, H., Wu, X., Li, F., Liu, Z., Li, T., & Yin, Y. (2020). Branched-chain amino acids, especially of leucine and valine, mediate the protein restricted response in a piglet model. *Food and Function*, *11*(2), 1304–1311. https://doi.org/10.1039/c9fo01757g

Zhou, X., Zhang, H., He, L., Wu, X., & Yin, Y. (2018). Long-term L-Serine administration reduces food intake and improves oxidative stress and Sirt1/NFκB signaling in the hypothalamus of aging mice. *Frontiers in Endocrinology*, *9*(AUG). https://doi.org/10.3389/fendo.2018.00476

Figure captions

Figure 1 SDS-PAGE electrophoretic pattern of Almond, Beef and Insect in undigested (raw) and digested form (OG indicate oral-gastric and OGD indicate oral-gastric-duodenal digestion). The figure includes each negative digestion control, i.e. the proteins treated in the same OG and OGD mixtures, but without enzymes. Negative digestion control bands are more intense due to the interference of the digestion mixtures.

Figure 2 Solubilized protein percentage (as referred to starting raw material) after oral-gastric (OG) and oral-gastric-duodenal (OGD) digested samples calculated by both Kjeldahl and total amino acids method and protein degree of hydrolysis (DH %) in almond (A), beef (B) and insect (I). Different letters mean statistically different samples ($p < 0.05$; one-way ANOVA, Tukey's-b test was used).

Figure 3 Heat map of the amino acid data in raw, orally gastric digested (OG) and orally gastric and duodenal digested (OGD) samples of almond (A), beef (B) and Alphitobius diaperinus insect (I). In the subsequent rows, red hues represent decreased concentrations and blue hues increased concentrations. Colour intensity increases proportionally to the magnitude of the change.

Figure 4 Principal components obtained for the amino acid composition of the in vitro oral gastric digested almond (A-OG), beef (B-OG) and Alphitobius diaperinus insect (I-OG) and the enterohormone secretion of active ghrelin (pg/ml), PYY (pg/ml) and CCK (ng/ml) after treating pig duodenum with these digestions. Pig proximal duodenum is the first segment reached after OG digestion where no pancreatin action occurs. Thus, this duodenum segment was treated with the OG digesta.

Figure 5 Principal components obtained for the amino acid composition of the *in vitro* oral gastric and duodenal digested almond (A), beef (B) and Alphitobius diaperinus insect (I) and the enterohormone secretion of total ghrelin, PYY, tGLP-1 (pg/ml) after treating human colon with these digestions.

Figure 6 Principal components obtained for the amino acid composition of the raw almond (A), beef (B) and Alphitobius diaperinus insect (I) samples and 3 and 20 hours food intake (kcal) after administer to the rats a 300 mg protein/ Kg body weight dose of these samples.

Table 1 Lipids and Protein percentage (on dry weight) in raw samples

Sample Name	Lipid content (% dw) (Soxhlet)	Protein content (% dw) (Kjeldahl)
Almond	56.7 ± 0.2	22.3 ± 0.4
Beef	6.6 ± 0.1	77.4 ± 0.1
Lesser mealworm	28.5 ± 0.5	58.8 ± 0.2

Table 12 Relative percentages of fatty acids detected in almond and lesser mealworm larvae oils. Fatty acids were determined as methyl esters after methylation in acidic media performed in duplicate. Relative coefficient of variation (CV %) ranged from 2 % to 10 %.

Fatty Acid	Raw almond	Raw Lesser mealworm
C12:0	0.1	n.d.
C14:0	0.5	1.1
C15:0	0.1	0.2
C16:0	7.1	27.2
C16:1 cis-9	0.9	n.d.
C18:0	2.4	6.8
C18:1 cis-9	59.4	34.3
C18:2n-6 (LA)	29.6	28.0
C18:3n-3 (ALA)	n.d.	2.3
Σ SFA	10.1	35.4
Σ MUFA	60.3	34.3
Σ PUFA	29.6	30.3

LA: Linoleic Acid; ALA: α-Linolenic Acid; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids.

Table 2~~3~~ Total amino acid profile in raw samples. Significant differences with one-way ANOVA, Tukey's-b test were evaluated ($p < 0.05$).

g/100 g dry weight			
Amino Acid	raw almond	raw beef	raw lesser mealworm
Ala	1.1±0.1	4.438±0.003	4.1±0.1
Arg	2.8±0.2	6.1±0.2	2.41±0.01
Asp	2.8±0.2	7.37±0.02	5.1±0.1
Cys	0.6±0.1	2.6±0.1	1.27±0.01
Glu	6.9±0.3	12.57±0.05	7.0±0.1
Gly	1.53±0.02	4.7±0.2	2.7±0.1
His	0.7±0.1	3.4±0.2	1.5±0.1
HPro	0.0	0.9±0.1	0.0
Ile	0.73±0.03	3.3±0.1	2.08±0.05
Leu	1.7±0.1	7.2±0.1	3.96±0.04
Lys	0.47±0.01	3.4±0.2	1.3±0.1
Met	0.21±0.01	2.56±0.02	0.96±0.02
Phe	1.5±0.1	4.4±0.3	3.1±0.1
Pro	1.1±0.1	3.8±0.2	3.70±0.03
Ser	1.20±0.02	4.18±0.01	3.04±0.07
Thr	0.79±0.02	4.8±0.1	2.77±0.02
Trp	0.11±0.01	0.29±0.02	0.3344±0.0003
Tyr	0.86±0.01	3.7±0.4	4.99±0.04
Val	0.97±0.01	4.0±0.1	3.10±0.01
Total amino acids	26.0±0.7	83.52±0.03	53.3±0.2
total protein content[a]	22.3±0.7	71.81±0.03	45.8±0.2

[a] Derived from total amino acid content considering the molecular mass of the residual amino acid moieties (i.e. without a water molecule)

Table 34 Solubilized percentage of lipids (as referred to starting raw material) after oral-gastric (OG) digested and oral-gastric-duodenal (OGD) digested samples.

Sample Name	Solubilized lipids after OG (%)	Solubilized lipids after OGD (%)
Almond	20±2	30±1
Lesser mealworm	16.0 ±0.2	23±1

Table 45 Relative molar percentages determined by ^{1}H NMR of the different glycerides and free fatty acids present in solubilized lipid fractions of lesser mealworm and almond before (RAW) and after both simulated oral-gastric (OG) and oral-gastric-duodenal (OGD) digestion. Coefficient of variations (CV %) max 5 %.

Lipid class	*Lesser mealworm RAW*	*Lesser mealworm after OG*	*Lesser mealworm after OGD*	*Almond RAW*	*Almond after OG*	*Almond after OGD*
1,2-DG	2.8	3.9	1.0	2.6	2.9	6.9
2-MG	0.7	1.4	0.0	0.0	0.2	3.6
1,3-DG	2.1	2.4	1.4	1.4	2.1	2.9
1-MG	3.6	8.8	17.6	1.5	3.7	17.2
TG	67.9	24.5	3.5	94.5	78.4	30.1
FFA	22.8	59.1	76.5	0.0	12.7	39.4

[1] Simulated gastro-intestinal fluid.

Table 6 Summary of main protein attribution and number of peptides identified for each digested sample.

Sample	Main Identified Proteins (code-name)	n. of Peptide Identified per protein	Coverage per protein %
ALMOND Oral-gastric digested	• Q43607- Prunin 1 • A0A4Y1S2I9- RmlC-like cupins superfamily protein • A0A5E4EZP4- Vicilin	133 53 20	75 45 17
ALMOND Oral-gastric-duodenal digested		101 28 5	60 36 8
BEEF Oral-gastric digested	• A0A1K0FUF3- Myoglobin • Q5KR49- Tropomyosin • P68138- Actin • Q9BE40- Myosin	18 20 56 138	66 64 55 53
BEEF Oral-gastric-duodenal digested		2 8 25 79	18 16 37 21
INSECT Oral-gastric digested	• D6W8Q9-Pupal cuticle protein • A0A0L7QKC9- Actin • P80681-Larval cuticle protein	6 19 2	42 35 26
INSECT Oral-gastric-duodenal digested	• A0A084VLA6-Actin • A0A482VEP8- Uncharacterized protein • P80681- Larval cuticle protein	14 3 5	31 30 17

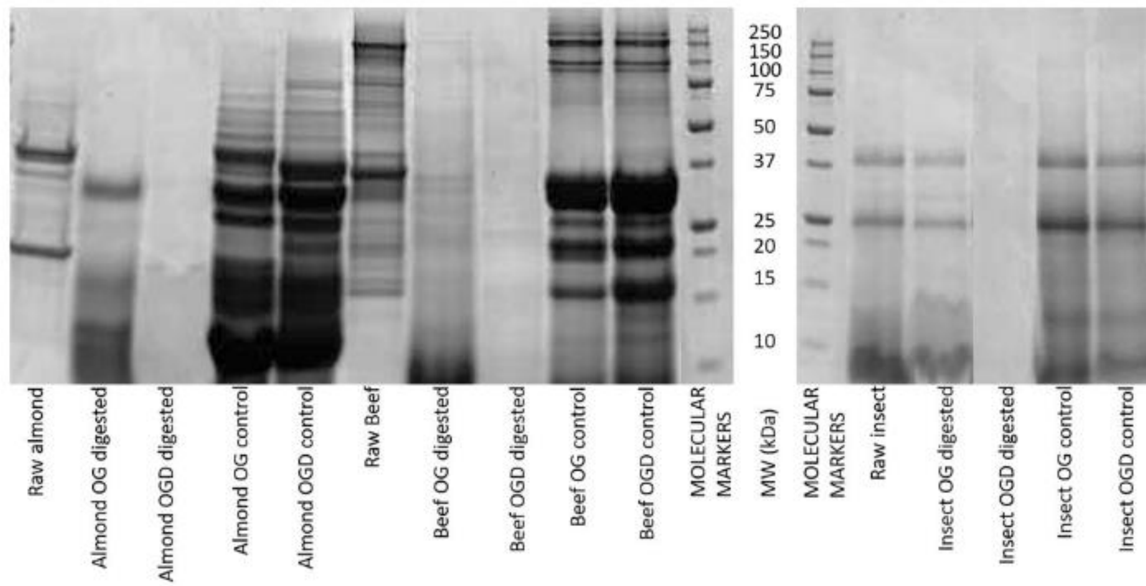

Fig. 1. SDS-PAGE electrophoretic pattern of Almond, Beef and Insect in undigested (raw) and digested form (OG indicate oral-gastric and OGD indicate oral-gastric-duodenal digestion). The figure includes each negative digestion control, i.e. the proteins treated in the same OG and OGD mixtures, but without enzymes. Negative digestion control bands are more intense due to the interference of the digestion mixtures.

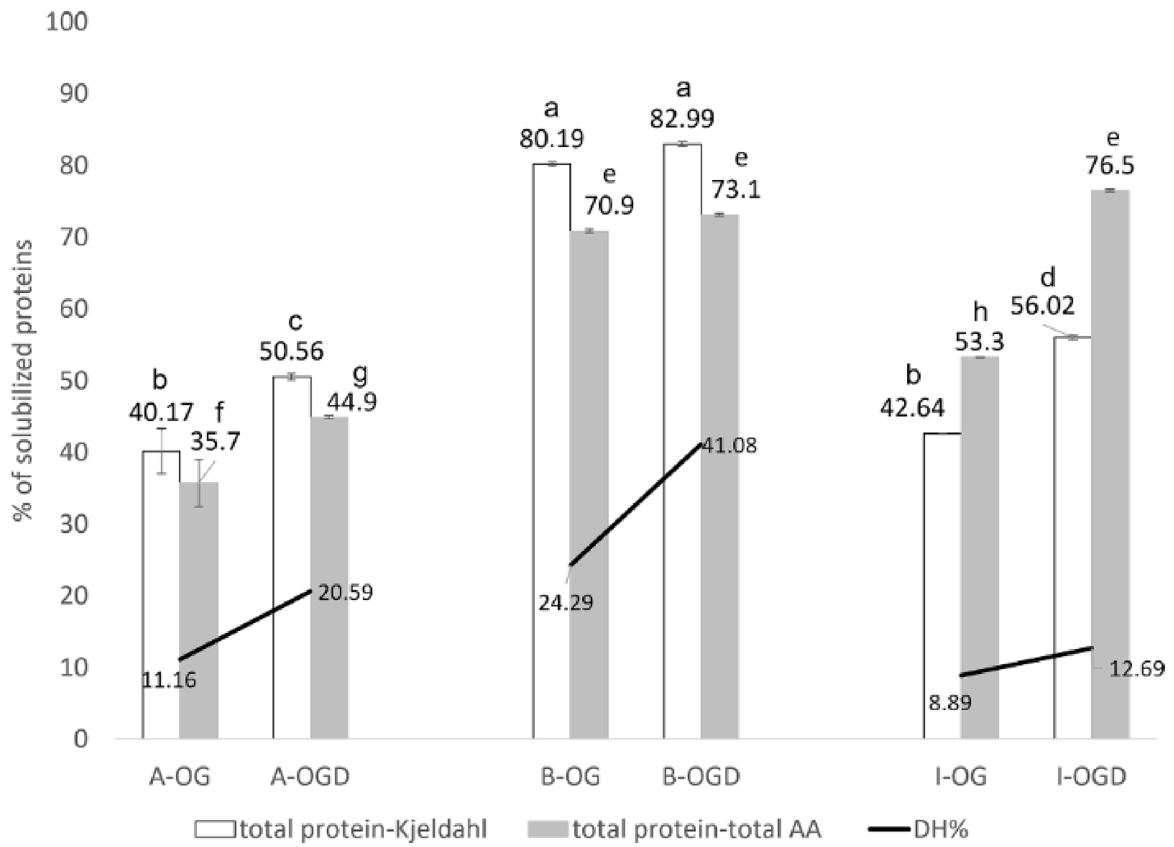

Fig. 2. Solubilized protein percentage (as referred to starting raw material) after oral-gastric (OG) and oral-gastric-duodenal (OGD) digested samples calculated by both Kjeldahl and total amino acids method and protein degree of hydrolysis (DH %) in almond (A), beef (B) and insect (I). Different letters mean statistically different samples ($p < 0.05$; one-way ANOVA, Tukey's-b test was used).

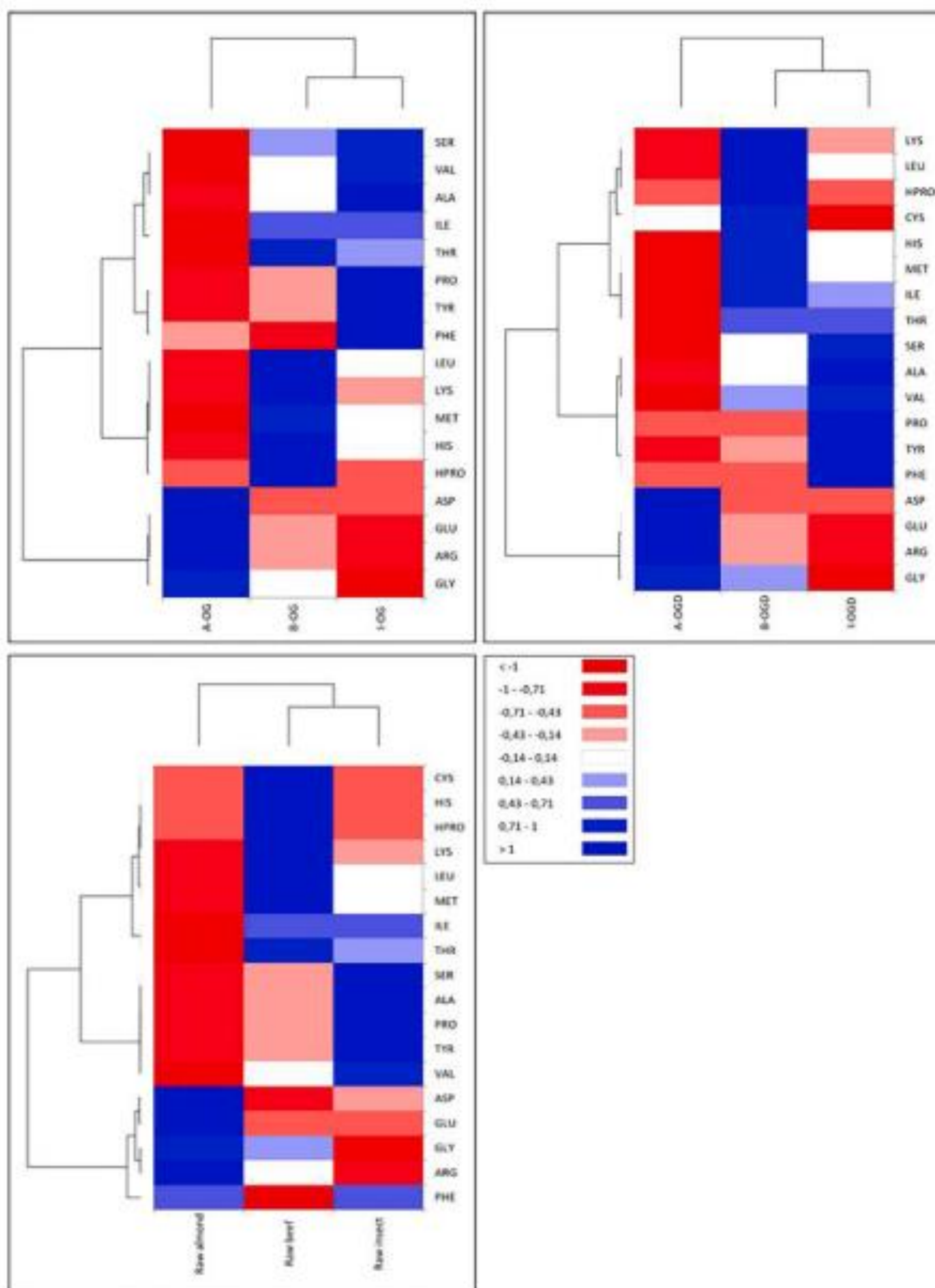

Fig. 3. Heat map of the amino acid data in raw, orally gastric digested (OG) and orally gastric and duodenal digested (OGD) samples of almond (A), beef (B) and *Alphitobius diaperinus* insect (I). In the subsequent rows, red hues represent decreased concentrations and blue hues increased concentrations. Colour intensity increases proportionally to the magnitude of the change.

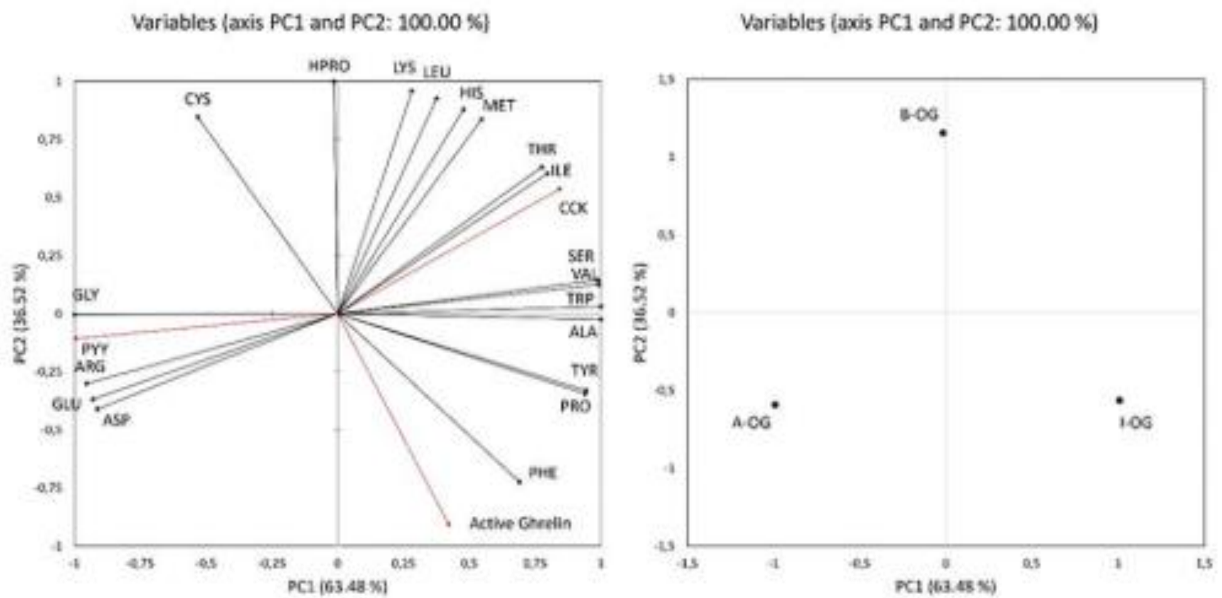

Fig. 4. Principal components obtained for the amino acid composition of the *in vitro* oral gastric digested almond (A-OG), beef (B-OG) and *Alphitobius diaperinus* insect (I-OG) and the enterohormone secretion of active ghrelin (pg/mL), PYY (pg/mL) and CCK (ng/mL) after treating pig duodenum with these digestions. Pig proximal duodenum is the first segment reached after OG digestion where no pancreatin action occurs. Thus, this duodenum segment was treated with the OG digesta.

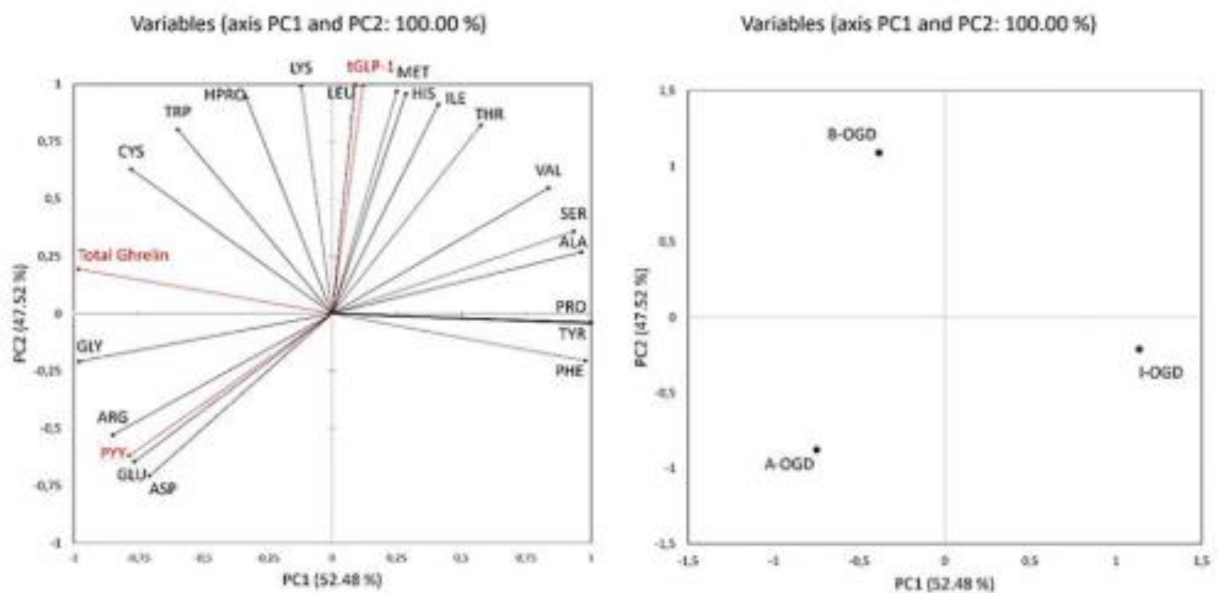

Fig. 5. Principal components obtained for the amino acid composition of the *in vitro* oral gastric and duodenal digested almond (A), beef (B) and *Alphitobius diaperinus* insect (I) and the enterohormone secretion of total ghrelin, PYY, tGLP-1 (pg/mL) after treating human colon with these digestions.

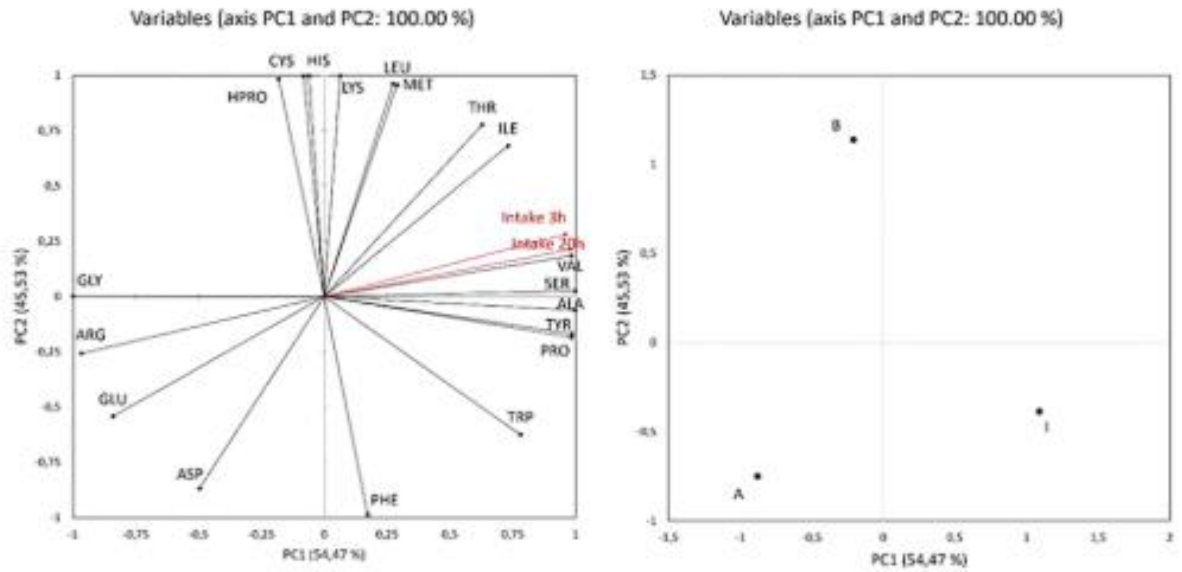

Fig. 6. Principal components obtained for the amino acid composition of the raw almond (A), beef (B) and *Alphitobius diaperinus* insect (I) samples and 3 and 20 h food intake (kcal) after administer to the rats a 300 mg protein/ Kg body weight dose of these samples.