



In vitro gastroduodenal and jejunal brush border membrane digestion of raw and roasted tree nuts

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ABSTRACT

Heat treatments induce chemical/physical modifications, which may affect the stability to enzymatic digestion and consequently the allergenicity of food proteins to a varying extent, depending on the time/temperature regimen. Herein, we evaluated the stability to digestion of whole tree nut (walnuts, hazelnuts and almonds) allergens in a food digestion model reflecting the real one by, taking into consideration the allergen-containing processed (roasted) food. To this aim, whole raw and roasted tree nuts were subjected to *in vitro* digestion combining the harmonized oral-gastric-duodenal digestion models with brush border membrane enzymes (BBM) to simulate the jejunal degradation of peptides. The degradation of allergens was monitored by integrated proteomic/peptidomic and bio-informatic tools. Roasting increased digestibility of tree nuts, since very few peptides were detected in digested samples (< 6.5 kDa fraction). After BBM digestion step, the degradation of peptides was enhanced in roasted walnuts and hazelnuts compared to the raw counterpart. Conversely, almond allergens showed a different behaviour, since the presence of resistant peptides was more evident for roasted almonds, probably because of the hydrolysis of high molecular weight aggregates generated during roasting. Our results provide new insight into the relationship between thermal processing and metabolic fate of tree nut allergens, highlighting the importance of investigating the digestion stability of whole allergenic food, rather than purified proteins.

1. Introduction

Tree nuts are a common cause of IgE-mediated adverse reactions worldwide, affecting up to 4.5% of the population, with increasing prevalence (Geiselhart, Hoffmann-Sommergruber, & Bublin, 2018; McWilliam et al., 2015; Stiefel et al., 2017). Overall, 70–90% of fatal anaphylactic reactions reported in United States between 2001 and 2007 were caused by the accidental consumption of peanut and tree nuts; among these, 18–40% were caused by tree nuts (Clark, Anagnostou, & Ewan, 2007; McWilliam et al., 2015). Hazelnuts (*Corylus avellana*) are the major cause of tree nut sensitization with great geographic and age related variations in terms of allergenic determinants and relative severity of the symptoms (Datema et al., 2015; Geiselhart et al., 2018). Almonds (*Prunus dulcis*) and walnuts (*Juglans regia* and *nigra*) are the primary cause of tree nut allergy in the UK, with lower prevalence in the rest of Europe (Geiselhart et al., 2018; Weinberger & Sicherer, 2018). The major tree nut allergens are seed storage proteins belonging to the cupin family (vicilins and legumins) and to the

prolamin superfamily (2S albumins, nsLTPs), or are pollen related allergens (Bet v 1- homologues, profilins), regulatory proteins and lipophilic gene products (Roux, Teuber, & Sathe, 2003). In particular, the WHO/IUIS reports eight food related allergens for hazelnut, eight and three for English and black walnut, respectively, and six for almond (<http://www.allergen.org/>).

Tree nuts are consumed raw or roasted finding application in food manufacturing companies as ingredients in pastry, confectionery, non-dairy milk and meat-based products. Thermal processing is known to induce physical-chemical modifications of proteins (Teodorowicz, Van Neerven, & Savelkoul, 2017). Downs, Baumert, Taylor, and Mills (2016) recently identified Maillard adducts occurring in the cupin (7S and 11S) fraction of roasted English walnut. The broad dynamic range of concentration, typical of seed storage proteins, challenges the characterization of Maillard adducts in less abundant proteins as the prolamins (2S and nsLTP), underlying the importance of monitoring allergen specific thermal induced modifications (Downs, Baumert, et al., 2016). The protein structural modifications can modulate either

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increasing (neo-allergens) or reducing the allergenic potential. In fact, the stability of allergens to gastrointestinal digestion (pH and enzymes) and the way allergens are presented to the immune system might be affected by thermal treatment, although a direct correlation between digestibility and allergenicity does not seem to exist (Verhoeckx, Bøgh, Dupont, & Egger, 2019). The minimum number of amino acids in the peptide sequence to elicit an allergic reaction (epitopic sequence) was found to be about nine (Bøgh & Madsen, 2016; De Angelis, Bavaro, Forte, Pilolli, & Monaci, 2018; Naegeli et al., 2017). De Angelis et al. (2018) showed a reduced potential allergenicity of heat and pressure treated almonds after simulated gastrointestinal digestion evaluated through the absence of previously characterised epitopes (De Angelis et al., 2018). Similarly, we demonstrated that roasting reduced the potential allergenicity of peanuts since no intact IgE-binding epitopes survived the oral-gastric-duodenal and brush border membrane enzymes (BBM) digestion (Di Stasio et al., 2020). The studies described above were carried out according to the Infogest standardised model of the *in vitro* static digestion (Brodkorb et al., 2019; Minekus et al., 2014). This method simulates the digestion of a food product rather than that of isolated proteins enabling the investigation of the protein behaviour in their real matrix, within a complex macromolecular interacting system. We implemented the Infogest model by adding a final digestion step using jejunal BBM enzymes. These enzymes includes oligopeptidases (endo- and exo-peptidases), lipases and glycosidases (Di Stasio et al., 2017; Di Stasio et al., 2020; Mamone et al., 2015; Picariello et al., 2015) promoting the uptake of nutrients by further reducing the size of macromolecules released by the upstream digestion steps into absorbable units. BBM enzymes are mainly located in vesicles on the surface of the gut epithelium and can be shed in the periapical space of enterocytes by action of the biliopancreatic secretions (Picariello, Ferranti, & Addeo, 2016).

In the present study, we compared the digestibility of raw and roasted walnuts, hazelnuts and almonds through simulated oral-gastro-duodenal and BBM digestion. Proteomic and peptidomic techniques were employed complementarily to identify and characterize the polypeptides surviving the digestion process. *In silico* investigation enabled the identification of potential epitopes within their natural matrices before and after roasting.

2. Materials and methods

2.1. Chemicals

Raw and roasted walnuts (var. Cilena), hazelnuts (var. Campana) and almonds (var. Italia) were provided by Besana S.p.A. (Milano, Italy) as de-shelled, peeled and coarsely grounded nuts. Tree nuts were roasted for 15 min at 170 °C according to the commercial nut-roasting Besana S.p.A. Digestive enzymes (salivary α -amylase, pepsin, trypsin, chymotrypsin, pancreatic α -amylase, pancreatic lipase and bile salts), all chemicals used for the preparation of digestion buffers (potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, and calcium chloride), Tris-HCl, urea, HPLC grade solvents and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St Louis, MO, USA). BBM vesicles were purified from porcine jejunum as previously described by (Picariello et al., 2015). Egg lecithin was from Lipid Products (Redhill, UK). Reagents for electrophoresis analysis were from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. *In vitro* gastroduodenal-BBM digestion of raw and roasted tree nuts

The simulated oral, gastric and duodenal digestion of grounded tree nuts was carried out according to Minekus et al. (2014). Immediately after duodenal digestion, the pH was adjusted to 7.2 and samples were incubated with BBM vesicles containing small intestinal hydrolases for 6 h at 37 °C. The activity of intestinal BBM aminopeptidase N activity

was 13 mU/ μ L, with enzymatic units (U) defined as the enzyme amount hydrolyzing 1.0 μ mol/min of L-leucine p-nitroanilide substrate (3.0 mM concentration) at pH 8.0 and 25 °C (Picariello et al., 2015; Di Stasio et al., 2017). Finally, digests were centrifuged at 4,500 g for 30 min and cooled in an ice-cold bath. Supernatants and pellets, containing soluble and insoluble digested polypeptides, respectively, were separately collected and rapidly purified or used for subsequent analysis. Hereafter in the text, oral and gastro-duodenal digestion, before and after hydrolysis with BBM enzymes, will be referred to as OGD and OGD-BBM, respectively.

2.3. SDS-PAGE analysis

SDS-PAGE was carried out under non-reduction conditions, using a Mini-PROTEAN cell system (Bio-Rad). OGD and OGD-BBM soluble digesta samples (100 μ L) were precipitated for 30 min in ice cold bath with trichloroacetic acid (TCA) up to 30% (w/v) final concentration. After centrifugation (4,000 g for 20 min), the resulting pellet was washed with 1 mL of -20 °C cold acetone (3 times) for removing TCA and then dissolved in 50 μ L of Laemli buffer (0.125 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 0.02% bromophenol blue). OGD and OGD-BBM insoluble digesta samples were extracted with 1 mL of 7 M urea buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). A 10 μ L aliquot of each sample was combined with 10 μ L of Laemli buffer and then loaded on precast 12% gel (Bio-Rad). After migration, gel was stained with Silver blue (Coomassie Brilliant Blue G-250) and processed by LABScan scanner and ImageQuant TL software (Amersham Bioscience/GE Healthcare, Uppsala, Sweden). The electrophoretic profiles of soluble and insoluble tree nut digesta were compared to those of undigested proteins extracted as previously described (Di Stasio et al., 2017). Protein bands were manually excised, in-gel destained, Cys-reduced/alkylated with iodoacetamide and hydrolyzed with proteomic grade modified trypsin (Pierce, Rockford, IL, USA) overnight at 37 °C, as previously described (Di Stasio et al., 2017). Resulting tryptic peptides were two-fold extracted with 50% acetonitrile (v/v) containing 2.5% (v/v) formic acid and finally vacuum-dried.

2.4. LC-MS/MS analysis

An aliquot of the soluble digesta fraction was dialyzed by 500–1,000 MWCO tubes (Spectra Por Float-A-Lyzer G2 Dialysis Device, Spectrum Laboratories Inc., USA) against PBS (Phosphate-Buffered Saline) buffer. After desalting with C18 spin column (Pierce C18 Spin Column, Thermo Scientific, San Jose, CA, USA), samples were analyzed by LC-MS/MS using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific), coupled online with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were suspended in 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 mm i.d. pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C18 column (2 mm, 15 cm-75 mm) 3 mm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in Milli-Q water; eluent B was 0.1% formic acid (v/v) in acetonitrile. The column was equilibrated at 5% B. Peptides were separated applying a 4–40% gradient of B over 60 min. The flow rate was 300 nL/min. The mass spectrometer was operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an m/z scan range of 350–1600. 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 $1/10^6$ 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 10 s was applied. Ions with one or more than six charges were excluded.

2.5. MS/MS data processing

MS/MS data were elaborated using the MaxQuant software (<https://maxquant.org>), restricting the search to *Juglandaceae*, *Corylus avellana* and *Prunus dulcis* databases for walnuts, hazelnuts and almonds, respectively, which were extracted from the UniprotKB (downloaded on December 2019). Database searching parameters for identification of SDS-PAGE protein bands were the following: methionine oxidation and pyroglutamic for N-terminus glutamine as variable protein modifications; carbamidomethylcysteine as a constant modification; mass tolerance value of 10 ppm for precursor ion and 0.01 Da for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavage up to 2. To identify the peptides that survived digestion, database searching parameters were the same above, except for no modification of cysteine residues included and no proteolytic enzyme selected. The false discovery rate and protein probabilities were calculated by Target Decoy PSM Validator, working between 0.01 and 0.05 for strict and relaxed searches, respectively. Proteins were considered confidently identified based on at least three sequenced peptides.

2.6. Data visualization

Raw data were visualized using PepView, an in house developed software. PepView has been realised in JAVA (<http://www.java.com>) and it uses JFreeChart (<http://www.jfree.org/jfreechart/>) to display charts. Starting from normalized intensities elaborated (in the current version) by the MaxQuant software, PepView provides a comparative detailed graphical representation of sequenced peptides arising from the digestion for each protein in various experimental conditions. At each amino acid residue along the protein sequence, the height of the bars is proportional to the intensity of peptides overlapping that position. Visualization produced by PepView has been further integrated with information obtained from a database of epitopes.

2.7. Bioinformatics analysis for assessing IgE binding

Peptides arising from OGD and OGD-BBM digestion of raw and roasted tree nuts were screened in a free epitope database and prediction resource (IEDB) (<https://www.iedb.org/>), in order to detect linear epitopes encrypted in the sequenced peptides that survived digestion. The IEDB parameters used for the analysis were: i) linear epitopes, ii) searching epitopes homologous with exact match (BLAST) and iii) human as host.

3. Results

3.1. *In vitro* OGD and OGD-BBM digestion of raw and roasted tree nuts

The bio-accessibility of the allergenic proteins in tree nuts was assessed using an *in vitro* static digestion model followed by proteomic and peptidomic analysis of the digesta. The Infogest OGD model (Minekus et al., 2014; Brodtkorb et al., 2019), was further implemented with jejunal BBM enzymes for simulating the final step of chyme breakdown (Di Stasio et al., 2017; Di Stasio et al., 2020; Mamone et al., 2015; Picariello et al., 2015). Protein digestion was monitored before and after BBM hydrolysis by proteomics (SDS-PAGE and LC-MS/MS-based analysis). The occurrence of insoluble proteins entrapped in the starchy matrix was also explored by SDS-PAGE analysis of residue pellets after extraction with denaturing buffer (Figs. 1–3). Soluble low molecular weight (MW) peptides which escaped protein purification and SDS separation were dialyzed and analysed by peptidomics (LC-MS/MS analysis) (Figs. 4 and 5).

The detailed workflow employed for this investigation is schematized in Supplementary Fig. 1. Table 1 shows an overview of tree nut allergens that survived the *in vitro* digestion and detected by proteomics (soluble and insoluble fraction) and peptidomics analysis (soluble

fraction). The characterization of digesta samples of grounded walnuts, hazelnuts and almonds is described separately below.

3.1.1. Proteomic analysis of digested walnuts

Crude protein extracts from raw and roasted walnuts were preliminary analysed with proteomic techniques (Fig. 1). As previously reported for peanut flour (Di Stasio et al., 2017; Mamone et al., 2018), mono-dimensional SDS-PAGE did not allow a complete separation of the individual protein isoforms. Protein bands within the 45–66 kDa MW range contained legumin A-like and legumin B-like. The Jug r 2 and Jug r 4 allergens migrated in the 55–70 kDa MW range. The 2S albumin was detected in the region < 21 kDa. Vicilin (Jug r 6) and 11S globulin 1-like and 2-like were spread out in the gel with different MW size, likely due to aggregation or to a variable protein post-synthetic processing (Supplementary Table 1). Interestingly, SDS-PAGE (under non-reducing condition) showed the occurrence of high MW aggregates (97–200 kDa) that contained cupin family proteins (vicilins and legumins) (Supplementary Table 1).

In vitro digestion of raw walnuts resulted in the virtually complete degradation of all the proteins, either in the raw or in the roasted walnut extracts, while a neo-formed broad band appeared at MW 14–6.5 kDa in both OGD and OGD-BBM soluble fractions (Fig. 1A). Upon tryptic in-gel digestion and MS/MS analysis, peptides from vicilin (Jug r 2 and Jug r 6), 2S albumin (Jug n 1) and oleosin, this last detected only in the OGD-BBM sample, were identified in this band. The analysis of the insoluble fractions revealed only a faint band at ~ 7 kDa, identified as vicilin- (Jug r 2 and Jug r 6) and 2S albumin- (Jug n 1) derived polypeptides that remained entrapped in the pellet of OGD raw walnuts (Table 1, supplementary Table 2). A protein band at the lower edge of the gel also dominated the SDS-PAGE profile of the soluble fractions from OGD and OGD-BBM digests of roasted walnuts; a similar band was also barely detectable in the insoluble counterparts (Fig. 1B). In addition to those found in soluble raw walnut (i. e. Jug r 6, Jug r 2, Jug n 1, oleosin), fragments of other allergenic proteins such as legumin A-like, legumin-B-like, 11S globulin (Jug r 4), and other isoforms of vicilin were detected in the OGD soluble fraction of roasted walnuts by LC-MS/MS analysis. However, after BBM digestion, soluble vicilin was no longer detected as it was likely hydrolysed into shorter fragments. The insoluble OGD-BBM digesta contained only fragments of Jug r 2 and Jug n 1 (Table 1, supplementary Table 2). These findings are consistent with previous results, showing the ability of BBM peptidases to hydrolyse even large-sized polypeptides (> 25–30 amino acid residues), though with a slower kinetics compared to small peptides (Picariello et al., 2016).

3.1.2. Peptidomic analysis of digested walnuts

In vitro digestion of walnut produced a complex variety of soluble polypeptides, which were characterized by LC-MS/MS analysis and search engine assisted matching. Overall, 668 and 775 peptides were sequenced in OGD and OGD-BBM digested raw walnuts, respectively (Fig. 4A). For roasted walnuts, the number of sequenced peptides drastically decreased to 188 and 90 in OGD and OGD-BBM, respectively. A detailed list of the parent proteins identified in digested walnuts is reported in Supplementary Table 3. Overall, 13 common proteins were identified through sequencing of peptides released by digestion of raw and roasted walnuts, except for oleosin that was not detected in the OGD-BBM roasted walnuts (Table 1, Supplementary Table 3). Fig. 5A tracks the pathway of degradation of the main allergens during walnut digestion, along with normalised abundance, expressed on the graph as intensity and determined as sum of the MS/MS intensities for all the sequenced peptides belonging to each protein, as computed by the MaxQuant software. In raw walnuts, legumin A-like, legumin B-like, 11S globulin and Jug r 4 allergens were the most extensively digested allergens (Table 1, supplementary Table 3) either before or after BBM digestion. Accordingly, these allergenic proteins were not detected by SDS analysis of the raw walnut digesta, so we can

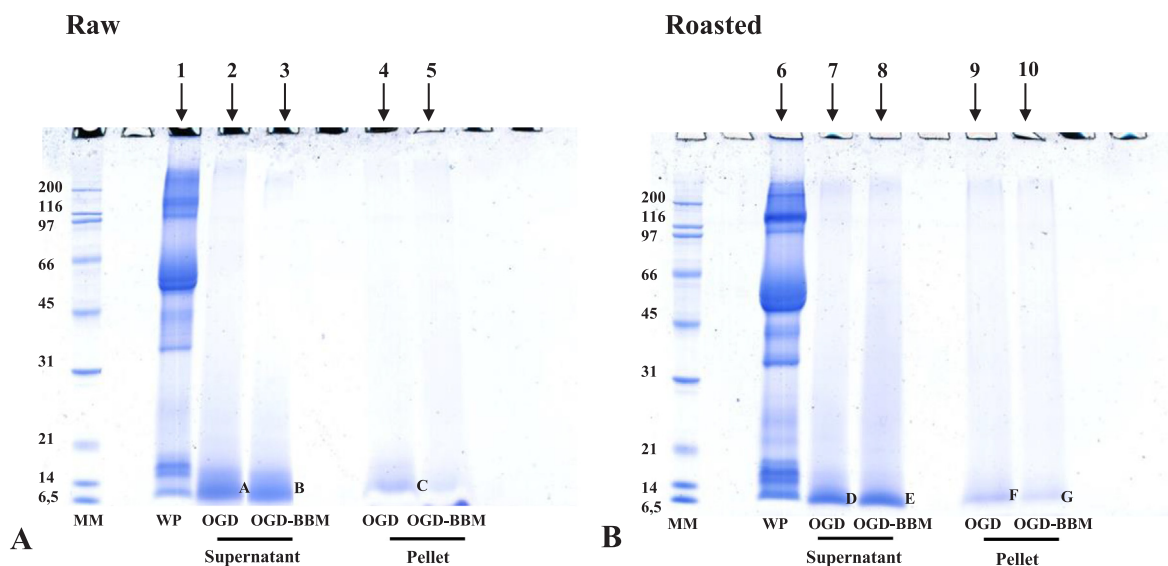


Fig. 1. SDS-PAGE analysis, under no-reducing condition, of digesta from raw and roasted walnuts. MM: Molecular Marker; WP (lane 1): Whole protein extracts from raw (panel A) and roasted (panel B) walnuts were run as reference control; OGD: oral gastric and duodenal digesta (supernatant and pellet) from raw (lanes 2 and 4- panel A) and roasted (lanes 7 and 9- panel B) walnuts; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (lanes 3 and 5, panel A) and roasted (lanes 8 and 10, panel B) walnuts. Bands, excised from the SDS gel, were analysed by LC-HR-MS/MS and identified using MaxQuant software (Supplementary Table 2).

assume that legumin A-like, legumin B-like, 11S globulin and Jug r 4 were practically entirely digested into small sized peptides (Fig. 1A). Vicilin (Jug r 6), 2S albumin (Jug n 1) and oleosin were detected in the soluble digests of raw walnuts (Table 1, supplementary Table 3) by both SDS-PAGE and peptidomic analysis, supporting the greater stability of these proteins to gastrointestinal digestion (Fig. 1A). The intensity of peptides arising from digestion of roasted walnuts was significantly decreased compared to the raw ones. These changes were accompanied by the formation of stable polypeptides (> 6.5 kDa) detected by SDS-PAGE as intense and faint bands in OGD and OGD-BBM digests, respectively (see SDS-PAGE, Fig. 1B). The resulting peptidomic data of

soluble fractions were better visualized using an in-house developed software (PepView), which compares the resulting protein maps individually and matches the sequences with known epitopes (Supplementary Fig. 2). Graphic processing by PepView disclosed few differences before and after BBM digestion of raw walnuts. For instance, the PepView profile of Jug r 4 from raw OGD walnuts, showed a total of 133 peptides with a 54.8% protein coverage (Supplementary Fig. 2); after BBM, the number of peptides increased to 181 with a protein coverage increased to 56%. Intensity of peptides arising from digested roasted walnuts significantly decreased. The PepView of Jug r 4 from roasted OGD and OGD-BBM walnuts showed a much lower number of

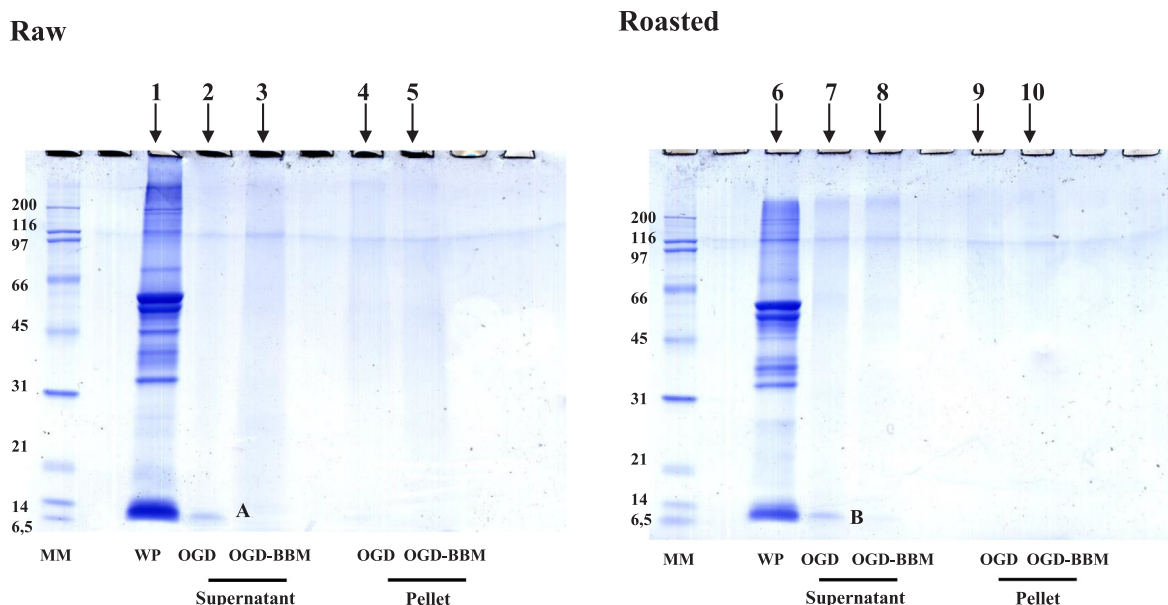


Fig. 2. SDS-PAGE analysis, under non-reducing condition, of digesta from raw and roasted hazelnuts. MM: Molecular Marker; WP (lane 1): Whole protein extracts from raw (panel A) and roasted (panel B) hazelnuts were run as reference control; OGD: oral gastric and duodenal digesta (supernatant and pellet) from raw (lanes 2 and 4, panel A) and roasted (lanes 7 and 9, panel B) hazelnuts; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (lanes 3 and 5, panel A) and roasted (lanes 8 and 10, panel B) hazelnuts. Bands, excised from the SDS gel, were analysed by LC-HR-MS/MS and identified using MaxQuant software (Supplementary Table 2).

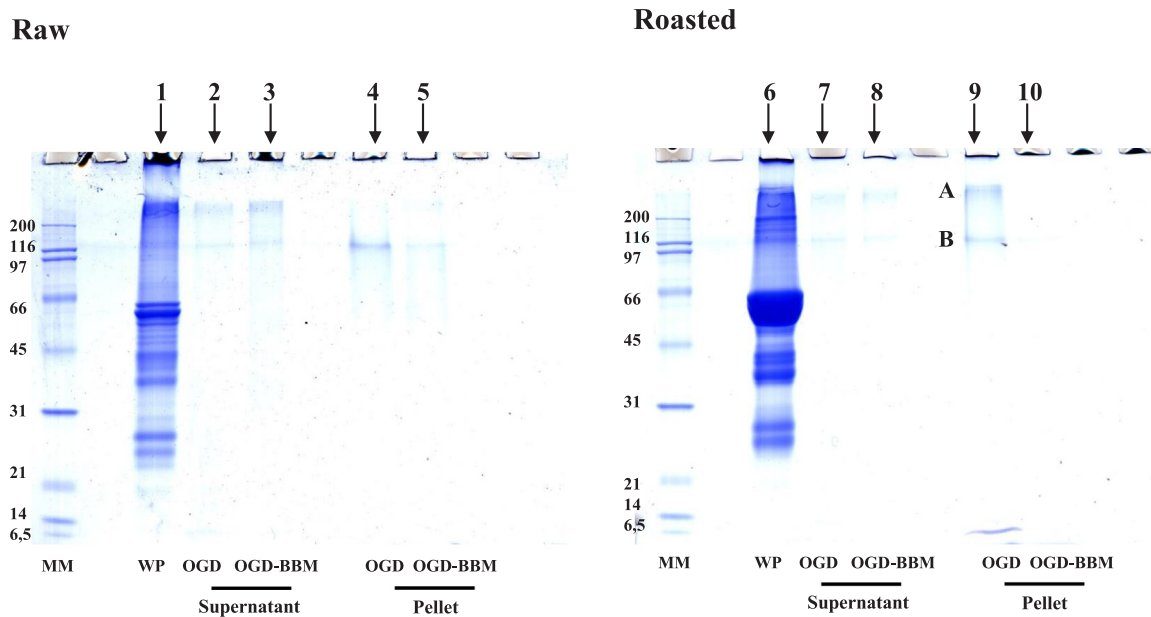


Fig. 3. SDS-PAGE analysis, under non-reducing condition, of digesta from raw and roasted almonds. MM: Molecular Marker; WP (lane 1): Whole protein extracts from raw (panel A) and roasted (panel B) almonds were run as reference control; OGD: oral gastric and duodenal digesta (supernatant and pellet) from raw (lanes 2 and 4, panel A) and roasted (lanes 7 and 9, panel B) almonds; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (lanes 3 and 5, panel A) and roasted (lanes 8 and 10, panel B) almonds. Bands, excised from the SDS gel, were analysed by LC-HR-MS/MS and identified using MaxQuant software (Supplementary Table 2).

identified peptides (5 and 4, respectively) compared to the raw counterpart, indicating that after roasting most of the proteins were extensively hydrolysed into very small peptides or free amino acids not detected by MS analysis (Supplementary Fig. 2). The same trend was observed for Jug r 2, Jug r 6 and Jug n 1 (Supplementary Fig. 2). The digested raw walnuts contained immunogenic peptides, as assessed by matching with the sequences reported in the IEDB database (Supplementary Fig. 2; Supplementary Table 4). In particular, seven linear epitopes for Jug r 4, five for Jug n 1 and eight for Jug r 2 were resistant to gastrointestinal digestion (Archila et al., 2015; Maleki et al., 2011; Robotham et al., 2009; Robotham, Teuber, Sathe, & Roux, 2002; Rosenfeld et al., 2012). However, after roasting, the intensity of these peptides decreased and some of them were no longer detected, highlighting how thermal treatment can affect the potential immunogenic properties (Supplementary Table 4).

3.1.3. Proteomic analysis of digested hazelnuts

SDS-PAGE (under non-reducing conditions) of undigested extracts from hazelnuts revealed three dominant bands with molecular mobility of approximately 56.8, 52.6 and 10 kDa, which were identified by LC-MS/MS as the 7S vicilin (Cor a 11 allergen), the 11S globulin-like (Cor a 9 allergen) and the 2S albumin (Cor a 14 allergen), respectively. In agreement with Nitride et al. (2013), 11S globulin-like protein, consisting of two isoforms, were detected between 45.4 and 34.8 kDa. The high MW (~200 kDa) aggregates were identified as Cor a 9 and Cor a 11 allergens (Supplementary Table 1). The simulated digestion of raw and roasted hazelnut resulted in an extensive degradation of the proteins evidenced by the substantial absence of protein bands in the relevant SDS-PAGE lanes (Fig. 2 panel A and B). Only a faint band was detected at ~9 kDa in the OGD digests, which contained fragments of 2S albumin (Cor a 14), 7S globulin (Cor a 11) and oleosin (Cor a 13), as assessed by LC-MS/MS analysis of the in-gel tryptic hydrolysate. Oleosin was detectable exclusively in the raw digesta, suggesting that the heat treatment significantly reduced its stability. The analysis of OGD-BBM digesta of raw and roasted hazelnuts showed the absence of detectable bands on the gel.

3.1.4. Peptidomic analysis of digested hazelnuts

Overall, 178 and 208 peptides were identified by LC-MS/MS analysis in the soluble fraction of OGD and OGD-BBM digesta of raw hazelnuts. Similarly to walnut, roasting increased the digestibility of hazelnut resulting in a decreased number of identified peptides (68 sequenced peptides) in both OGD and OGD-BBM (Fig. 4B). The peptides identified in digesta of raw and roasted hazelnuts belonged to seven common allergenic proteins (Supplementary Table 3). Interestingly, no fragment of 2S albumin was detected in the digesta of roasted hazelnuts after hydrolysis with BBM enzymes. The relative abundances of the allergens identified in OGD and OGD-BBM digesta, inferred from the sum of the intensity of relevant peptides, are compared in Fig. 5B for both raw and roasted hazelnuts. In general, the 11S globulin and the 7S vicilin are the most abundant allergenic proteins. The degree of digestion gradually increased from OGD to OGD-BBM and was much higher for raw than for roasted hazelnuts. Similar digestibility behaviour was observed for other allergens Cor a 14, Cor a 13, Cor a 8 and Cor a 1. In line with proteomic results (Fig. 2, Table 1), peptidomics suggested that all the hazelnut allergens were virtually completely hydrolysed into small polypeptides (< 6.5 kDa) after OGD-BBM. Supplementary Fig. 3 reports the PepView analysis of digested hazelnut allergens. The sequenced peptides of Cor a 9 from raw hazelnuts after OGD and OGD-BBM covered 43.5% (88 peptides) and 40.8% (105 peptides) of the protein, respectively. After roasting the number of identified peptides of Cor a 9 drastically dropped down to 29 (32.5% coverage) and 37 peptides (27.8% coverage) in OGD and OGD-BBM, respectively. A similar trend was observed also for other storage proteins, including Cor a 11 (7S globulin or 48-kDa), Cor a 1, Cor a 8, Cor a 14 and Cor a 13. Interestingly, Cor a 14 was particularly affected by roasting, since no fragments deriving from this protein were detected in the OGD-BBM digests. The exploration on IEDB database demonstrated that previously established immunogenic peptides of hazelnut allergens were resistant to gastroduodenal digestion. In detail, digests contained six IgE-binding sequences from Cor a 9, two from Cor a 1, one each from Cor a 8 and Cor a 11 (Archila et al., 2015; Bohle et al., 2005; Elsayed, Holen, & Dybendal, 1989; Jahn-Schmid et al., 2005; Robotham et al., 2009; Schulten et al., 2011). Similar to walnut, most of these epitopes were not

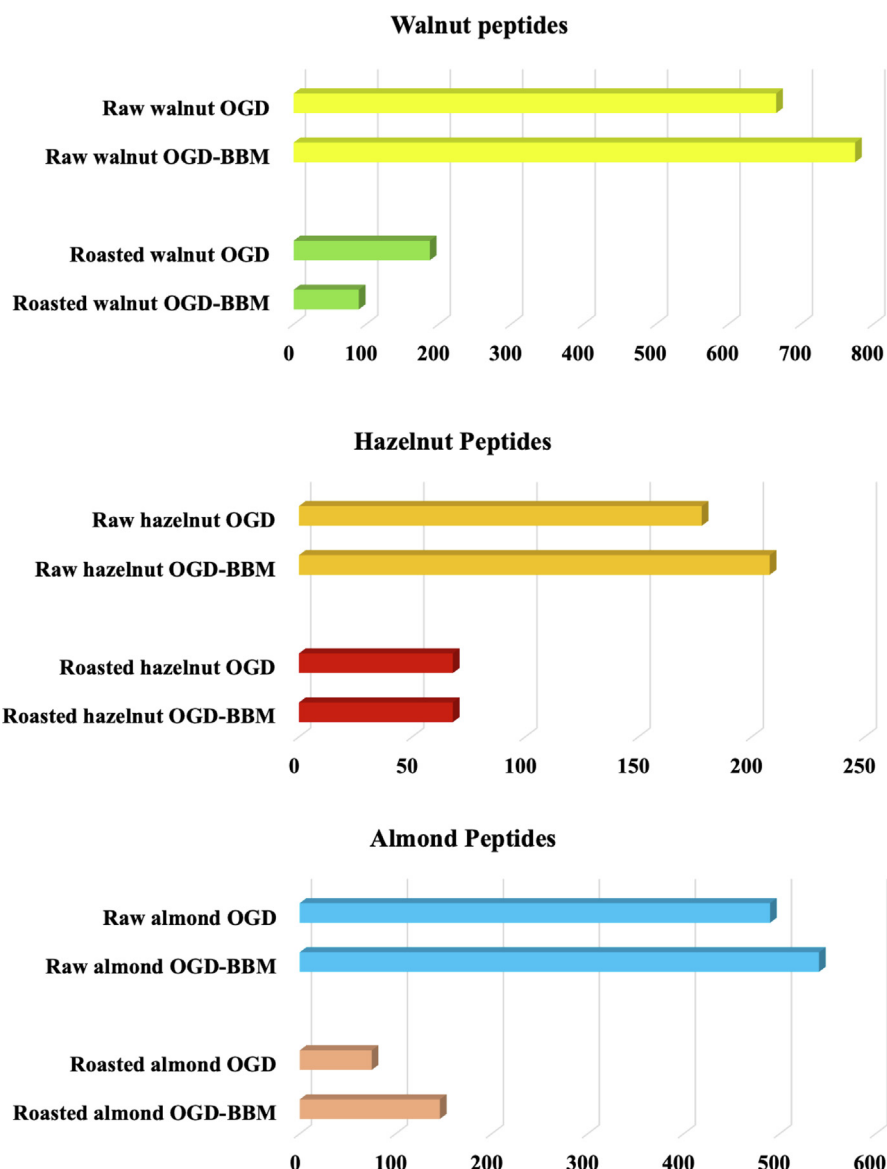


Fig. 4. Histograms representing the number of total peptides (MW < 6.5 kDa) resulting from OGD and OGD-BBM digestion of raw and roasted tree nuts through peptidomics analysis.

longer detected after BBM digestion of roasted hazelnut, due to extensive proteolysis (Supplementary Table 4, Supplementary Fig. 3).

3.1.5. Proteomic analysis of digested almonds

In analogy to previous reported matrices, the digestion of raw and roasted almonds was monitored by SDS-PAGE in comparison to protein extracts from undigested samples (Fig. 3). Amandin (known as Pru du 6), the most abundant allergen of almonds, was identified at 60 kDa along with relevant isoforms detected between 50.6 kDa and 23.7 kDa in both raw and roasted almonds. The bands at MW ~ 200 kDa contained cupin family proteins similarly to walnuts and hazelnuts (Fig. 3A and 3B, Supplementary Table 1). Both OGD and OGD-BBM digestion led to a practically complete disappearance of the protein bands in both raw and roasted almonds. Notably, the insoluble fractions contained high MW aggregates (~305 kDa and ~ 105 kDa) in roasted almond, which also survived the OGD digestion at scant amounts. These aggregates, analysed by LC-MS/MS after in-gel trypsinolysis, contained isoforms of amandin (Pru du 6, Pru du 6.0101 and Pru du 6.0201) as well as other important allergens, such as vicilin-like proteins (Table 1, Supplementary Table 2). Also in this case, the lack of these protein

aggregates in the OGD-BBM extracts (Fig. 3B) should be attributed to the further endoproteolytic action of intestinal peptidases.

3.1.6. Peptidomic analysis of digested almonds

OGD and OGD-BBM digestion of raw and roasted almonds produced a complex multitude of soluble peptides. Overall, 490 (OGD) and 541 (OGD-BBM) peptides were sequenced in raw almonds. Also in almond the number of sequenced peptides drastically decreased after roasting, down to 75 and 146 peptides in OGD and OGD-BBM, respectively (Fig. 4C). The majority of these peptides belonged to the Pru du 6 allergen. Peptides from Pru du 6.0101 and Pru du 6.0201 isoforms were also detected but to a lower intensity (Fig. 5C). Vicilin, 11S globulin, 7S globulin, oleosin and LTP peptides were detected in the soluble fractions of digested raw and roasted almonds (Table 1, Supplementary Table 3). Roasted OGD peptides did not contain fragments of vicilin, which in contrast was detected in the insoluble fraction by SDS-PAGE (Fig. 3B, Table 1, Supplementary Tables 2 and 3). The graphical representation of the intensity of main almond allergens, inferred from the respective survived peptides, is displayed in Fig. 5C. In raw almond, the intensity of the Pru du 6 allergen decreased from OGD to OGD-BBM

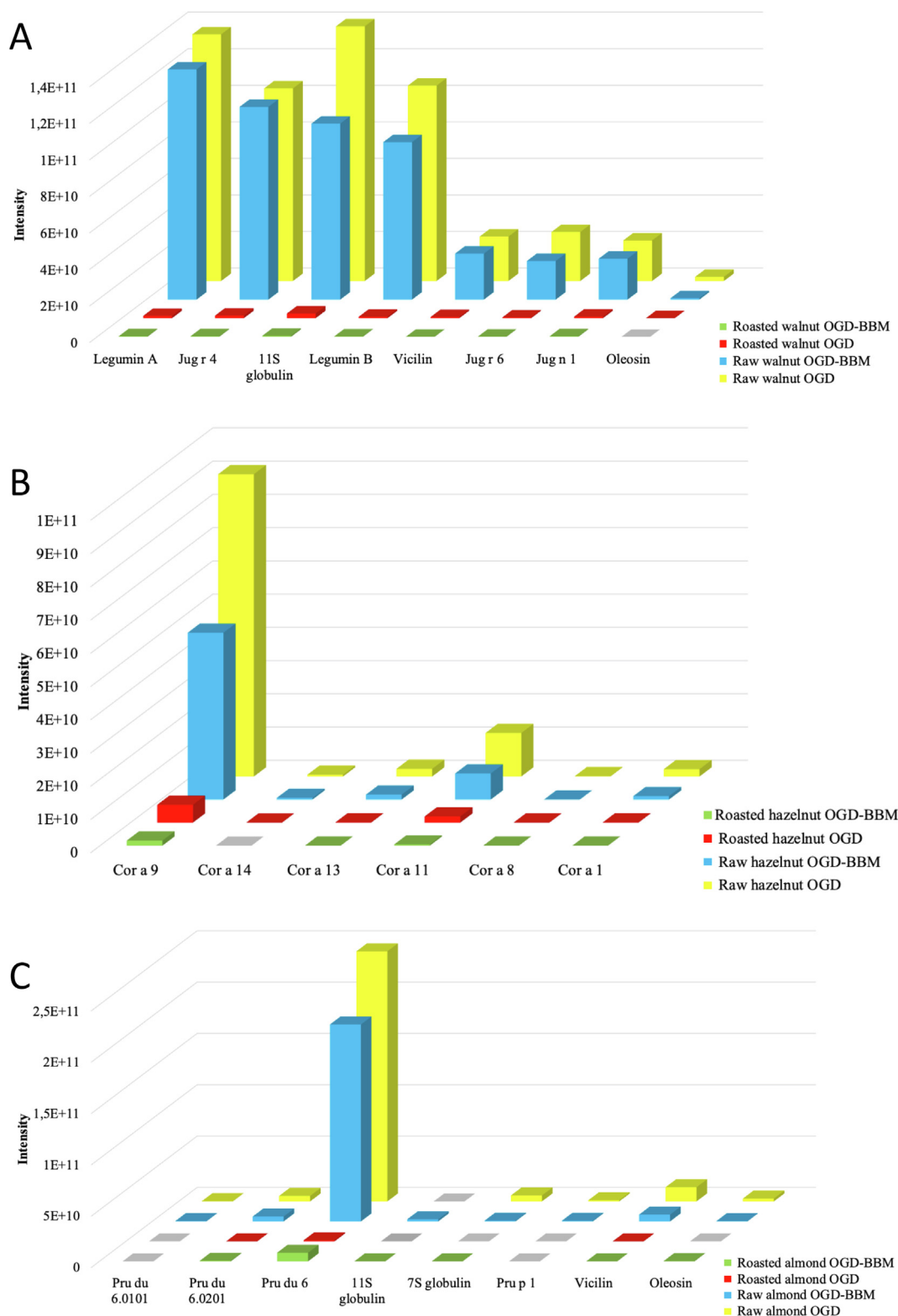


Fig. 5. Graphical representation of the sum of peptide intensities (Supplementary Table 3) of each allergens identified in raw and roasted walnuts (A), hazelnuts (B) and almonds (C), before and after BBM digestion.

by 21%. A drastic reduction of Pru du 6 was observed after roasting, although its total intensity slightly increased after BBM digestion, probably as a consequence of the BBM digestion of high MW insoluble aggregates detected in OGD digests. The digestion course of almond allergens was graphically represented with the PepView software (Supplementary Fig. 4). The OGD and OGD-BBM digests mapped Pru du

6 for 49% and 55.8% with 88 and 113 sequenced peptides, respectively. After roasting, the number of Pru du 6 peptides dropped to 28 and 64 sequenced peptides in OGD and OGD-BBM with coverage of 30.6% and 43.3%, respectively. Isoforms of Pru du 6 exhibited a similar trend. Surprisingly, differently from walnut and hazelnut, we observed that the sequence coverage and number of peptides of OGD-BBM digests

Table 1

Summary of the OGD- and OGD-BBM- resistant proteins identified using proteomic (SDS-PAGE) and peptidomic (Shotgun) approach of raw and roasted tree nuts.

	RAW						ROASTED					
	Shotgun ^a		SDS-PAGE ^b				Shotgun ^a		SDS-PAGE ^b			
	OGD ^c	OGD-BBM ^c	Soluble digesta		Insoluble digesta		OGD ^c	OGD-BBM ^c	Soluble digesta		Insoluble digesta	
			OGD ^d	OGD-BBM ^d	OGD ^d	OGD-BBM ^d			OGD ^d	OGD-BBM ^d	OGD ^d	OGD-BBM ^d
WALNUTS	Legumin A ¹ (151)	Legumin A(192)	—	—	—	—	Legumin A (50)	Legumin A (22)	Legumin A (7 kDa)	Legumin A (7 kDa)	Legumin A (7 kDa)	—
	Jug r 4 ² (133)	Jug r 4 (181)	—	—	—	—	Jug r 4 (46)	Jug r 4 (20)	—	—	Jug r 4 (7 kDa)	—
	11S globulin ³ (113)	11S globulin (127)	—	—	—	—	11S globulin (37)	11S globulin (20)	11S globulin (7 kDa)	11S globulin (7 kDa)	11S globulin (7 kDa)	—
	Legumin B ⁴ (107)	Legumin B (120)	Jug r 6 (7 kDa)	Jug r 6 (7 kDa)	Jug r 6 (7 kDa)	—	Legumin B (29)	Legumin B (29)	Legumin B (7 kDa)	Legumin B (7 kDa)	Legumin B (7 kDa)	Jug r 2 (7 kDa)
	Vicilin ⁵ (76)	Vicilin (76)	Jug r 2 (7 kDa)	Jug r 2 (7 kDa)	Jug r 2 (7 kDa)	—	Vicilin (17)	Vicilin (5)	—	—	Legumin B (7 kDa)	—
	Jug r 6 ⁶ (71)	Jug r 6 (76)	—	—	—	—	Jug r 6 (18)	Jug r 6 (6)	Jug r 6 (7 kDa)	Jug r 6 (7 kDa)	—	—
	Jug r 2 ⁷ (62)	Jug r 2 (59)	—	—	—	—	Jug r 2 (11)	Jug r 2 (5)	Jug r 2 (7 kDa)	Jug r 2 (7 kDa)	Jug r 6 (7 kDa)	—
	Legumin B ⁸ (47)	Legumin B (49)	—	—	—	—	Legumin B (14)	Legumin B (7)	Jug r 2 (7 kDa)	Jug r 2 (7 kDa)	Jug r 2 (7 kDa)	Jug n 1 (7 kDa)
	Vicilin ⁹ (40)	Vicilin (41)	Jug n 1 (7 kDa)	Jug n 1(7 kDa)	—	—	Vicilin (8)	Vicilin (3)	Legumin B (7 kDa)	Legumin B (7 kDa)	Legumin B (7 kDa)	—
	11 s globulin ¹⁰ (28)	Jug n 1 (19)	—	Oleosin (7 kDa)	—	—	11 s globulin (5)	11 s globulin (3)	Vicilin (7 kDa)	—	Legumin B (7 kDa)	—
	Jug n 1 ¹¹ (16)	Oleosin (12)	—	—	—	—	Jug n 1 (5)	Jug n 1 (4)	Vicilin (7 kDa)	—	—	—
	Oleosin ¹² (10)	—	—	—	—	—	Oleosin (3)	—	Jug n 1 (7 kDa)	Oleosin (7 kDa)	Oleosin (7 kDa)	—
HAZELNUTS	Cor a 9 ¹³ (105)	Cor a 9 (129)	—	—	—	—	Cor a 9 (35)	Cor a 9 (47)	—	—	—	—
	Cor a 11 ¹⁴ (51)	Cor a 11 (51)	Cor a 11 (9 kDa)	—	—	—	Cor a 11 (20)	Cor a 11 (14)	Cor a 11 (9 kDa)	—	—	—
	Cor a 1 ¹⁵ (7)	Cor a 13 (12)	—	—	—	—	Cor a 1 (5)	Cor a 1 (2)	—	—	—	—
	Cor a 13 ¹⁶ (6)	Cor a 8 (1)	Cor a 13 (9 kDa)	—	—	—	Cor a 13 (5)	Cor a 13 (4)	—	—	—	—
	Cor a 8 ¹⁷ (3)	Cor a 14 (5)	—	—	—	—	Cor a 8 (1)	Cor a 8 (1)	—	—	—	—
	Cor a 14 ¹⁸ (2)	—	—	—	—	—	Cor a 14 (1)	—	Cor a 14 (9 kDa)	—	—	—
ALMONDS	Pru du 6.0101 ¹⁹ (77)	Pru du 6.0101 (119)	—	—	—	—	Pru du 6.0101 (31)	Pru du 6.0101 (39)	—	—	Pru du 6.0101 (305 kDa; 125 kDa; 6.0201 (305 kDa; 125 kDa)	—
	Pru du 6.0201 ²⁰ (90)	Pru du 6.0201 (115)	—	—	—	—	Pru du 6.0201 (26)	Pru du 6.0201 (107)	—	—	Pru du 6.0201 (305 kDa; 125 kDa)	—
	Pru du 6 ²¹ (88)	Pru du 6 (113)	—	—	—	—	Pru du 6 (28)	Pru du 6 (64)	—	—	Pru du 6 (305 kDa; 125 kDa)	—
	Pru du 6.0101 ²² (76)	Pru du 6.0101(117)	—	—	—	—	Pru du 6.0101(30)	Pru du 6.0101(38)	—	—	Pru du 6.0101 (305 kDa; 125 kDa)	—
	Vicilin ²³ (25)	Vicilin (28)	—	—	—	—	Vicilin (2)	Vicilin (3)	—	—	Pru du 6.0101 (305 kDa; 125 kDa)	—
	Vicilin ²⁴ (22)	Vicilin (26)	—	—	—	—	Vicilin 1)	Vicilin (5)	—	—	Vicilin (305 kDa; 125 kDa)	—
	Vicilin ²⁵ (17)	Vicilin (15)	—	—	—	—	Vicilin (1)	Vicilin (1)	—	—	Vicilin (305 kDa; 125 kDa)	—
	Vicilin ²⁶ (15)	Vicilin (18)	—	—	—	—	—	Vicilin (1)	—	—	—	—
	Pru p 1 ²⁷ (4)	Pru p 1(3)	—	—	—	—	—	Vicilin (1)	—	—	—	—
	11S globulin ²⁸ (73)	11S globulin (94)	—	—	—	—	11S globulin (22)	11S globulin (55)	—	—	Vicilin (305 kDa)	—
	7S globulin ²⁹ (13)	7S globulin (9)	—	—	—	—	—	7S globulin (2)	—	—	Vicilin (125 kDa)	—
	Cupin ³⁰ (8)	Oleosin (4)	—	—	—	—	Oleosin (1)	Cupin (1)	—	—	—	—
	Oleosin ³¹ (7)	Oleosin (3)	—	—	—	—	—	Oleosin (5)	—	—	—	—
	Oleosin ³² (4)	Oleosin (1)	—	—	—	—	LTP (1)	—	—	—	Cupin (125 kDa)	—
	Oleosin ³³ (2)	LTP (3)	—	—	—	—	—	Oleosin (1)	—	—	—	—
	LTP ³⁴ (5)	—	—	—	—	—	—	LTP (1)	—	—	—	—

(continued on next page)

Table 1 (continued)

RAW						ROASTED					
Shotgun ^a		SDS-PAGE ^b				Shotgun ^a		SDS-PAGE ^b			
		Soluble digesta		Insoluble digesta				Soluble digesta		Insoluble digesta	
OGD ^c	OGD-BBM ^c	OGD ^d	OGD-BBM ^d	OGD ^d	OGD-BBM ^d	OGD ^c	OGD-BBM ^c	OGD ^d	OGD-BBM ^d	OGD ^d	OGD-BBM ^d

Protein Accession number: ¹A0A2I4F6R4; ²Q2TPW5; ³A0A2I4EG83; ⁴A0A2I4GEH1; ⁵A0A2I4DYE9; ⁶A0A2I4E5L6; ⁷Q9SEW4; ⁸A0A2I4GEI2; ⁹A0A2I4F3W3; ¹⁰A0A2I4F669; ¹¹Q7Y1C2; ¹²G8H6H9; ¹³A0A0A0P7E3; ¹⁴Q8S4P9; ¹⁵Q9FPK4; ¹⁶Q84T91; ¹⁷Q9ATH2; ¹⁸D0PWG2; ¹⁹Q43607; ²⁰E3SH29; ²¹Q43608; ²²E3SH28; ²³A0A5E4E2P4; ²⁴A0A5E4EE27; ²⁵A0A5E4E7H8; ²⁶A0A5E4FV72; ²⁷B6CQT0; ²⁸A0A4Y1S2I9; ²⁹A0A5E4EAH6; ³⁰A0A4Y1QPI1; ³¹A0A5E4ET55; ³²A0A5E4GEY2; ³³Q43804; ³⁴A7Y7K3.

^a Supplementary Table 3.

^b Supplementary Table 2.

^c The number between brackets represent the number of sequenced peptides by LC-MS/MS.

^d The value between brackets represent the MW of SDS-PAGE gel (see Figs. 1–3).

increased compared to OGD of roasted almonds. This finding was likely a consequence of the splitting of larger peptides by action of BBM enzymes, which degraded the insoluble aggregates of roasted almonds. Related to this point, the bioinformatic matching of almond epitopes in the IEDB database revealed that the IgE-binding peptides of almonds (QNQLDQVPRRFLAG and QEEGRQEEQQGQQ from Pru du 6 and Pru du 6.0101 respectively) (Willison et al., 2011; Willison et al., 2013) were prevailing in the OGD-BBM digesta of roasted almonds, likely because they arose from digestion of high MW aggregates (Supplementary Table 4, Supplementary Fig. 4).

4. Discussion

In the present study, we assessed how thermal processing and food matrix may affect the allergen stability of walnut, hazelnut and almond throughout simulated gastrointestinal digestion. A deterministic correlation between inherent digestion stability and allergenicity does not appear to exist (Verhoeckx et al., 2019); however, the analysis of the peptides surviving the digestion can provide information about the resistance of known epitope sequences. A recent finding of Hoh et al. (2020) highlights the importance of local IgE response in the gastrointestinal tissue. These authors demonstrated that the gastrointestinal tissue is an important reservoir of IgE-producing B cells.

The harmonized *in vitro* static model of human digestion implemented by use of BBM enzymes, in conjunction with proteomic and peptidomic analysis, provided information about tree nut polypeptides surviving proteolysis, with special emphasis about those harbouring IgE-binding sequences. SDS-PAGE analysis has been long considered as the main tool for monitoring the disappearance or the stability of target allergens after *in vitro* digestion (Wickham, Faulks, & Mills, 2009). Combined to immunochemical techniques, electrophoresis provides evidence about the capability of digested allergen to preserve immunogenic properties.

By SDS-PAGE, we observed a different behaviour of walnut, hazelnut and almond allergens to digestion, since walnuts seemed to contain more stable allergens in comparison to almonds and hazelnuts. Digestion with the Infogest method released large fragments (> 6.5 kDa) of Jug r 6, Jug r 2 and Jug n 1, which were detected in the soluble and/or insoluble fractions. This finding was partly consistent with previous reports, because allergen fragments with MW different from those here identified were previously observed (Downs, Baumert, et al., 2016; Downs, Simpson, et al., 2016). Such discrepancy is likely an effect of the food matrix, which may increase or decrease the stability of allergen proteins, as already demonstrated for peanuts (Di Stasio et al., 2017; Di Stasio et al., 2020). Roasting critically affects the

allergen stability; in fact, beside Jug r 6, Jug r 2 e Jug n 1, fragments of other allergens such as legumin A-like, 11S globulin, legumin B-like and vicilin were detected as well in both soluble and insoluble fraction. It is plausible that, differently from hazelnuts and almonds, roasting potentiated the resistance of walnut proteins to proteases, as consequence of the heat-induced modifications (i.e. covalent modification, proteins aggregation, Maillard reaction) (Downs, Simpson, et al., 2016; Toda, Heilmann, Ilchmann, & Vieths, 2014; Vanga & Raghavan, 2017). The electrophoretic analysis of raw and roasted walnuts OGD-BBM digestion showed that large fragments (~6.5 kDa) were slightly affected by BBM enzymes. Although not detected in the insoluble fraction of walnut and hazelnut digesta, it cannot be excluded that high MW aggregates (> 100 kDa) were not extracted by the combined action of a chaotropic agent and the sonication (Sayers et al., 2016). Similarly, we cannot rule out that soluble peptides with low ionisation efficiency and carrying heat-induced modifications (i.e. Maillard) escaped the identification by MS/MS analysis. An appropriate pipeline of analysis for such difficult peptides is required.

Hazelnut allergens appeared to be more susceptible to gastrointestinal proteases compared to walnut allergens. Polypeptides derived from Cor a 11, Cor a 13 and Cor a 14 occurred in the soluble fraction of OGD digesta, though as barely detectable bands, congruently with previous results (Korte, Bräcker, & Brockmeyer, 2017). BBM affected the stability of hazelnut allergens, degrading them into peptides, which escaped the SDS-PAGE detection. After roasting, OGD digestion generated soluble Cor a 11 e Cor a 14 fragments, which were further degraded by BBM digestion. It is worth noting that, compared to almonds and walnuts, a lower number of hazelnut protein sequences is catalogued in the UniprotKB database, probably affecting the number of peptides identified.

An interesting trend was observed for almond allergens. After digestion, both OGD and OGD-BBM, no allergen was detectable by SDS-PAGE (De Angelis et al., 2018; Mandalari et al., 2014). Conversely, roasting induced the formation of insoluble high MW aggregates, which were identified as isoforms of Pru du 6 allergen and cupin family proteins (vicilin). However, these aggregates were hydrolysed by BBM and no longer detectable by SDS-PAGE.

Therefore, considering the results of the SDS-PAGE analysis alone, we could state that almond and hazelnut allergens are poorly stable to gastrointestinal digestion; whereas walnut allergens endure partly gastrointestinal hydrolysis. These assumptions would not take into consideration the intrinsic limitations of the applied electrophoretic analytical conditions, which is unable to detect low MW polypeptides (< 6.5 kDa, in the case in point). For this reason, the metabolic fate of peptides, which might include linear epitopes, has been poorly

investigated so far. Although a given allergen can be hydrolysed by gastric and intestinal proteases (disappearing from SDS-PAGE gel detection), it is likely that peptides deriving from digestion might be then stable to peptidases, thus preserving the immunogenic sequences. In the case of the gliadin proteins, although highly unstable to pepsin digestion, the resulting peptides (ranging between 1,000 and 5,000 Da) are resistant to intestinal peptidases and, therefore, capable of triggering innate and adaptive immune response in celiac disease subjects (Mamone et al., 2007). Though the etiopathology of celiac disease is different from that of IgE-mediated food allergy, the behaviour of gliadins highlights the importance of studying the peptide fraction in the digestive systems, in order to enlarge the inventory of polypeptides able to reach intact the gut epithelium and related immunocompetent tissue.

IgE-binding epitopes consist of a minimum of 9–15 amino acids (~0.9–1.5 kDa) (Naegeli et al., 2017). Bøgh and Madsen (2016) underlined that a minimum 30 amino acids (~3.5 kDa) corresponding to two epitopes are necessary for cross-linking with two antibody paratopes and triggering the allergic reaction. The *in silico* evaluation of the linear epitopes (continuous) surviving the gastrointestinal digestion has been the final focus of this work. A single conformational epitope is reported in IEDB.org for the tree nuts studied (Willison et al., 2013). Conformational epitopes (discontinuous) rely on the stability of the three-dimensional structure of the protein to be recognised and are highly susceptible of chemical and physical alteration. They are of particular interests for aero-allergy and skin contact allergy, while are of lower importance in food allergy as they are destabilised during food processing and gastrointestinal digestion.

LC-MS/MS analysis of digested tree nuts revealed a large number of peptide (with MW between 700 and 3500 Da), which survived the gastroduodenal and BBM digestion. Interestingly, we found that several peptides were potentially immunogenic since they encrypted linear IgE-binding sequences. However, the intensity of these peptides drastically decreases after roasting suggesting that thermal treatment might reduce the toxicity as also previously demonstrated for peanuts (Di Stasio et al., 2020). Noteworthy, roasting seems to affect differently the stability of IgE-binding epitopes of almond allergens, since stable peptides harbouring immunogenic sequences were released only after BBM digestion. To the best of our knowledge, a detailed inventory of peptides arising from digestion of walnuts and hazelnuts has been never compiled. Compared to previous studies about the peptidomic analysis of gastrointestinal digesta of almonds (De Angelis et al., 2018), our results highlight the presence of known immunogenic sequences that are released after hydrolysis with BBM endo- and exo-peptidases, probably by the action on the high MW aggregates trapped in the insoluble residue. Such discrepancy might be due to a different bioinformatic tool employed for database searching. This observation underlines the need of harmonizing the procedures of peptidomic analysis in order to render comparable the outcomes of different laboratories on similar food matrices.

5. Conclusion

The comprehensive characterization of protein digestomes is challenging due to many drawbacks. A clear prerequisite a successful characterization of a digestome is the efficient of extraction of protein and peptide targets from the food matrix. Herein, our observations about digestibility of tree nut proteins rely on extraction conditions including chaotropic agents and sonication, which are known to maximize the extraction of proteins and peptides from food matrices. The analysis of free amino acids and di-tripeptides present in the soluble fraction of raw and roasted digested tree nuts may contribute to assess conclusively the digestibility of differently treated (e.g. raw or roasted) protein sets.

Based on the evidence obtained from analysis of soluble and insoluble fractions of tree nut digesta, we can conclude that roasted tree

nuts are more digestible than the raw ones, also leading to the reduction or destruction of known epitopes of the most abundant allergens. Although digestion may reduce the immunogenicity of tree nut allergens, several peptides preserve intact IgE-binding sequences. To this regard, we point out how the characterization of food digests should not be limited to the high MW fragments (detectable by electrophoretic analysis), but rather also to small-to-medium sized polypeptides, which might harbour epitope sequences. Although immunogenicity is distinct from allergenic potential, the lack of comprehensive peptidomic characterization might in part explain why some highly labile food proteins are actually established food allergens. Food allergy is the multifactorial response to multiple individual and environmental factors. On the other hand, unquestionably, the digestion stability contributes to increase the probability that a food protein (or its derived peptide) can sensitize and elicit an adverse reaction, because in addition to the contact with skin and respiratory/oral mucosa, also the intestinal route of exposure to non-self antigens becomes accessible. It is therefore important the characterization of resistant peptides harbouring IgE-binding sequences, useful to design strategies for the prevention, management and treatment of food allergies.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2020.109597>.

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