Study of the effect of sprouting on chickpea flour's proteome and digestibility using an *ex vivo* gastro-duodenal and jejunal digestion with brush border membrane enzymes.

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Abstract:
The demand for sustainably produced protein is increasing proportionally with the world population and is prompting a dietary shift toward vegetable proteins. Vegetable proteins have lower digestibility and biological value compared to animal derived counterparts. We explored sprouting of chickpea seeds, selected as a pulse model, as a tool for improving digestibility. Protein evolution induced by the sprouting process was assessed by proteomics. An *ex vivo* model of gastroduodenal and jejunal digestion was applied to assess the bioavailability of the protein digests. Proteins from chickpea sprouts showed a greater susceptibility to digestion. Peptides with potential immunoreactivity and bioactivity were catalogued in both digested chickpea sprouts and seeds using an *in-silico* approach.

Keywords: Chickpea, sprouting, germination, *ex vivo* digestion, human gastric and duodenal juice, brush border membrane enzymes, discovery mass spectrometry

Abbreviations:

ACN acetonitrile

AMBIC ammonium bicarbonate

CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate

CnS chickpea seeds flour

CS chickpea sprouts flour

DTT dithiothreitol

FA formic acid

FAN Free amino nitrogen

GAPC cytosolic glyceraldehyde-3-phosphate dehydrogenase

IAA Iodoacetamide

IEF isoelettrofocusing

IPG immobilized pH gradient

LC-HR-MS/MS liquid chromatography high resolution mass spectrometry

MWCO molecular weight cut-off

NAD(H) nicotinamide adenine dinucleotide (NAD)

NADP(H) nicotinamide adenine dinucleotide phosphate (reduced form)

NOPA α-amino nitrogen

RP-HPLC reverse phase high resolution liquid chromatography

SDS sodium dodecyl sulfate

SDS-PAGE Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

TFA Trifluoroacetic acid

1. Introduction

The consumption of animal derived food products is foreseen to double by 2050 alongside the word population that will reach 9.5 billion (Henchion et al., 2017). In western countries, animal derived ingredients are the major source of valuable proteins for their high digestibility and the good balance in terms of essential amino acids (Gilani et al., 2012; Tomé, 2013). The 28% of the European land is currently dedicated to livestock production. Among the agricultural practices, livestock farming is the principal contributor of terrestrial biodiversity loss, soil acidification, air and water pollution (Leip et al., 2015). Bovine and ovine meat products are responsible for the emission of 0.06 - 0.12 kg of CO_2 per grams of protein compared to legumes generating less than 0.02 kg of CO_2 per gram of protein (Gardner et al., 2019). The concern around global climate change is forcing a dietary shift to more sustainable sources of proteins to be used as ingredients in the food production chain.

Pulses are a sustainable alternative protein source also high in fibers, vitamins, and minerals. The increased consumption of legumes is supported by the Food and Agriculture Organization (FAO), which declared 2016 the international year of pulses. Pulses represent a source of proteins for low-income consumers and for vegan, vegetarian, and flexitarian consumers (Jukanti et al., 2012). The consumption of pulses alongside cereals provides a complete support of amino acids, which is comparable to a diet including meat and dairy products (FAO, 2015; Jukanti et al., 2012). Soybean, because of the high protein content (43.2% dry weight), finds large application in the production of meat and dairy substitute and feeds (Verma et al., 2013). However, the production of soybean is mainly focused in Brazil and America, and it is responsible for deforestation, water pollution and loss of biodiversity (Fearnside, 2001; Henchion et al., 2017; Lima et al., 2019). The exploration of alternative pulses to soybean like lentils, peas and chickpeas is required.

South and south East Asia are the world's largest producer of pulses with chickpea (*Cicer arietinum*) representing the third legume crop produced globally after dry beans and peas (Boukid, 2021; Gupta et al., 2017). Chickpea seeds contain around 22% of proteins (El-Adawy, 2002). Legume proteins have low digestibility because of anti-nutritional factors (trypsin inhibitors, lectines, phytic acid) and a low concentration of sulfur containing essential amino acids (El-Adawy, 2002; R. Wang & Guo, 2021). Thermal (boiling) and non-thermal (germination) processes have been found as effective treatments to improve the nutritional quality of chickpeas (El-Adawy, 2002). On the contrary, soaked and boiled chickpea proteins more resistant to simulated gastroduodenal digestion (Ribeiro et al., 2017; Tavano & Neves, 2008). Protein aggregation, occurring during heating, may be responsible for the reduces accessibility of the enzymes. Germination is a traditional, non-thermal process during which the embryo resumes to grow. The chemical composition of the seed changes. Antioxidant polyphenols, vitamin and minerals (magnesium) are found to increase significantly, while the protein profile and structure changes (Gupta et al., 2017; Li et al., 2016). Seeds accumulate enormous amount of storage proteins, which during germination are degraded to supply nitrogen, carbon, and sulfur to the embryo (Fujiwara et al., 2002; J. Wang et al., 2007). During germination, new proteins, particularly enzymes, can be synthesized. Sprouting positively improved the bio-accessibility of polyphenols and flavonoids (Gupta et al., 2017) and efficiently reduced the concentration of trypsin inhibitors and lectins in chickpea sprouts, improving the digestibility compared to the seeds (El-Adawy, 2002). In germinating seeds, the affinity of proteases inhibitors toward enzymatic targets decreases and inactive proenzymes of quiescent seeds are enzymatically converted into active proteases, which start the mobilization of storage proteins.

Cupin and prolamin superfamilies represent the dominant storage protein of legumes. Together with plant defense-system-proteins, cupins and prolamins are classified as type 1 allergens in legumes and cereals, as they can trigger IgE and IgG mediated allergic reactions in sensitized subjects (Gupta et al., 2017). Enzymatic hydrolysis has been described effective in reducing the immunoreactivity of chickpea proteins (Clemente et al., 1999; Sormus de Castro Pinto et al., 2009).

Short-term germination proved effective in reducing the allergenic potential of peanut, rice and soybeans (Li et al., 2016; Wu et al., 2012; Yamada et al., 2005).

In this study, the protein expression between chickpea seeds and sprouts was compared by proteomic approaches. The susceptibility to gastrointestinal digestion of spouted chickpeas and the bioavailability of the free amino acids, di- and tripeptides was evaluated using an *ex vivo* static digestion model (Ulleberg et al., 2011). The model was implemented with brush border enzymes to simulate the peptide degradation at the small intestine absorptive surface. This pool of enzymes finalizes the digestion of macronutrients. Peptide sequences resistant to digestion were mapped by mass spectrometry and evaluated *in silico* to identify potential IgE-epitopes and bioactive sequences.

2. Materials and Methods

2.1 Materials

The chemicals and solvents, including chloroform, methanol, borate buffer, acetone, acetonitrile (ACN), dithiothreitol (DTT), Iodoacetamide (IAA), ammonium bicarbonate (AMBIC) and MilliQ water, were purchased from SIGMA (St. Luis, MO, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Carlo Erba Reagents (Milan, Italy). Trypsin was from Promega (Madison, WI, USA). Reagents for electrophoresis analysis were purchased from Bio-Rad (Milan, Italy).

2.2 Sprouting procedure

Raw and sprouted chickpeas were provided by Molino Quaglia (Vighizzolo d'Este, Italy). Chickpeas were sprouted in an industrial sprouting plant (Buhler Pargem, Buhler AG, Uzwil, Switzerland) as described by (Marengo et al., 2017). Sprouted chickpeas were dried at 50°C for 10 hours until reaching a final moisture lower than 10% and were stored at room temperature. When required, chickpea samples (sprouted or unsprouted) were ground into flour (0.25 mm particle size) in a pin mill (Buhler).

2.3 Protein extraction and fractionation

Proteins of chickpea seeds were extracted adapting a method developed for pea seeds, with some modifications (Rubio et al., 2014b).

Samples (1:10, w:v) were defatted by stirring for 30 minutes at 20-22 °C the chickpea flours in a solution of chloroform and ethanol (2:1, v:v). The suspension was centrifuged at 400g for 20 minutes and the supernatant was removed. The flours were air dried by overnight evaporation of the solvents. For fractionation of protein subfamilies, 1 g of defatted flour was suspended in 200 mM borate buffer (pH 8.0) (1:10, w:v). Samples were stirred for 12 hours at 22-22 °C and centrifuged at 4000 g for 20 min. Proteins were precipitated by adjusting the pH at 4.5 using 50% aqueous acetic acid. The resulting pellet contained the 11S globulin fraction. The supernatant was dialysed against MilliQ water using 3500-MW cut-off (MWCO) tubes (Fischer Scientific, Italy) for about 24 hours at 20-22°C. The solution was centrifuged at 4000 g for 20 minutes to obtain a pellet containing the 7S vicilin like protein fraction and a clear supernatant containing the albumin fraction.

2.4 SDS-PAGE analysis

Proteins were separated into a 12% polyacrylamide gel (Bio-Rad) under reducing. The running buffer was 192 mM glycine, 25 mM Tris and 0.1% SDS. Analysis was carried out at room temperature, and constant voltage (100 V). Proteins were visualized with Coomassie® Brilliant blue G 250 staining. The gel was imaged with a scanner and processed using the LABScan software 3.00 (Amersham Bioscience). For proteomic analysis, protein bands were manually excised, de-stained with acetonitrile/25mM AMBIC (1:1, v:v) and dried under vacuum after dehydration in acetonitrile. Gel pieces were rehydrated with 20 µL of a 12 ng/µL trypsin solution in 50 mM AMBIC for 45

minutes on an ice-cold bath. The excess of trypsin solution was discarded, and the protein bands were incubated overnight at 37°C. The tryptic peptides were extracted in 100 µL of 50% ACN containing 2.5% (v:v) FA and dried using a Speed-Vac device.

2.5 2-D IEF/SDS-PAGE analysis

For two-dimensional electrophoresis (2-DE) analysis, aliquots of chickpea flour proteins were quantified with the Bradford assay and precipitated in 1 mL of −20°C cold acetone. The protein pellets (100 µg/400 µL) were dissolved in IPG strip rehydration buffer [8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% v/v Pharmalytes pH 4.0–10.0 and traces of bromophenol blue]. Immobiline Dry Strips (pH 4–7, 11 cm) were rehydrated overnight in an Immobiline Dry-Strip Reswelling Tray (Amersham Pharmacia). Isoelectrofocusing (IEF) was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). IEF was carried out at pI 4–7. The program run was 1000 V for 1 h and 3500 V for 16 h. After focusing, proteins were Cys-reduced for 15 min in equilibration buffer (6 M Urea, 30% glycerol, 2% SDS, 2% DTT), and alkylated for 15 min with 2.5% iodacetamide. SDS-PAGE in the second dimension was performed as described in section 4.4 using a 15% acrylamide concentration to enhance the resolution of the low molecular weight region. For proteomic analysis, protein bands were manually excised and processed as described in the previous paragraph.

2.6 *Ex vivo* gastrointestinal digestion with human enzymes

The simulated static gastro-intestinal digestion was carried out using human gastric and duodenal juices as the source of enzymes and was performed based on the standardized INFOGEST consensus model (Brodkorb et al., 2019) with some modifications (Asledottir et al., 2019). Human gastrointestinal juices were collected from human volunteers according to Ulleberg et al., 2011 (Ulleberg et al., 2011). The aspiration of humans was performed at Lovisenberg Diaconale Hospital, Oslo and approved by the Norwegian Ethical Committee (2016). The pepsin and trypsin activities, besides concentration of bile salts in the gastro-intestinal juices, were assayed according to the INFOGEST protocol. All samples were processed in parallel. The oral phase was bypassed. Briefly, 10 mg of flour were solubilized in 0.5 mL of MilliQ water and divided in two sub-samples. The gastric digestion was carried for 1 hour in a water bath at 37°C, under continuous shaking, while the duodenal digestion was performed for 2 hours at 37 ° C as described for the gastric phase.

2.7 Jejunal digestion with brush border membrane enzymes

The brush border membrane enzymes (BBM) were purified as described by (Picariello et al., 2015). The aminopeptidase N activity and the total peptidase activity were determined and corresponded to 879 µU/µL, and 1,018 µU/µL, respectively (Asledottir et al., 2019). Briefly, 200 µL of duodenal digest was brought to pH 7 and incubated to a ratio of 1:70 (BBM enzyme:peptide substrate), corresponding to 13 µU/µL peptidases-to-substrate ratio. The digestion was carried for 2 hours at 37°C and interrupted by boiling the sample and stored at -80°C until further analysis.

2.8 High pressure liquid chromatography

HPLC chromatograph was an HP 1100 Agilent modular system equipped with a diode array detector (Palo Alto, CA, USA).

For chickpea protein analysis, extracted proteins were acidified with TFA to a final concentration of 0.1% (v/v). Before the supernatant injection, samples were subjected to centrifugation (4000 g x 10 min). Reverse phase (RP)-HPLC analysis was carried out using a 204CP54 C4 Vydac (250 X 4.6 mm, 5µM, Grace, Milan, Italy) column (Hesperia, CA, USA). Solvent A was 0.1% TFA in water (v/v). After 10 min of isocratic elution using 25% solvent B (0.1% TFA in acetonitrile, v/v), a 25-70% B gradient ramp was applied for 60 min, at a flow rate of 1.0 ml/min. The column effluents were monitored by UV detection at λ = 220 and 280 nm.

The analyses of the de-proteinised samples were performed with the same apparatus on a 218TP54 C18 Grace Vydac (2.1x25 mm, 5µm). The injection volume was 50 µL, and the flow was set at 0.2 mL/min. Analyte elution was performed with a linear gradient from 15 to 100% of 0.07% TFA (v/v) in ACN (eluent B) in 70 min. Each sample was analysed in triplicate.

2.9 α-amino nitrogen determination

The content of free amino nitrogen in the samples was determined using the Enzytec™ Alpha-amino Nitrogen kit by R-Biopharm (E2500 R-Biopharm AG, Germany) following the manufacturer instructions. The primary amino groups are derivatised by o-phthaldialdehyde (OPA) and N-acetyl cysteine (NAC) to form isoindoles. The result was reported as mg of α-amino nitrogen per mg of sample. The iCubio i-Magic M9 (Origlia S.r.L, Italy) was set to perform the enzymatic reaction in full automatization and the absorbance was read at 340 nm.

For determining the amount of free amino nitrogen in the flours, samples were suspended in water (1:10, w:v) and placed in the ultrasonic bath at approximately 20 °C for 30 minutes. The solution was centrifuged at 4000 g for 30 minutes and the supernatant was de-proteinised to a 20% trichloroacetic acid (TCA) final volume. After precipitation for 30 minutes at 20°C, the solution was centrifuged at 4000g for 30 minutes and the supernatant was neutralised to a pH 7 using sodium hydroxide (NaOH) before analysis. The sample procedure was applied to quantify α-amino groups, mainly represented by free amino acids and oligopeptides, released after gastroduodenal and after jejunal digestion. All samples were assayed in triplicate and absorbance values were averaged.

2.10 Preparation of proteins and peptides for mass spectrometry analysis

Prior to MS analysis, peptide digests were desalted using C18 Zip-Tip pre-packed micro-columns (Millipore, Bedford, MA, USA), previously equilibrated with 0.1% TFA and eluted with 50% acetonitrile (v/v) containing 0.1% TFA (v/v).

2.11 LC-HR-MS/MS analysis

LC-HR-MS/MS analysis was performed by using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography equipment (Thermo Scientific). Samples were loaded through a 5mm long 300 µm id pre-column (LC Packings, USA) and separated by an EASYSpray™ PepMap C18 column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent C was 0.1% FA (v/v) in water; eluent D was 0.1% FA (v/v) in 80% (v/v) ACN. The column was equilibrated at 5% D. Peptides were separated applying a 4–40% gradient of D over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/z* scan range of 350 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of $1x10^6$ ions and a maximum ion injection time (IT) of 120 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. To prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or over six charges were excluded.

Spectra were processed using Peaks Studio (Bioinformatics Solutions, Waterloo, ON, Canada). A specific database was generated for the analysis of the mass spectrometry data. The database included both Uniprot and NCBI sequences for *Cicer arietinum*, downloaded on 16/10/2019. Dbtoolkit was used to customize the database and remove duplicated sequences (Bromilow et al., 2017; Martens et al., 2005). Homo sapiens protein sequences, downloaded the 16/10/2019 from Uniprot, were also included in the database to increase the confidence of identification of the peptides.

PEAKS Studio (version 6.0, Bioinformatics Solution Inc., Waterloo, Canada) was used for database searching applying the following parameter: Methionine oxidation and pyroglutamic for N-terminus glutamine as variable modifications; cysteine carbamidomethylation as a constant modification for electrophoresis isolated proteins or no modifications for products of gastrointestinal digestion; mass tolerance value of 8 ppm and 0.02 Da for precursor and MS/MS fragment ions, respectively; no proteolytic enzyme specificity for products of digestion; trypsin, with up to 2 missed cleavage for the identification of protein bands/spots. The false discovery rate was set at 0.1%. Proteins were considered confidently identified based on at least four sequenced peptides.

2.12 Statistical analysis of data

All statistical analysis was performed using R Statistical Software (version 4.0.4; R Foundation for Statistical Computing, Vienna, Austria). Data were compared by one-way ANOVA, followed by the Tukey–Kramer post hoc test ($\alpha = 0.05$).

3. Results

3.1 Proteomic characterization of the chickpea seed and sprout flours.

During sprouting, the storage proteins are degraded to meet the amino nitrogen requirement of the growing embryo, and novel proteins associated particularly with photosynthesis are expressed. The chemical characterization of the flours from spouted chickpea has been the subject of a previous study (Marengo et al., 2017). The crude protein concentration of the chickpea seeds flour (CnS) and chickpea sprouts flour (CS), which was determined by Kjeldhal analysis, corresponded to 18.6 ± 0.3 and 20.2 ± 0.1 gr per 100 gr of flour, respectively. Free amino nitrogen (FAN) released during sprouting was determined after solubilization in water and deproteinization to remove protein/peptides. The α-amino nitrogen (NOPA) concentration of the chickpea sprouts was found to be two times higher compared to chickpea seeds (9.8 ± 0.2 mg of NOPA per 100 gr of CnS flour and 20.5 ± 0.6 mg of NOPA per 100 gr of CS flour).

The first aim of this work was to characterize the protein profile of chickpea sprouts compared to chickpea seeds by a proteomic approach, with particular interest in the seed storage proteins. The 11S globulin, 7S vicilin and the 2S albumin were fractionated by isoelectric precipitation from both samples and analysed by 1D electrophoresis alongside crude protein extracts (Figure 1) (Chang et al., 2011a, 2012). The profile of the most abundant proteins did not differ qualitatively between chickpea seeds and sprouts. Proteins were assigned on the 1D-gel according to a previous literature study (Chang et al., 2011b) and the identity of few bands was further confirmed by mass spectrometry analysis of the in-gel trypsin digests. The identifications are reported in Table S1, which highlights the complexity of each band containing a variable number of co-migrating proteins. The 11S globulin appeared the most abundant protein in both flours as also confirmed by the analysis of the isolated fraction (line 2 and line 6 in Figure 1). The profile of the 7S vicilin and the 2S albumin remained almost unaltered after sprouting. Interestingly, the electrophoretic profile under reducing conditions of the 7S vicilin-enriched fraction showed several polypeptide chains in the relative mobility region between 15 to 66 kDa. The 7S polypeptides with relative mobility <50 kDa may be generated by proteolytic post-translational events that have been described previously for other legume seeds such as pea (Gatehouse et al., 1981; Rubio et al., 2014a). One major band with relative mobility of ~24 kDa was detected on the 1D-gel for the 2S albumin fraction. Several faint bands on the gel suggested additional co-precipitating polypeptides. A fragment of 2S albumin in the band 5 with approximate molecular mobility of 20 kDa was identified by mass spectrometry. The 2S albumin undergoes post-translational proteolysis, like the one described for the 11S globulin. The profile of chickpea 2S appeared different from pea. Unlike chickpea that exhibits a single 2S electrophoretic band, the pea profile showed three main electrophoresis-revolved proteoforms that were identified as 2S albumin by mass spectrometry (Rubio et al., 2014a).

Qualitative differences in protein expression between CnS and CS were evident when comparing the profiles of the crude protein extracts. A larger number of bands in the high relative mobility region (50-70 kDa) and the medium region (25-30 kDa) (line 1 and line 5 in Figure 1) were detected in CnS compared to CS.

The 2D-electrophoretic map of the seed protein extracts appeared similar to 2D gel maps available in literature (Singh et al., 2016), confirming the complexity of the chickpea proteome. The 7S-vicilin showed the typical microheterogeneity pattern associated with the glycosylation (Figure 2, panel a). The 11S globulin under reducing conditions showed the expected presence of the two subunits, namely acidic and basic, generated by a non-specific post-translational event and chemical reduction of the inter-chains' disulphide bond. The same patterns were also detected in the analysis of sprouted chickpea proteins (Figure 2, panel b). However, differences between seeds and sprouts were observed in the acidic pI/medium molecular mobility region and in the high molecular mobility region (showed by a circle in Figure 2, panel b). A larger number of spots could be detected in the CS flour in the medium molecular weight (Mr) region. These newly generated proteins may be associated with the germination. The mass spectrometry analysis of selected spots (Figure S2 and Table S2) allowed the identification of fragments of high-molecular mobility proteins such as the 11S legumin, the 7S vicilin and the lipoxygenase, generated by proteolytic events taking place during seed germination. Compared to CnS, a lower number of spots of 7S and lipoxygenase were detectable in the high Mr of the CS flour gel (Figure 2, panel b). A series neoformed protein spots in CS was identified by mass spectrometry analysis of the in-gel trypsinized spots, among which the UTP-glucose-1-phosphate uridylyltransferase. This enzyme, located in cell wall components, has been also described in germinated barley (malt) and takes part to the pathway of carbohydrate metabolism, particularly sucrose formation (Fearnside, 2001; Lima et al., 2019). The protein was found to be upregulated during germination in Chinese wheat seeds (Yu et al., 2014). The ribulose bisphosphate carboxylase large chain, also known as RuBisCO, is associated to photosynthesis and plant growth. It has been previously identified in sugar beet seeds during germination (El Amrani et al., 1997). The cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) is a key enzyme in glycolysis and regulation of the plant development (Luo et al., 2020). The malate dehydrogenase converts malate to oxaloacetate using NAD(H) or NADP(H) as a cofactor. It is involved in early chloroplast development and it has been identified in germinating pumpkin cotyledons (Schreier et al., 2018; Yamaguchi et al., 1987).

To collect additional information on the extracted/isolate proteins and the low molecular weight polypeptides (Mr < 14 kDa) likely generated during germination, which escaped the electrophoretic analysis, protein extracts were also analyzed by reverse phase chromatography (Figure 2S). The main peak corresponded to the 11S globulin as also confirmed by the profile of the isolated 11S globulin (Figure 2S, panel b). The dominant form of 7S vicilin co-eluted with the 11S globulin, while the less abundant polypeptides, also detected in the electrophoretic profile, slithered on the column possibly because of glycosylation (Figure 2S, panel c). Differently from the electrophoretic profile, two main peaks were detected on the chromatographic profile of the 2S fraction. This may be attributed to at least two isoforms of the 2S albumin in chickpea, with similar molecular mobility but different hydrophobicity. No qualitative differences could be detected between the two flours, indicating substantial stability of 2S albumin toward the germination events.

3.2 Analysis of the digestome by free amino nitrogen determination.

The flour samples were subjected to an *ex vivo* gastro-duodenal static digestion method based on the use of gastric and duodenal aspirated fluids collected from healthy volunteers as the source of enzymes, and further implemented with the jejunal digestion step carried out with brush border membrane enzymes purified from porcine small intestinal mucosa.

Free amino nitrogen (FAN) was determined on the deproteinized digests before and after the action of the brush border membrane enzymes (Figure 3). The sprouting contributed by 10% to the

amount of α-nitrogen compared to the seed flour digests. The increment was consistent before and after the action of the brush border membrane enzymes (Figure 3). Compared to the gastro-intestinal digestion products, the brush border membrane enzymes increased by 40% the amount of FAN, confirming that these enzymes are the key players in finalizing the digestion, releasing free amino acids, di- and tri- peptides that are available for absorption (Picariello et al., 2015). Their implementation in the INFOGEST model is essential when studying *in vitro* protein digestion and amino acids bioavailability. Further studies are required to standardize and harmonize this crucial step. The C18 RP-HPLC profile of the de-proteinised solutions (Figure 3S) confirmed the release of free aromatic amino acids from both CnS and CS flours (Figure 3 panel a and c). After jejunal stage, new peaks ascribable to di-and tripeptides appeared in both digested flours. These were less abundant in CS flour. In the profile of CnS digests, a peak eluting at ~37 minutes showed high resistance to the duodenal-jejunal digestion (figure 3S panels a and b). The peak was absent in the CS flour digest already during the duodenal phase (Figure 3S panels c and d), showing a prompt digestion or the missed formation of this peptide. The larger number of soluble oligopeptides in CnS flour compared to CS flour may reflect a lower digestibility for the first sample. The peak area of the aromatic amino acids analyzed across the 4 samples duodenal and jejunal digests of CnS and CS flour did not show significant differences (analyses were performed in triplicate).

This result is in line with a previous study comparing the essential amino acid profile of chickpea seed flour and chickpea sprout flour after 24 and 48 hours of germination (Fernandez & Berry, 1988). Lysin was the only essential amino acid showing a significant ($p<0.05$) increase after germination.

3.3 Peptidomic analysis of the digestome

The peptides surviving the *ex vivo* gastro-duodenal-jejunal digestion were analyzed by mass spectrometry using a curated non-redundant database that enhanced the confidence of identification. NCBI and Uniprot that are the two major repository of protein sequences, are undergoing an extensive reviewed to suppress all segmented, journal-scan and miss-annotated sequences. The continuous updating of the databases is a best practice to avoid the deposit of obsolete information, which may lead to miss-interpretation of mass spectrometry data that rigorously dependent on them. The Uniprot database (16/10/2019) at the time of the analysis contained 31236 protein sequences for *Cicer arietinum*, of which only 50 were reviewed sequences, while NCBI contains 35679 protein sequences. The two databases were combined for a total number of 66915 sequences. The 54% of the sequences were removed using DBToolKit as redundances. The *Cicer arietinum* database used for processing the data comprised 36134 sequences. The database was implemented with the *Homo sapiens* Uniprot database, including 1475809 (16/10/2019) sequences, to identify the peptides derived from the autolysis of endogenous proteins (digestive enzymes).

The germination appeared to improve the digestibility of the chickpea proteins. In fact, lower number of fragments could be identified in the CS flour compared to the CnS counterpart (Table S3 and S4; and Figure S5). These data are also supported by the FAN analysis that resulted 10% higher in CS. The peptides identified in the CS flour were associated with protein involved in embryogenesis and germination. The abscisic acid (ABA)-responsive protein ABR18-like is generated in the late embryo development and was previously identified and characterized in several germinating seeds among which pea, barley and rice (Dure et al., 1989; Ho et al., 1992; Mundy & Chua, 1988; Walker-Simmons, 1987). This protein has been associated with plant growth and development, and in response mechanisms to environmental stresses (Ho et al., 1992). The concentration of the protein is reported to increase during germination and decrease during desiccation. The elongation factor 1-delta 2-like are engaged in translation machinery and were found to be upregulated during wheat seed germination (Gallie et al., 1998). Similarly, the alcohol dehydrogenase was found in germinating pea, lentil, and kidney beans (Leblová et al., 1973). It

appears to have a major contribution on carbohydrates metabolism when the germination of rice occurs in partially oxygenated water (Takahashi et al., 2014).

Peptides derived from two isoforms of the non-specific lipid transfer protein (*ns*-LTP) (Uniprot accession number: O23758 and A0A1S2Z3T1) could only be identified in the digested CS flour. The *ns*-LTP is associated with many biological processes, including seed germination and maturation (D'Agostino et al., 2019; Liu et al., 2015). The protein was found to be more abundant during germination in the *Euphorbia lagascae*, as involved in either recycling endosperm lipids or protecting cotyledons from proteases, which are naturally released during programmed cell death (Eklund & Edqvist, 2003).

3.4 *In silico* analysis of the peptidome

Peptide sequences collected at the end of the jejunal digestion of both CnS and CS flours were manually evaluated to search digestion-resistant IgE-binding and bioactive sequences.

3.4.1 *In silico* evaluation of IgE binding sequences

Double-blind placebo control food challenges using isolated proteins, involving one thousand four hundred patients, found chickpeas responsible for the 2.2% of the allergic reaction in the Indian population (Sangita P. Patil, Pramod V. Niphadkar, 2001). Cultural and religious reasons make a high percent of the Indian population vegetarian, with pulses being the main constituent of their diet. In Spanish children, legumes, also including chickpea, were responsible for allergic reactions after in oral food provocation (Martínez San Ireneo et al., 2008).

The seed storage proteins 11S globulin, 7S vicilin and the 2S albumin are relevant allergenic proteins in other pulses such as soy (Katz et al., 2014), peanut (Mueller et al., 2014) and lentil (Pascual et al., 1999). (Clemente et al., 1999; Sormus de Castro Pinto et al., 2009)Several resistant peptides derived from these proteins were identified in both CnS and CS flours. The *in-silico* evaluation of the digest peptide sequences highlighted the presence in both samples of precursor peptides of 5 IgE/IgG binding sequences LEPDH (IEDB epitope ID 913606), NRIESEG (IEDB epitope 913850), PDNRIE (IEDB epitope 913893), and RIESEG (IEDB epitope ID 914030) described in the 11S soybean legumin (Kern et al., 2019). None of the identified sequences contained peanut and lentil IgE/IgG binding sequences.

The germination process, by degrading the proteins to short fragments, contributes to a reduced immunoreactivity (Li et al., 2016). The sprouted soybean proteins were found not to induce anaphylactic shock symptoms in BALB/c mouse models after oral sensitization compared to raw soybean proteins (Yang et al., 2015). Similarly, short-term germinated peanuts were found to have a decreased immunoreactivity by ELISA and western blotting analysis using a polyclonal rabbit anti-Ara h 1. Overall, a lower number of peptides could be detected in the digests from CS compared to CnS flours.

To date, the WHO/IUIS database (http://www.allergen.org/) includes only one allergenic protein for the chickpea. The Cic a 1.0101 is a late embryogenesis protein recognized by ImmunoCAP and western blotting analysis, using IgE serum antibodies of Spain patients with positive skin prick test (SPT). Peptide sequences belonging to a late embryogenesis protein (E7BSD7) were identified in the digest of both chickpea seeds and sprouts. These peptides were precursors of the IgE-binding sequence VGSHPIG (IEDB epitope 914230) identified by the serum of subjects with soy bean sensitization (Kern et al., 2019).

An additional 26 kDa protein, identified as an albumin with hemagglutination property, was retrieved mining the COMprehensive Protein Allergen REsource (COMPARE) database (https://comparedatabase.org/), which catalogues updated protein sequences with described IgE binding capacity (last update 29/01/2021). The Basic Local Alignment Search Tool (BLAST) search against the UNIPROT database allowed retrieving two homologue protein sequences

belonging to the chickpea proteome: a lectin protein C-25 (R9TPI6, 95.2% identity) and an albumin-2-like protein (A0A3Q7K771, 86.9% identity). The three protein sequences were aligned using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the peptides identified in both digests and inferred by the Peaks studio software to the albumin-2-like protein, were mapped onto to sequences to find common peptides (Figure S6). Despite the high homology shared between the sequences, all identified peptides were unique to the albumin-2-like protein. The manual evaluation of the *de novo* sequenced peptides, allowed the identification only in the digest of CS flour of two peptides with sequence NDKYVLL and TLDYWP belonging to the IgE-binding hemagglutinin proteins (Table S7). Contrasting information are available in literature regarding the relation between lectin synthesis and germination. Lectins have been identified in wheat, rye, and bean seeds during germination. They appear to be involved in a defense mechanism of the embryo (Koval'chuk, 1999; Peumans et al., 1982). The concentration was found to increase significantly after 6 days of sprouting in lentil seeds (Cuadrado et al., 2000). Our data are in line with these observations since resistant peptides could only be identified in the digest of CS flour. A similar study on white kidney beans found the concentration of lectin to reduce during germination (Savelkoul et al., 1994). A dynamic study, targeting the lectin may be required to address specifically this issue and understand the role of germination in enhancing or reducing the allergenicity of chickpea.

3.4.2 *In silico* evaluation of bioactive sequences

The antioxidant properties of chickpea hydrolysates has been demonstrated by previous studies and was associated to tri- and tetrapeptide sequences (Ghribi et al., 2015). Peptides shorter than 5 amino acid residues (m/z <300) were not monitored under our analytical conditions since they suffer from matrix background and low confidence of identification. ALEPDHR, TETWNPNHPEL, FVPH and DHG antioxidant sequences could be identified in both CnS and CS flour digests.

4. Conclusions

Germination induces significant changes in the chickpea seed proteome, which are reflected in the digestome composition with reduced peptides derived from seed storage proteins and increased number of peptides belonging to germination related processes. The peptide sequence information collected in both samples enabled the identification of common sequences with potential antioxidant activity and potential IgE-binding capability. Immunoassays and cell-based readouts with sera of allergic subjects can be set up to assess whether the germination process effects the allergenic potential of the ingredients.

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Figure captions:

Figure 1 SDS-PAGE analysis (15% acrylamide) of crude protein extracts and protein fractions from chickpea seed (CnS) and chickpea sprouts (CS). Lane 1: proteins extracted from CnS; lane 2: 11S legumins isolated (CnS); lane 3: 7S vicilin isolated from (CnS); lane 4: 2S albumin isolated from (CnS); lane 5: proteins extracted from CS; lane 6: 11S legumins isolated from CS; lane 7: 7S vicilin isolated from CS; lane 8: 2S albumin isolated from CS. The bands of interest that were subjected to trypsin digestion and mass spectrometry analysis are indicated with numbers.

Figure 2 2D IEF/SDS-PAGE (15% acrylamide; Coomassie staining) comparison of (a) proteins extracted from chickpea seed flour (CnS) and (b) proteins extracted from sprouted chickpea flour

(CS). Protein extracts only separated on the 2nd dimension (SDS-PAGE) were included on each gel to guide the localization of the proteins on the 2D map. Spots from the region indicated by the circle were excised, in-gel digested with trypsin and identified by mass spectrometry. The enlargement of the 2Dmap with spot numbers and relevant identification are reported as supplementary Figure S1 and Table S1.

Figure 3 Box plot of mg of α-amino nitrogen determined on 10 mg of digested flour ($p<0.01$). The soluble digests after duodenal and duodenal + jejunal digestions were de-proteinised, and the FAN was determined. Samples were analyzed in triplicates. The chromatographic profiles of the de-proteinised digest are reported in Figure S3.

Supplementary figure captions:

Figure S1: Enlargement of CS flour's 2D map. The in-gel trypsin digested spots are indicated with numbers on the figure. Protein entries identified by mass spectrometry are report in Table S1.

Figure S2 C8-RP-HPLC separation. Panel a) crude protein extracts; Panel b) 11S globulin isolated fraction; panel c) 7S vicilin fraction and panel d) 2S albumin fraction. Black trace (220 nm): chickpea seed flour (CnS); red trace (220 nm): proteins extracted from sprouted chickpea flour (CS).

Figure S3 C18-RP-HPLC separation of the de-proteinised digests analyzed for the FAN determination. Panel a) CnS after duodenal digestion; Panel b) CnS flour after duodenal and jejunal digestion; panel c) CS flour after duodenal digestion d) CS flour after duodenal and jejunal digestion. The black trace was detected at 220 nm; the black dotted trace was detected at 280 nm. 1) free tyrosine; 2) free tryptophan. The retention time of the aromatic amino acid was confirmed by analyzing standards under the same conditions Figure S4.

Figure S4 C18-RP-HPLC separation of standard aromatic amino acids. Peak 1) tyrosine, peak 2) tryptophan. The black trace was detected at 220 nm; the black dotted trace was detected at 280 nm.

Figure S5 Graphical representation of the peptides identified by mass spectrometry onto the protein sequence. The legumin Q9SMJ4 was selected as an exemplar protein. Panel a) digestome of CnS flour; panel b) digestome of CS flours.

Figure S6 Protein sequence alignment of the COMPARE chickpea allergen with Uniprot retrieved sequences (panel a). The identified peptides in the digests of CnS (panel b) and CS (panel c) flour were inferred to an albumin 2- like protein (A0A3Q7K771).

Supplementary Table:

Table S1 Proteins identified by HPLC-ESI-MS/MS analysis of the in-gel trypsin digested bands excised from the 1D SDS PAGE in figure 1.

Table S2 Proteins identified by HPLC-ESI-MS/MS analysis of the in-gel trypsin digested bands excised from the 2D map in figure 2, panel b.

Table S3 Proteins identified by HPLC-ESI-MS/MS analysis of gastro-duodenal and jejunal digestion of the CnS flour.

Table S4 Proteins identified by HPLC-ESI-MS/MS analysis of gastro-duodenal and jejunal digestion of the CS flour.

Table S5 Peptide sequences identified by HPLC-ESI-MS/MS analysis of gastro-duodenal and jejunal digestion of the CnS flour.

Table S6 Peptide sequences identified by HPLC-ESI-MS/MS analysis of gastro-duodenal and jejunal digestion of the CS flour.

Table S7 de novo sequenced peptides identified in the digestome of the chickpea sprout flour inferred to the IgE binding hemagglutinin protein indexed in COMPARE database.

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Figure 1

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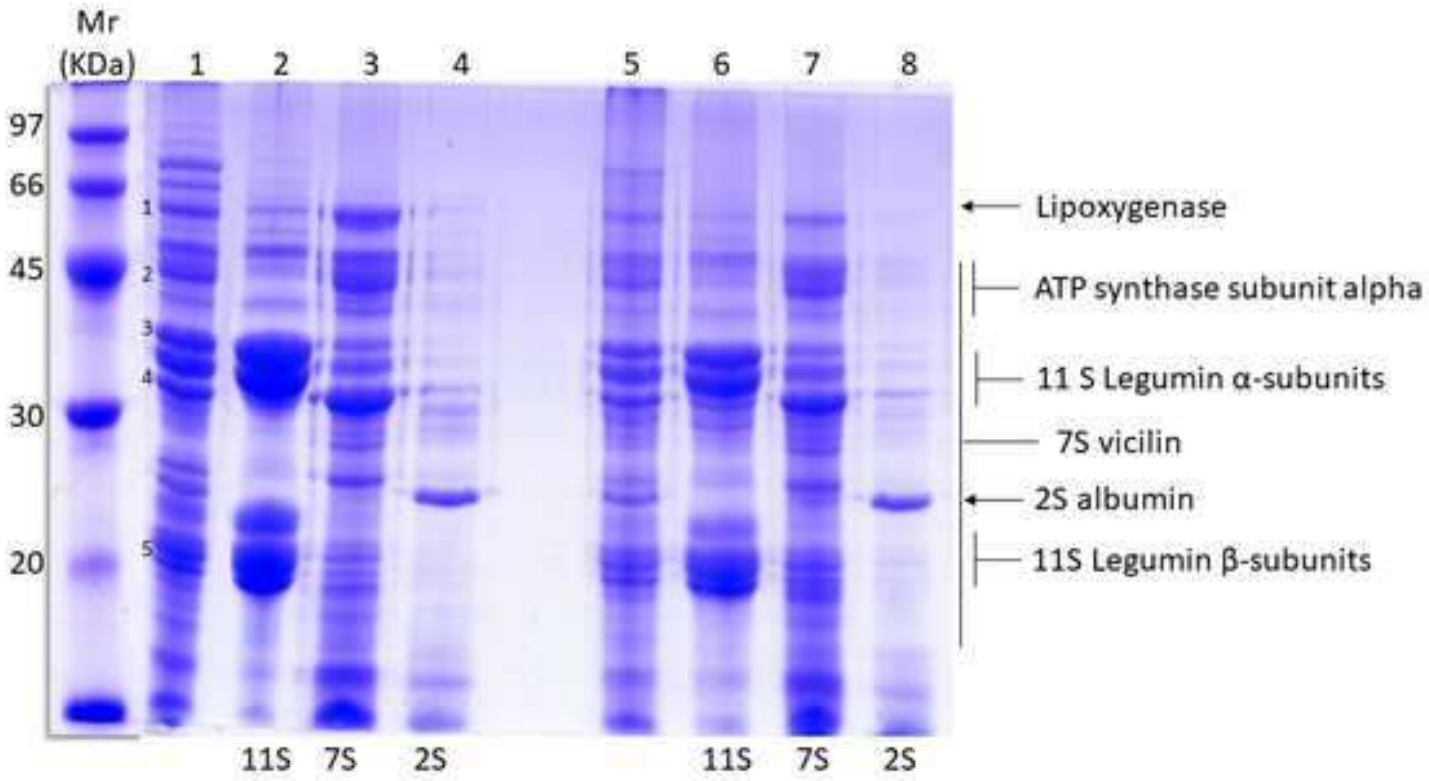

Figure 2

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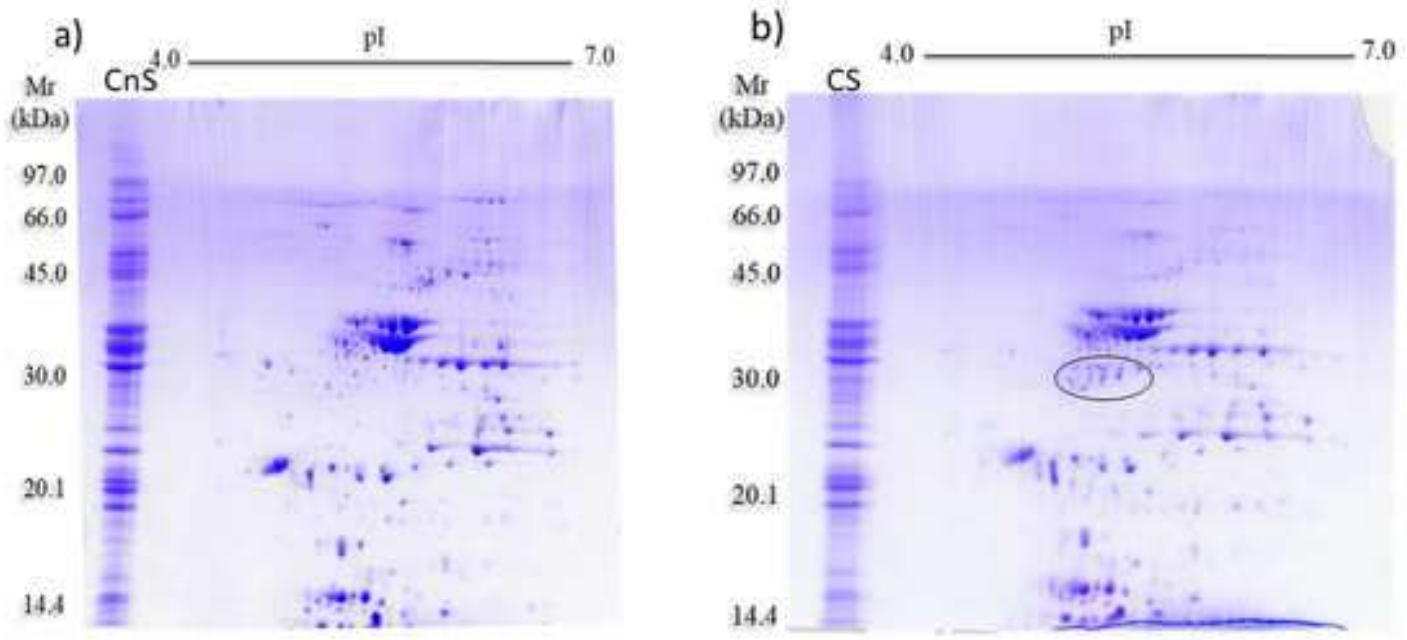

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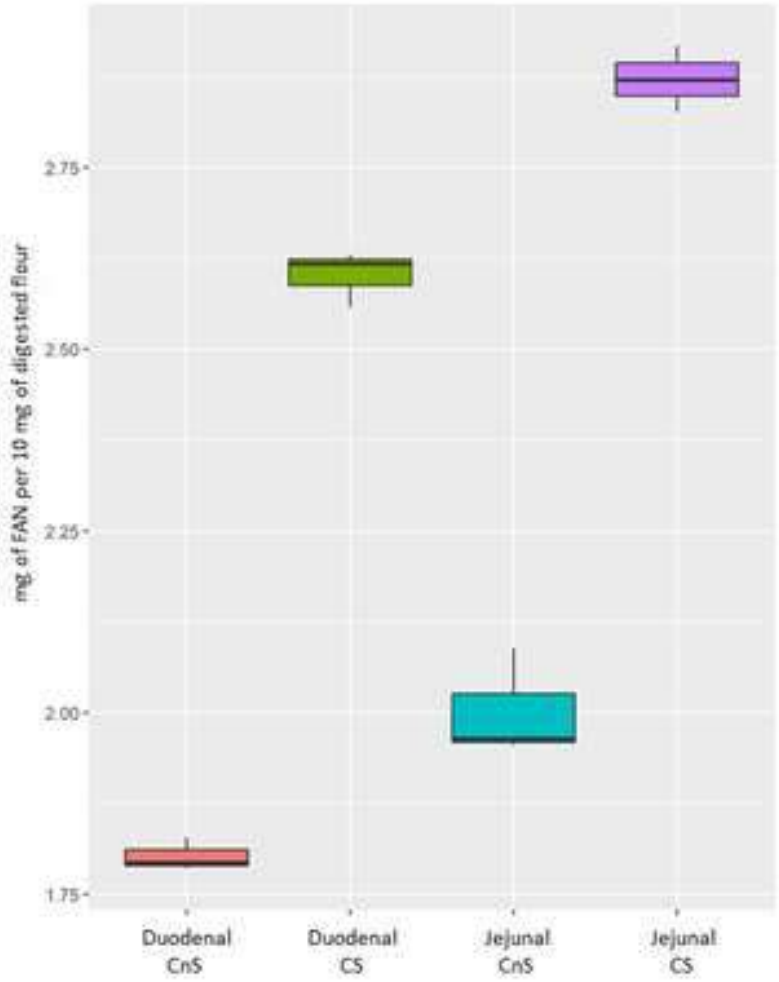

figure S1

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table S1

table S2

table S3 - S7

Graphical Abstract

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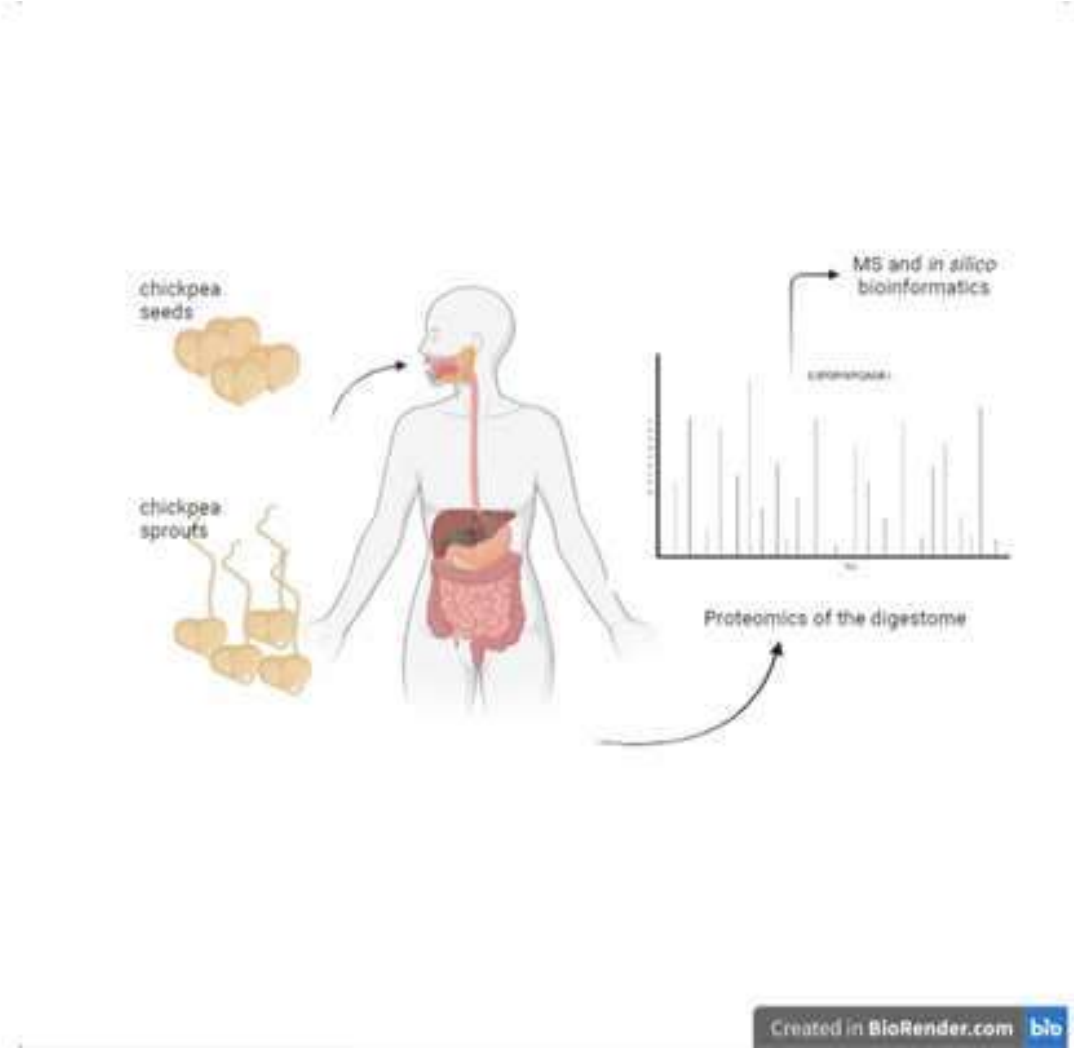

Author contributions

Chiara Nitride: Conceptualization, investigation, formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing; **Gerd Elisabeth Vegarud**: Conceptualization and Writing - Review & Editing; **Irene Comi**: investigation and Writing - Review & Editing; **Tove G. Devold**: investigation and Writing - Review & Editing; **Arne Røseth**: investigation and Writing - Review & Editing; **Alessandra Marti:** Writing - Review & Editing; **Stefania Iametti:** Writing - Review & Editing; **Gianfranco Mamone**: investigation and Writing - Review & Editing; **Gianluca Picariello**: investigation and Writing - Review & Editing; **Fabio Alfieri**: investigation; **Maria Adalgisa Nicolai**: investigation; **Clare Mills**: Writing - Review & Editing and **Pasquale Ferranti**: Conceptualization, formal analysis- Review & Editing

Declaration of interest

On behalf of all authors, the corresponding author declared that there is no conflict of interest.