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Comparative analysis of eliciting capacity of raw and roasted peanuts: the role of gastrointestinal digestion

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ABSTRACT

This study investigated the simultaneous impact of food matrix and processing on the food allergy eliciting capacity of peanuts in a physiologically relevant context. Whole raw and roasted peanuts 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. SDS-PAGE and HPLC analysis showed that roasting increased digestibility of peanuts and this trend was even more evident after BBM degradation. The eliciting properties of raw and roasted peanuts were assessed by Rat Basophil Leukemia assay in the presence of sera from peanut-allergic patients. As general features, the BBM digestion reduced allergenicity of roasted peanuts compared to the raw counterpart, suggesting that intestinal peptidases effectively contribute to further destroy specific domains of peanut allergens. These findings provide new and more realistic insights in the stability of peanut allergens within their natural matrix.

1. Introduction

Allergic reaction to peanuts is an important public health concern that affects an estimated 1% of young children and 0.6% of adults within the Western population, with an increasing prevalence (Sicherer & Sampson, 2010). Seed storage proteins are the triggering factor of the allergic immune response to peanut whose symptoms may range from mild reactions to life-threatening anaphylactic shock in susceptible subjects (Zhuang & Dreskin, 2013). Among sixteen peanut allergens catalogued by the Word Health Organization and International Union of Immunologic Societies Subcommittee (WHO/IUIS) (www.allergen.org) so far, Ara h 1, Ara h 3, Ara h 2 and Ara h 6 have been established as the most clinically relevant allergens (Palladino & Breiteneder, 2018).

Peanuts are often consumed after roasting in westernized countries, while boiling and frying are the most widespread processing methods in Asia, Africa and China (Boulay et al., 2008). Protein allergens may be differently affected by heat treatments, eventually leading to epitope destruction or to formation of IgE-binding neo-epitopes (Cabanillas et al., 2015; Rao, Tian, Fu, & Xue, 2018). In general, the heat-induced conformational changes might expose formerly hidden antigenic sites, thus altering the susceptibility to gastrointestinal digestion (Bavaro et al., 2018; De Angelis, Bavaro, Forte, Pilolli, & Monaci, 2018; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009; Sathe, Teuber, & Roux, 2005).

Previous studies demonstrated that the dry-roasting process, unlike boiling or frying, increases the IgE-binding to peanut (Beyer et al., 2001; Blanc et al., 2011; Maleki, Chung, Champagne, & Raufman, 2000; Zhang et al., 2018). Detailed investigation on purified allergens from roasted peanuts showed that heating differently affected the allergenic potential of individual allergens, reducing the degranulation capacity of Ara h 2 while significantly increasing that of Ara h 1 (Vissers et al., 2011). Taking into account the possible correlation between digestion stability and allergenic potential, the behaviour of peanut allergens during various *in vitro* digestion steps has been the subject of extensive studies, with controversial outcomes (Vissers, Wichers, & Savelkoul, 2012). Most of these studies have been carried out on purified or extracted allergens, highlighting the high resistance of Ara h 2 and Ara h 6 or, conversely, the great susceptibility of Ara h 1 and Ara h 3 to gastric and/or duodenal proteases (Bogh & Madsen, 2016; Koppelman, Hefle, Taylor, & de Jong, 2010; Pekar, Ret, & Untersmayr, 2018). However, this approach could be misleading, since the interaction of allergens

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with food matrix components naturally occurring in whole foodstuff (e.g. polysaccharides, lipids, other proteins, protease inhibitors) may greatly affect the dynamics of gastrointestinal digestion (Bogh et al., 2012; Vissers et al., 2012). In a previous study, we exploited the harmonized *in vitro* oral-gastro-duodenal digestion sequential model completed with a brush border membrane (BBM) step to track the metabolic fate of allergens in whole peanuts (Di Stasio et al., 2017). In this frame, proteomics and immunochemical assays provided novel information on the impact of food matrix on enzymatic degradation of peanuts, demonstrating that large fragments of Ara h 3 and small-size peptides of Ara h 1 – both undetected previously - survived the *in vitro* human digestion. Interestingly, resistant Ara h 3 fragments still harboured IgE-binding sequences, which survived unaltered (Di Stasio et al., 2017).

Generally, the immunogenic potential of peanut allergens has been primarily evaluated with ELISA and immunoblotting assays, using sera from patients suffering from IgE-mediated allergy to peanut (Yunginger et al., 2000). The elicitation properties of allergens have also been investigated with cellular assays based on the type I allergenic reaction, measuring the IgE cross-linking and binding to human high-affinity receptor for IgE (FcεRI) that lead to activation of mast cells and basophils (Knol, 2006). Expressing constitutively human α, β, and γ chains of FcεRI, humanized Rat Basophil Leukemia (RBL) cells are able to bind IgE from the sera of allergic individuals and are susceptible to activation in an allergen-specific manner (Wiegand et al., 1996). Humanized RBL cells have been exploited to evaluate peanut allergenicity, demonstrating that purified Ara h 2 and Ara h 6 were more potent triggers than Ara h 1 and Ara h 3 (Blanc et al., 2009).

The elicitation capacity of peanut allergens undergone simulated gastrointestinal digestion within their whole food matrix has not been investigated so far. We sought to assess the *in vitro* allergenicity of whole raw and roasted peanuts after simulated gastro-duodenal and BBM digestion, monitoring degradation of allergens by electrophoretic and chromatographic techniques and evaluating the effect of peanut digestion-resistant polypeptides by RBL degranulation test.

2. Materials and methods

2.1. Chemicals

Peeled raw and roasted peanut seeds (*cv Virginia*) were provided by Besana (Milano, Italy). Peanuts were roasted according to canonical industrial process: 170 °C for 15 min. Human salivary amylase, pepsin, trypsin, chymotrypsin, bile salts, Tris-HCl, urea, ammonium bicarbonate (Ambic), HPLC- grade solvents, phosphatidylcholine and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St Louis, MO, USA). BBM enzymes were purified from porcine jejunum as previously described 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 oral-gastroduodenal-BBM digestion of whole raw and roasted peanuts

Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions (Minekus et al., 2014). Ground raw and roasted peanuts (100 mg) were suspended in 207 μL SSF (pH 7.0) including human salivary amylase (1500 U/mL) to reproduce the oral phase. Subsequently, SGF including porcine pepsin (3300 U/mg, final concentration of 12 mg/mL) and egg lecithin liposomes (0.17 mM final concentration) were incorporated and pH was adjusted to 2.7. Simulated gastric digestion was left to occur 2 h at 37 °C. Afterwards, pH was raised to 7.0 using 1 N NaOH. To simulate the duodenal digestion, the gastric digest was diluted with SIF containing bile salts (10 mM in the final mixture, measured as cholic acid), bovine α-chymotrypsin (25 U/mL), porcine trypsin (100 U/mL TAME), pancreatic α-amylase (200 U/mL) and pancreatic lipase (2000 U/mL). Following incubation at 37 °C for 2 h, sample was further subjected to simulated small intestinal digestion with BBM enzymes (1.02 mU/μL peptidase activity), 4 h at 37 °C, after adjusting pH to 7.2. Reaction was stopped by 5 min immersion in a boiling water bath. Prior to biological assays, we verified by reversed phase (RP)-HPLC that the peptide profiles were not affected significantly by the heating treatment used to stop digestion.

2.3. Purification of soluble and insoluble digesta samples

After simulated gastrointestinal digestion, peanut digests were immediately centrifuged at 10.000*g* for 30 min. Supernatants (soluble fraction) and pellets (insoluble fraction) were separated and individually processed as follows:

(i) an aliquot of the soluble fraction was collected and stored at −20 °C for RP-HPLC analysis and RBL assay, whilst the remain sample was precipitated for 30 min in an ice cold bath with trichloroacetic acid (TCA) up to 30% (w/v) final concentration; the resulting pellet was washed with 1 mL of −20 °C cold acetone (three-fold) for removing TCA and finally analyzed by SDS-PAGE.

(ii) insoluble fraction was extracted with 1 mL Urea buffer (7 M Urea, 1 M Tris-HCl, pH 8.5) for 2 h at 37 °C; after centrifugation the supernatant was collected, while the pellet was further suspended in 1 mL 2% SDS, 20 mM DTT. Sample was incubated for 2 h at 37 °C and then centrifuged at 5000*g* for 40 min. Both Urea-and SDS-extract samples were analysed by SDS-PAGE.

2.4. SDS-PAGE

SDS-PAGE was performed on a Tetra-cell Mini-PROTEAN systems (Bio-Rad, Town, Country). Raw and roasted peanut digests were dissolved in 50 μL Laemli buffer (0.125 M Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 0.02% bromophenol blue) and 10 μL of the resulting solution was loaded onto precast 12% acrylamide gel (Bio-Rad). Electrophoresis was carried out under non-reducing conditions, omitting β-mercaptoethanol or dithiothreitol in the Laemli buffer. Urea and 2% SDS extracts of polypeptides were analysed using the same conditions. Undigested peanut proteins, extracted from raw and roasted ground peanut as described in Di Stasio et al. (2017), were run as reference controls. Protein bands were visualized with silver blue (Coomassie Brilliant Blue G-250) and digitalized using a LABScan scanner (Amersham Bioscience/GE Healthcare, Uppsala, Sweden). Protein bands were characterized by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) according to Di Stasio et al. (2017).

2.5. RP-HPLC

Soluble peptides arising from digestion of raw and roasted peanut were fractionated by RP-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with Diode Array Detector (DAD). Digests (50 μL) were diluted with 0.1% TFA and separated by C18 column (Aeris PEPTIDE, 3.6 μm, 250 × 2.10 mm i.d., Phenomenex, Torrance, CA, USA). Eluent A was 0.1% TFA (v/v) in Milli-Q water; eluent B was 0.1% TFA (v/v) in acetonitrile. The column was equilibrated at 10% B. Peptides were separated applying a linear 10–60% gradient of B over 60 min at a 0.2 mL/min flow rate. Chromatographic separation was performed at 37 °C, using a thermostatic column holder. The column effluent was monitored at 220 nm with DAD.

2.6. Evaluation of eliciting capacity of peanut digests by humanized RBL cells

Patient's sera were collected during a Clinical trial (NCT02384707) authorized by a local Ethical Committee (CPP Ouest II). Recruitment was based on a clear clinic history of immediate allergic reaction after

ingestion of peanut and skin prick-tests (Supplementary Table 1). Specific IgE concentration of each sera against either raw or roasted peanuts was determined by ELISA (Denery-Papini et al., 2012). Twenty sera with specific IgE concentration ranged between 600 and 6000 ng/mL, were selected and used to constitute a pool (Supplementary Table 2). IgE to Ara h 2 were determined for 18/20 patients using the ImmunoCAP method (Phadia, Uppsala, Sweden) (Supplementary Table 1). IgE to Ara h 2 values were always above the cut-off value of 5.17 kU/L that predicts a positive oral food challenge and confirmed the peanut allergy (Klemans et al., 2013).

Soluble polypeptides arising from oral-gastrointestinal digestion of raw and roasted peanut were desalted by C18 Sep-pak (Waters, Milford, MA, USA). Protein/peptide concentration was determined using the Lowry assay kit (Bio-Rad). The potential allergenicity was evaluated using a RBL cell line expressing human FcεRI (clone RBL-SX38), kindly provided by Pr Kinet (Harvard Medical School, New York, USA) (Lupi et al., 2018). Cells were sensitized with the pool of human sera for 48 h prior to stimulation in Tyrode buffer containing 50% deuterium oxide with whole protein extracts and digests (0.1–1–10–100–1000 ng/ml) or 1 ng/ml anti-human IgE antibody as positive control (clone Le27 mouse anti-human IgE-Fc Region Antibody; 500 ng/ml, NBS-C Bioscience, Vienne, Austria). Proteolytic enzymes and buffers were also tested to avoid potential interference with the RBL assay. RBL degranulation was monitored by measurement of total β-hexosaminidase release, as described previously (Bodinier et al., 2008). Samples and controls were analyzed in triplicate in three (OGD) or four (OGD-BBM) independent experiments and the results expressed as a percentage of the β-hexosaminidase release induced by the samples compared to the release observed with anti-human IgE antibody. Means of the percentages of each of the three independent experiments were used to build degranulation curves linking the percentage of degranulation and for peanut proteins concentration. Bars represent standard error (SEM) in the independent experiments. In this model, allergens generate dose–response curves that can be fitted with four-parameter equation where EC50 and maximum describe respectively the sensitivity and the reactivity of the sensitized basophils. These two parameters allows the comparison of triggering potency of allergens (Santos & Lack, 2016). GraphPad Prism 5.0 software was used to determine four parameters equations. Degranulation curves were compared by Extra sum-of square F test ($p < 0.05$).

3. Results

3.1. Gastrointestinal digestion of whole raw and roasted peanuts

Raw and roasted peanuts were processed according the workflow diagram shows in Fig. 1. Simulated digestion was carried out on whole peanuts both raw and roasted, in order to evaluate the stability of allergens embedded within their natural food matrix. Oral, gastric and duodenal phases were physiologically reproduced according to the Infogest protocol (Minekus et al., 2014). The oral gastric-duodenal digestion (OGD) workflow was also integrated with porcine jejunal BBM hydrolases (OGD-BBM) as already described in previous studies from our group (Di Stasio et al., 2017; Gianfrani et al., 2015; Mamone et al., 2007; Mamone et al., 2015; Picariello et al., 2015). We used BBM digestion aimed to simulate the final step of chyme breakdown occurring at the level of the intestinal epithelial barrier. Although there is not yet a consensus about their use, we adopted conditions (peptidase activity, pH and incubation time) similar to those that have demonstrated physiological correspondence (Shan et al., 2002). OGD and OGD-BBM digests were purified from lipids and salts prior to protein analysis and *in vitro* allergenicity assays.

3.2. Characterization of digests by electrophoresis and liquid chromatography (RP-HPLC)

Soluble polypeptides arising from simulated OGD and OGD-BBM digestion of peanuts were individually analyzed by SDS-PAGE under non reducing conditions (Fig. 2). Protein extracts from both raw and roasted peanuts were run as reference control (lane 1 and 8, respectively). As expected, the electrophoretic profile was drastically modified by digestion, since the major protein bands disappeared producing polypeptide fragments at lower molecular weight. Digests from OGD raw (lane 2, panel A) and roasted (lane 9, panel B) peanuts exhibited significant differences, especially in term of intensity of bands. These differences became more evident following digestion with BBM enzymes (lanes 3 and 10), with only a band at 21 kDa and a much fainter one at 14 kDa surviving the complete digestion of roasted peanuts, thereby indicating an increased susceptibility of peanut allergens after heating. In accordance with previous identifications (Di Stasio et al., 2017), LC-MS/MS-based analysis of the tryptic peptides arising from the digestion-resistant bands revealed that OGD-BBM of raw polypeptides ranging between 10 and 21 kDa were fragments of Ara h 2 and Ara h 6 as well as Ara h 3 fragments. The 21 and 14 kDa bands detected in OGD-BBM digests of roasted peanuts were fragments of Ara h 3 and Ara h 2/Ara h 6, respectively (Supplementary Fig. 1).

In order to assay the presence of possible insoluble protein aggregates trapped in the starchy matrix (Gianfrani et al., 2017), the pellet of both OGD and OGD-BBM digests from raw and roasted peanuts were sequentially extracted with Urea-containing (lanes 4–5 and 11–12) and SDS/reducing buffer (lanes 6–7 and 13–14). Nevertheless, no protein band was detected in these latter extracts by SDS-PAGE

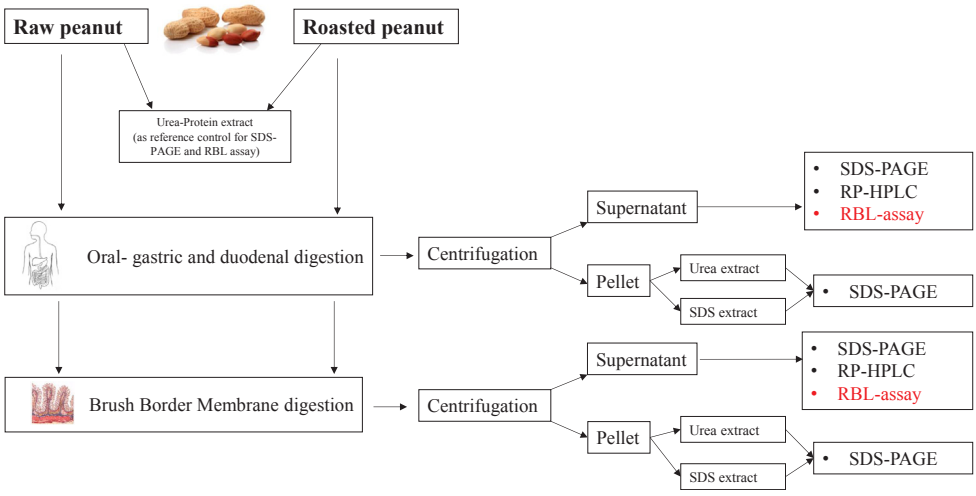


Fig. 1. Schematic workflow of the experimental approach employed for studying the digestibility and allergenicity of raw and roasted peanuts. Whole peanuts (without any pretreatment of kernels) were subjected to *in vitro* OGD and by OGD-BBM. After centrifugation, the soluble fractions (supernatant) were analyzed for eliciting allergen properties on RBL cells. Soluble fractions were also analyzed by SDS-PAGE and HPLC. The reside pellet of digesta samples were extracted by Urea and SDS buffer, and then analyzed by SDS-PAGE.

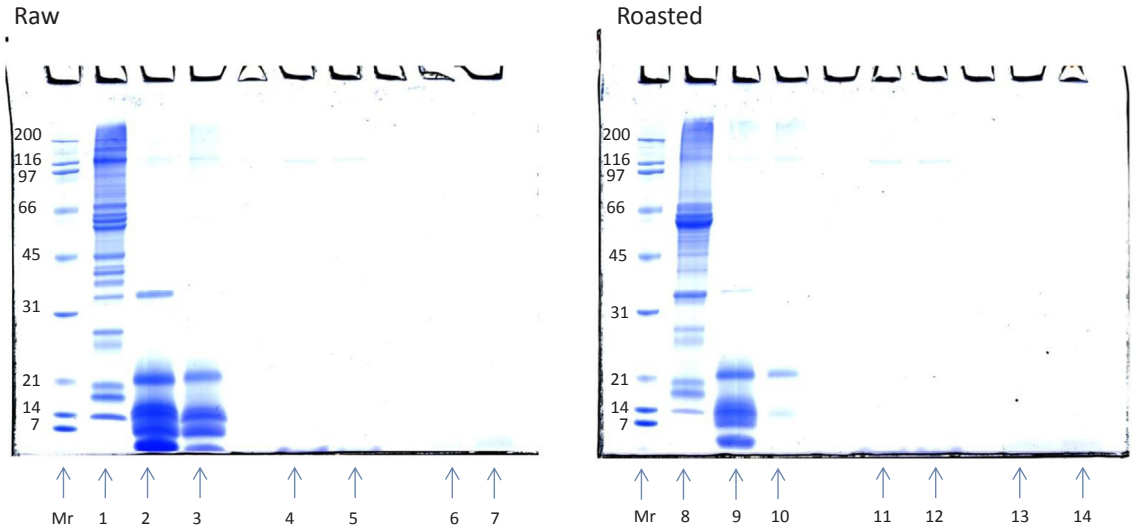


Fig. 2. Unreduced SDS-PAGE analysis of raw (panel A) and roasted (panel B) digesta peanuts. After centrifugation OGD and OGD-BBM supernatants were individually analysed. The residue pellets were subsequently extracted by 7 M Urea buffer followed by 2% SDS, 20 mM DTT. Lanes 2 and 9: supernatants of raw and roasted OGD peanuts; lanes 3 and 10 supernatants of raw and roasted OGD-BBM peanuts; lanes 4 and 11: Urea extract of pellets from raw and roasted OGD digestion; lanes 5 and 12: Urea extract of pellet from raw and roasted OGD-BBM digestion; lanes 6 and 13: SDS extract of pellet from raw and roasted OGD digestion; lanes 7 and 14: SDS extract of pellet from raw and roasted OGD-BBM digestion. Proteins extract from raw (lane 1) and roasted peanuts (lane 8), were run as reference controls.

analysis, indicating a substantially complete solubilization of hydrolyzed polypeptides, either in raw or roasted peanut digests.

In line with SDS-PAGE, the RP-HPLC analyses of soluble peptides arising from digestion confirmed the increased digestibility of roasted peanuts compared to the raw counterpart. Such a trend was even more evident after complete digestion, including the BBM degradation. In fact, the intensity of HPLC peaks clearly decreased either between raw and roasted peanuts or between OGD to OGD-BBM digests (Fig. 3).

3.3. Rat Basophil Leukemia assay

The RBL assay closely reflects the *in vivo* elicitation of an allergic reaction (Sun, Zhou, Zhou, Sun, & Che, 2015). Cells were sensitized with a pool of IgE from peanut-allergic patients. Only sera with comparable reactivity toward raw and roasted peanuts were selected (Supplementary Table 2). The pool of sera was then challenged by various dilutions of undigested proteins and digests from raw and roasted peanuts (protein concentration 1–1000 ng/mL range), as shown in Fig. 4. The cell response was determined monitoring the release of β-hexosaminidase as a marker of degranulation (Bodinier et al., 2008; Denery-Papini et al., 2012). Panel D of Fig. 4 displays the protein concentrations inducing 50% of the maximum release of mediator (EC50). No degranulation was observed when cells were challenged with control proteolytic enzymes or digestion buffers at the concentrations used for digestion (not shown).

A preliminary assay confirmed that undigested raw peanuts induced a release of β-hexosaminidase, which increased in parallel with the allergen concentrations. Roasting did not affect degranulation induced by undigested peanut proteins, since the EC50 difference observed between the reference sample (EC50 = 1.9 ng/mL) and the roasted one (EC50 = 2.8 ng/mL) was not statistically significant, indicating that the thermal treatment had no impact on the potential allergenicity of unhydrolyzed allergens (Fig. 4, panel A and D).

OGD digestion did not significantly change the degranulation capability of raw peanuts (Fig. 4, panel B and D). Similarly, the downstream BBM degradation had no detectable impact on the allergenicity of raw peanuts (Fig. 4, panel C and D). The degranulation induced by digested roasted peanuts showed an interesting trend: the remarkable increase of the EC50 value after OGD digestion (Fig. 4, panel B and D) was not confirmed by statistical analysis; conversely, the EC50 of the degranulation curves induced by OGD-BBM digests was significantly different from that induced by the raw counterpart (Fig. 4, panel C and D). Overall, these results indicated a reduction of allergenicity caused by digestion of roasted peanuts by BBM. In other terms, much higher concentrations of OGD-BBM roasted peanuts were required to induce an equivalent RBL degranulation, if compared to raw-OGD and roasted-OGD digests or unhydrolyzed peanut allergens.

4. Discussion

Many among the allergens share the common feature of stability to gastric and duodenal enzymes (Pekar et al., 2018). Although stability to digestion renders possible the contact of the intestinal mucosa with increased amounts of a food protein or derived immunoactive polypeptides, heat and digestion stability are poor predictors of the sensitization potential because skin, respiratory and oral mucosa are important routes of sensitization along with the intestinal tract (Herman & Ladics, 2018). On the other hand, elicitation of food allergy is expected to occur mostly through the gastrointestinal tract. Several physiologically relevant *in vitro* protocols have been devised to simulate human digestion for assessing the metabolic fate of allergens (Wickham, Faulks, & Mills, 2009). In this context, use of individual proteins (purified or recombinant form) makes it easier to track the pathway of degradation of allergens, but it precludes assessing the effect of the food matrix on protein digestibility.

An additional aspect that must be considered to understand the structural traits of food allergens at the molecular level is the impact of processing, especially thermal treatments that most foods undergo (Nowak-Wegrzyn & Fiocchi, 2009; Wickham et al., 2009). 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. Previous studies evaluated the “behaviour” of allergen exposed at high temperature and measured IgE-binding properties as well as the sensitization and elicitation capabilities. Even in this case, almost all investigations were limited to single purified allergens (Bogh & Madsen, 2016; Koppelman et al., 2010; Pekar et al., 2018). The stability of allergens within their natural matrix upon heat treatments as well as the elicitation properties of the resulting digestion products have been poorly explored (Prodic et al., 2018).

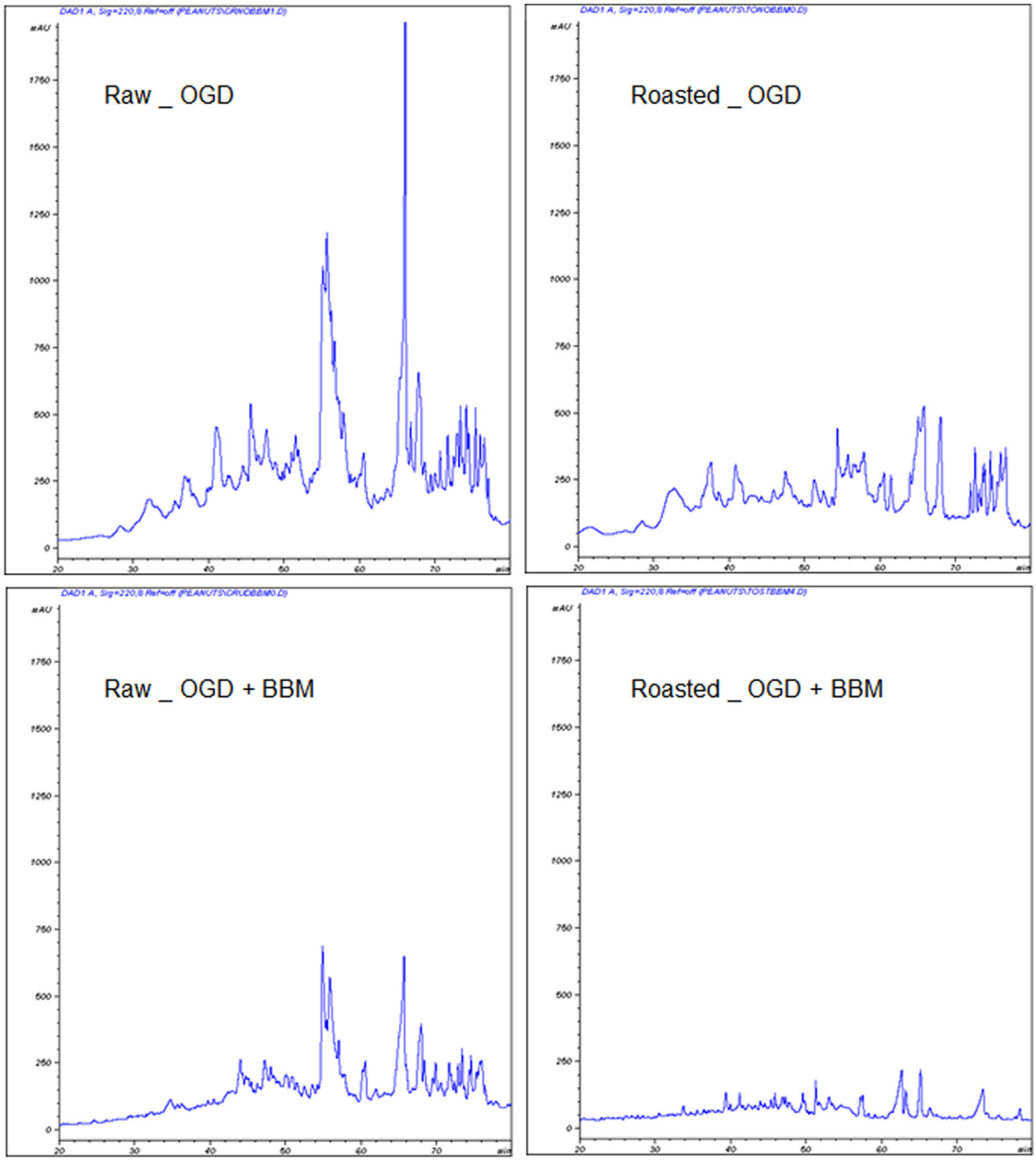
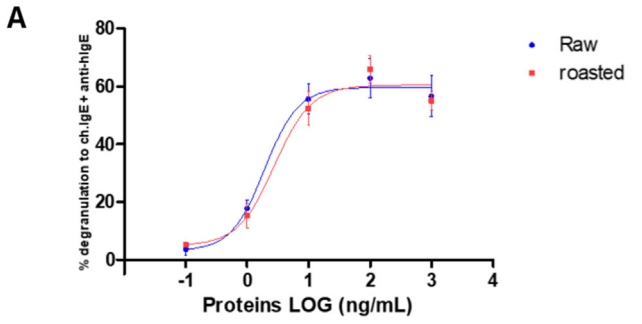
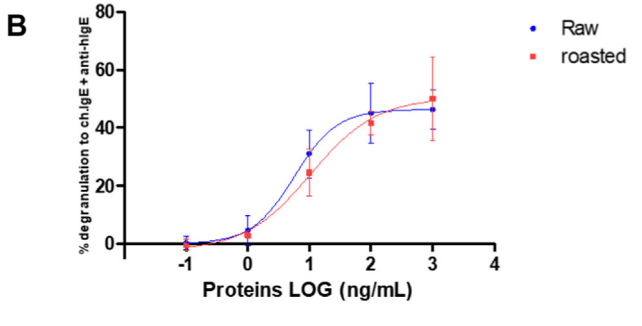


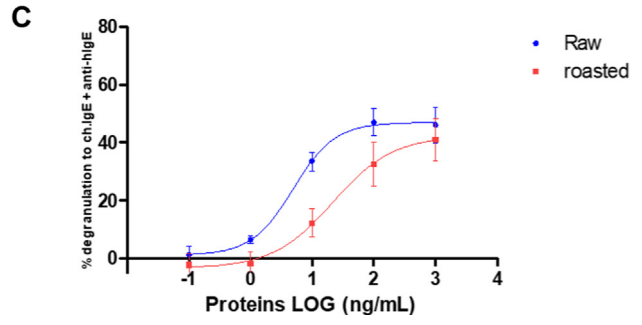
Fig. 3. HPLC comparison of soluble digested peanut proteins. Panel A and B: OGD digestion of raw and roasted peanuts respectively; panel C and D: OGD-BBM digestion of raw and roasted peanuts respectively.

Herein, we evaluated the stability to digestion and immunogenic potential of whole peanut allergens in a realistic context, taking into consideration the allergens-containing processed (roasted) food. To this purpose, we applied the *in vitro* digestion static model (Brodkorb et al., 2019; Minekus et al., 2014) (known as Infogest method). Since its first introduction in 2014, the Infogest (COST FA 1005 Infogest http://www.cost.eu/COST_Actions/fa/FA1005) method has been increasingly used, and it is now accepted as a physiologically relevant method suitable for determining the metabolic fate of a protein system (Egger et al., 2017). The Infogest protocol includes the oral, gastric and duodenal phase, while it omits mimicking the final physiological stage of peptide degradation occurring in the intestinal lumen prior to absorption. Enzymes involved in this last stage are those of the BBM which include a suite of oligopeptidases (endo- and *exo*-peptidses), lipases and oligosaccharidases. Together these enzymes tend to hydrolyze effectively those nutrient oligomers that survive the upstream digestion steps. BBM are mainly located on the surface of gut epithelium, but recent evidences also point at their release in the periapical space of enterocytes mediated by the biliopancreatic secretions (Hooton, Lentle, Monro, Wickham, & Simpson, 2015). Therefore, in order to evaluate the stability of peanut allergens in their natural matrix, we complemented the Infogest method with a BBM phase, using enzymatic condition previously assayed (Di Stasio et al., 2017; Mamone et al., 2015; Picariello, Ferranti, & Addeo, 2016) with demonstrated physiological consistence (Shan et al., 2002). However, since the use of BBM has not been harmonized so far, we assessed the stability to digestion either before (OGD) or after (OGD-BBM) incubation with BBM enzymes.

In line with previous characterizations (Di Stasio et al., 2017), the current results confirmed the digestion stability of large fragments of Ara h 3, Ara h 2 and Ara h 6 in raw peanuts. Electrophoretic and chromatographic analyses showed that roasting changed the susceptibility to gastrointestinal proteases and – even more strikingly - to BBM hydrolysis. In particular, only a major electrophoretic band, identified as an Ara h fragment 3 and a fainter one arising from Ara h 2/6,







D

	Undigested		OGD		OGD-BBM	
	Raw	*Roasted*	*Raw*	*Roasted*	*Raw*	*Roasted*
EC50% (ng/mL)	1.9	2.8 [a]	5.7	11	5.0 [b]	22 [a, b]
Max (%)	60%	60%	46%	51%	47%	42%

Fig. 4. Relative percentages of degranulation in RBL assay induced by OGD (panel B) and OGD-BBM (panel C) digested raw and roasted peanuts. Undigested raw and roasted peanuts (panel A) were used as reference control. Data represent the mean ± S.D. of four independent experiments. The Data points are mean of three (OGD) or four (OGD + BBM) independent determinations. Bars represent SEM. The EC50 values (as shown in panel D) were determined using GraphPad Prism 5.0. Data represent the mean ± SEM. [a, b] significantly different ($p < 0.05$).

survived to OGD-BBM digestion of roasted peanuts. Since heat treatment might cause a loss of protein solubility, an accurate investigation was carried out on the residual digestion pellets. In this case, no potentially harmful macro-peptide was detected, either in raw or roasted peanuts when the starchy matrix was sequentially extracted with chaotropic (by Urea) and SDS/reducing (by DTT) buffers, the latter particularly effective to extract large molecular polymers (Gupta, Khan, & Macritchie, 1993). Nevertheless, in general the careful extraction of digestion pellets with denaturing/reducing buffers, especially resulting from thermally treated food matrices, is strongly recommended when the effects of digests has to be assayed, in order to prevent possible loss of immunologically active polypeptides.

The effect of food processing and digestion on the allergenicity of peanut proteins was assessed by RBL assay, which provides predictive information on the ability of allergens to elicit an allergic reaction. In principle, the allergens exposed to heating (e.g. roasting, boiling, etc.) could contain epitopes with the capacity to target CD4+ T cells, but they may differently induce cross-linking of IgE and activate mast cells and basophils. Noteworthy, no significant differences were observed among undigested raw and roasted peanuts in terms of degranulation capability. Previous results demonstrated that Ara h 2/6 isoallergens were much more potent elicitors of basophil degranulation than Ara h 1. Also Ara h 3 has been reported to be a more potent allergen than Ara 1 (Blanc et al., 2009). Ara h 1 and Ara h 2 were contrariwise affected by thermal processes, since heating reduced the degranulation capacity of the Ara h 2/6 but significantly increased that of the Ara h 1 (Vissers et al., 2011). It is possible that the unchanged degranulation properties of undigested roasted compared to raw peanuts was the result of a balance of opposite effects on RBL, due to Ara h 2 / 6 decreased and Ara 1 increased activation.

A remarkable outcome of the current study was the confirmation that thermal treatments expose the peanut epitopes to gastrointestinal proteases, increasing the allergen degradation. RBL assay highlighted unequivocally that, the OGD digestion (according to the Infogest protocol) did not induce change in term of eliciting capacity of both raw or roasted peanuts. Noteworthy, the final step of degradation with BBM enzymes reduced the allergenicity of roasted peanuts, suggesting that BBM have a certain endoprotease activity and are effective to further destroy specific harmful protein fragments. Shorter peptides arising from OGD-BBM digestion of roasted peanuts exhibited a clearly diminished capability to crosslink IgE on basophils. In the case of raw peanuts, larger polypeptide fragments survived OGD digestion. For this reason, BBM enzymes had no evident effect because they mainly consist of peptidases hydrolyzing preferentially small-/medium-sized peptides (Woodley, 1994). The weak bands detected after OGD-BBM of the roasted peanuts are due to persistent fragments of Ara h 3 and Ara h 2/6, which, although lower, continue to exert an immunogenic effect. Our results are in partial agreement with those by Kroghsbo et al. (2014) who demonstrated that peanut roasting does not enhance allergenicity, though their investigations were carried out with rats (Kroghsbo et al., 2014). Although not strictly comparable with those obtained in animals due to the different experimental approach, our data support the conclusion by these authors in that whole roasted peanuts induced lower levels of RBL degranulation than extracts from roasted peanuts.

5. Conclusion

To the best of our knowledge, the eliciting properties of food allergens in their natural food matrix following the exposure to a process of real or simulated digestion have been never investigated before. The results of this study provide novel information about the relationship between structure and allergenicity of peanut proteins. To increase the physiological relevancy of our model we performed digestion on peeled peanut seeds, which are most largely consumed. On the other hand, digestibility of non-peeled peanuts as well as other tree-nuts (Mandalari et al., 2016) might be altered by the high content of polyphenols and other possible anti-nutritional factors (e. g. protease inhibitors) (Nepote, Grosso, & Guzman, 2002; Christman, Dean, Allen, Godinez, & Toomer, 2019).

Thermal processing drastically affects food protein structure and, hence, digestibility of food proteins. In turn, allergenicity significantly varies depending on the specific nature of the digests. In the case of peanuts, only digestion including the BBM stage appeared to destroy most of the epitopes of roasted peanuts, resulting in significantly lower RBL degranulation. Conversely, allergens of raw peanuts retained the

RBL degranulation potential due to an unmodified protein conformation and their relative resistance to digestion. These observations support on a molecular basis the importance of food processing and suggest molecular targets and technological strategies aimed to minimize the allergenicity of food. The digestion limited to the OGD stages did not evidence any differences in term of eliciting capacity. This finding emphasizes the importance of including the stage with BBM enzymes in the harmonized model of *in vitro* digestion as also indicated by Minekus et al. (2014) and Brodkorb et al. (2019). The *in vitro – in vivo* physiological correspondence should be validated in any case.

In perspective, it would be interesting to compare the eliciting properties of digested roasted peanuts to those of digested boiled or fried or autoclaved peanuts, in order to establish how different thermal processing may affects stability to digestion and allergenicity. To this purpose many factors should be taken into account such as the partial release of allergens in the boiling water, which can contribute to explain the reduced allergenicity of boiled peanuts.

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Declaration of Competing Interest

There are no conflicts to declare

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108758.

References

Bavaro, S. L., Di Stasio, L., Mamone, G., De Angelis, E., Nocerino, R., Canani, R. B., ... Monaci, L. (2018). Effect of thermal/pressure processing and simulated human digestion on the immunoreactivity of extractable peanut allergens. *Food Research International, 109*, 126–137. https://doi.org/10.1016/j.foodres.2018.04.021.

Beyer, K., Morrow, E., Li, X. M., Bardina, L., Bannon, G. A., Burks, A. W., & Sampson, H. A. (2001). Effects of cooking methods on peanut allergenicity. *Journal Allergy Clinical Immunology, 107*(6), 1077–1081. https://doi.org/10.1067/mai.2001.115480.

Blanc, F., Adel-Patient, K., Drumare, M. F., Paty, E., Wal, J. M., & Bernard, H. (2009). Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors. *Clinical & Experimental Allergy, 39*(8), 1277–1285. https://doi.org/10.1111/j.1365-2222.2009.03294.x.

Blanc, F., Vissers, Y. M., Adel-Patient, K., Rigby, N. M., Mackie, A. R., Gunning, A. P., ... Mills, E. N. (2011). Boiling peanut Ara h 1 results in the formation of aggregates with reduced allergenicity. *Molecular Nutrition and Food Research, 55*(12), 1887–1894. https://doi.org/10.1002/mnfr.201100251.

Bodinier, M., Brossard, C., Triballeau, S., Morisset, M., Guerin-Marchand, C., Pineau, F., ... Denery-Papini, S. (2008). Evaluation of an in vitro mast cell degranulation test in the context of food allergy to wheat. *International Archives of Allergy Immunology, 146*(4), 307–320. https://doi.org/10.1159/000121465.

Bogh, K. L., & Madsen, C. B. (2016). Food allergens: Is there a correlation between stability to digestion and allergenicity? *Critical Reviews in Food Science and Nutrition, 56*(9), 1545–1567. https://doi.org/10.1080/10408398.2013.779569.

Bogh, K. L., Nielsen, H., Madsen, C. B., Mills, E. N., Rigby, N., Eiwegger, T., ... Roggen, E. L. (2012). IgE epitopes of intact and digested Ara h 1: A comparative study in humans and rats. *Molecular Immunology, 51*(3–4), 337–346. https://doi.org/10.1016/j.molimm.2012.04.002.

Boulay, A., Houghton, J., Gancheva, V., Sterk, Y., Strada, A., Schlegel-Zawadzka, M., ... Rowe, G. (2008). A EuroPrevall review of factors affecting incidence of peanut allergy: Priorities for research and policy. *Allergy, 63*(7), 797–809. https://doi.org/10.1111/j.1398-9995.2008.01776.x.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Balance, S., ... Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Cabanillas, B., Cuadrado, C., Rodriguez, J., Hart, J., Burbano, C., Crespo, J. F., & Novak, N. (2015). Potential changes in the allergenicity of three forms of peanut after thermal processing. *Food Chemistry, 183*, 18–25. https://doi.org/10.1016/j.foodchem.2015.03.023.

Christman, L. M., Dean, L. L., Allen, J. C., Godinez, S. F., & Toomer, O. T. (2019). Peanut skin phenolic extract attenuates hyperglycemic responses in vivo and in vitro. *PLoS One, 14*(3), e0214591. https://10.1371/journal.pone.0214591.

De Angelis, E., Bavaro, S. L., Forte, G., Pilolli, R., & Monaci, L. (2018). Heat and pressure treatments on almond protein stability and change in immunoreactivity after simulated human digestion. *Nutrients, 10*(11), pii: E1679. https://doi.org/10.3390/nu10111679.

Denery-Papini, S., Bodinier, M., Larre, C., Brossard, C., Pineau, F., Triballeau, S., ... Moneret-Vautrin, D. A. (2012). Allergy to deamidated gluten in patients tolerant to wheat: Specific epitopes linked to deamidation. *Allergy, 67*(8), 1023–1032. https://doi.org/10.1111/j.1398-9995.2012.02860.x.

Di Stasio, L., Picariello, G., Mongiello, M., Nocerino, R., Berni Canani, R., Bavaro, S., ... Mamone, G. (2017). Peanut digestome: Identification of digestion resistant IgE binding peptides. *Food and Chemical Toxicology, 107*(Pt A), 88–98. https://doi.org/10.1016/j.fct.2017.06.029.

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., ... Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Research International, 102*, 567–574. https://doi.org/10.1016/j.foodres.2017.09.047.

Gianfrani, C., Camarca, A., Mazzarella, G., Di Stasio, L., Giardullo, N., Ferranti, P., ... Mamone, G. (2015). Extensive in vitro gastrointestinal digestion markedly reduces the immune-toxicity of Triticum monococcum wheat: Implication for celiac disease. *Molecular Nutrition and Food Research, 59*(9), 1844–1854. https://doi.org/10.1002/mnfr.201500126.

Gianfrani, C., Mamone, G., la Gatta, B., Camarca, A., Di Stasio, L., Maurano, F., ... Di Luccia, A. (2017). Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease. *Food and Chemical Toxicology, 101*, 105–113. https://doi.org/10.1016/j.fct.2017.01.010.

Gupta, R. B., Khan, K., & Macritchie, F. (1993). Biochemical basis of flour properties in bread wheats. I. Effects of variation in the quantity and size distribution of polymeric protein. *Journal of Cereal Science, 18*(1), 23–41. https://doi.org/10.1006/jcrs.1993.1031.

Herman, R. A., & Ladics, G. S. (2018). Allergenic sensitization versus elicitation risk criteria for novel food proteins. *Regulatory Toxicology and Pharmacology, 94*, 283–285. https://doi.org/10.1016/j.yrtph.2018.02.016.

Hooton, D., Lentle, R., Monro, J., Wickham, M., & Simpson, R. (2015). The secretion and action of brush border enzymes in the mammalian small intestine. *Reviews of Physiology Biochemistry and Pharmacology, 168*, 59–118. https://doi.org/10.1007/112_2015_24.

Klemans, R. J., Otte, D., Knol, M., Knol, E. F., Meijer, Y., Gmelig-Meyling, F. H., ... Pasmans, S. G. (2013). The diagnostic value of specific IgE to Ara h 2 to predict peanut allergy in children is comparable to a validated and updated diagnostic prediction model. *Journal of Allergy and Clinical Immunology, 131*(1), 157–163. https://doi.org/10.1016/j.jaci.2012.08.010.

Knol, E. F. (2006). Requirements for effective IgE cross-linking on mast cells and basophils. *Molecular Nutrition and Food Research, 50*(7), 620–624. https://doi.org/10.1002/mnfr.200500272.

Koppelman, S. J., Hefle, S. L., Taylor, S. L., & de Jong, G. A. (2010). Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: A comparative in vitro study and partial characterization of digestion-resistant peptides. *Molecular Nutrition and Food Research, 54*(12), 1711–1721. https://doi.org/10.1002/mnfr.201000011.

Kroghsbo, S., Rigby, N. M., Johnson, P. E., Adel-Patient, K., Bogh, K. L., Salt, L. J., ... Madsen, C. B. (2014). Assessment of the sensitizing potential of processed peanut proteins in Brown Norway rats: Roasting does not enhance allergenicity. *PLoS One, 9*(5), e96475. https://doi.org/10.1371/journal.pone.0096475.

Lupi, R., Denery-Papini, S., Claude, M., Tranquet, O., Drouet, M., Masci, S., & Larré, C. (2018). Thermal treatment reduces gliadin recognition by IgE, but a subsequent digestion and epithelial crossing permits recovery. *Food Research International, 118*, 22–31. https://doi.org/10.1016/j.foodres.2018.02.011.

Maleki, S. J., Chung, S. Y., Champagne, E. T., & Raufman, J. P. (2000). The effects of roasting on the allergenic properties of peanut proteins. *Journal of Allergy and Clinical Immunology, 106*(4), 763–768. https://doi.org/10.1067/mai.2000.109620.

Mamone, G., Ferranti, P., Rossi, M., Roepstorff, P., Fierro, O., Malorni, A., & Addeo, F. (2007). Identification of a peptide from alpha-gliadin resistant to digestive enzymes: Implications for celiac disease. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 855*(2), 236–241 [Epub 2007 May 18. PubMed PMID: 17544966.].

Mamone, G., Nitride, C., Picariello, G., Addeo, F., Ferranti, P., & Mackie, A. (2015). Tracking the fate of pasta (T. Durum semolina) immunogenic proteins by in vitro simulated digestion. *Journal of Agricultural and Food Chemistry, 63*(10), 2660–2667. https://doi.org/10.1021/jf505461x.

Mandalari, G., Vardakou, M., Faulks, R., Bisignano, C., Martorana, M., Smeriglio, A., & Trombetta, D. (2016). Food matrix effects of polyphenol bioaccessibility from almond skin during simulated human digestion. *Nutrients, 8*(9), https://doi.org/10.3390/nu8090568 568/1-568/17.

Mills, E. N., Sancho, A. I., Rigby, N. M., Jenkins, J. A., & Mackie, A. R. (2009). Impact of food processing on the structural and allergenic properties of food allergens. *Molecular Nutrition and Food Research, 53*(8), 963–969. https://doi.org/10.1002/mnfr.200800236.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an

international consensus. *Food and Function, 5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j.

Nepote, V., Grosso, N. R., & Guzman, C. A. (2002). Extraction of antioxidant components from peanut skins. *Grasas y aceites, 53*(4), 391–395.

Nowak-Wegrzyn, A., & Fiocchi, A. (2009). Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Current Opinion in Allergy Clinical Immunology, 9*(3), 234–237. https://doi.org/10.1097/ACI.0b013e32832b88e7.

Palladino, C., & Breiteneder, H. (2018). Peanut allergens. *Molecular Immunology, 100*, 58–70. https://doi.org/10.1016/j.molimm.2018.04.005.

Pekar, J., Ret, D., & Untersmayr, E. (2018). Stability of allergens. *Molecular Immunology, 100*, 14–20. https://doi.org/10.1016/j.molimm.2018.03.017.

Picariello, G., Miralles, B., Mamone, G., Sanchez-Rivera, L., Recio, I., Addeo, F., & Ferranti, P. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition and Food Research, 59*(5), 948–956. https://doi.org/10.1002/mnfr.201400856.

Picariello, G., Ferranti, P., & Addeo, F. (2016). Use of brush border membrane vesicles to simulate the human intestinal digestion. *Food Research International, 88*, 327–335. https://doi.org/10.1016/j.foodres.2015.11.002.

Prodic, I., Stanic-Vucinic, D., Apostolovic, D., Mihailovic, J., Radibratovic, M., Radosavljevic, J., ... Cirkovic Velickovic, T. (2018). Influence of peanut matrix on stability of allergens in gastric-simulated digesta: 2S albumins are main contributors to the IgE reactivity of short digestion-resistant peptides. *Clinical & Experimental Allergy, 48*(6), 731–740. https://doi.org/10.1111/cea.13113.

Rao, H., Tian, Y., Fu, W., & Xue, W. (2018). In vitro digestibility and Immunoreactivity of thermally processed peanut. *Food and Agricultural Immunology, 29*(1), 989–1001. https://doi.org/10.1080/09540105.2018.1499710.

Santos, A. F., & Lack, G. (2016). Basophil activation test: Food challenge in a test tube or specialist research tool? *Clinical Translational Allergy, 6*, 10. https://doi.org/10.1186/s13601-016-0098-7.

Sathe, S. K., Teuber, S. S., & Roux, K. H. (2005). Effects of food processing on the stability of food allergens. *Biotechnology Advances, 23*(6), 423–429. https://doi.org/10.1016/j.biotechadv.2005.05.008.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., ... Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. *Science, 297*(5590), 2275–2279. https://doi.org/10.1126/science.1074129.

Sicherer, S. H., & Sampson, H. A. (2010). Food allergy. *Journal of Allergy and Clinical Immunology, 125*(2 Suppl 2), S116–S125. https://doi.org/10.1016/j.jaci.2009.08.028.

Sun, N., Zhou, C., Zhou, X., Sun, L., & Che, H. (2015). Use of a rat basophil leukemia (RBL) cell-based immunological assay for allergen identification, clinical diagnosis of allergy, and identification of anti-allergy agents for use in immunotherapy. *Journal of Immunotoxicology, 12*(2), 199–205. https://doi.org/10.3109/1547691X.2014.920063.

Vissers, Y. M., Iwan, M., Adel-Patient, K., Stahl Skov, P., Rigby, N. M., Johnson, P. E., ... Wichers, H. J. (2011). Effect of roasting on the allergenicity of major peanut allergens Ara h 1 and Ara h 2/6: The necessity of degranulation assays. *Clinical & Experimental Allergy, 41*(11), 1631–1642. https://doi.org/10.1111/j.1365-2222.2011.03830.x.

Vissers, Y. M., Wichers, H. J., & Savelkoul, H. F. J. (2012). *Influence of food processing, digestion and the food matrix on allergenicity & cellular measures of allergenicity Multidisciplinary approaches to allergies.* Springer203–227.

Wickham, M., Faulks, R., & Mills, C. (2009). In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Molecular Nutrition and Food Research, 53*(8), 952–958. https://doi.org/10.1002/mnfr.200800193.

Wiegand, T. W., Williams, P. B., Dreskin, S. C., Jouvin, M. H., Kinet, J. P., & Tasset, D. (1996). High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. *The journal of immunology, 157*(1), 221–230.

Yunginger, J. W., Ahlstedt, S., Eggleston, P. A., Homburger, H. A., Nelson, H. S., Ownby, D. R., ... Zeiger, R. S. (2000). Quantitative IgE antibody assays in allergic diseases. *Journal of Allergy and Clinical Immunology, 105*(6 Pt 1), 1077–1084. https://doi.org/10.1067/mai.2000.107041.

Zhang, T., Shi, Y., Zhao, Y., Tang, G., Niu, B., & Chen, Q. (2018). Boiling and roasting treatment affecting the peanut allergenicity. *Annals of Translational Medicine, 6*(18), 357. https://doi.org/10.21037/atm.2018.05.08.

Zhuang, Y., & Dreskin, S. C. (2013). Redefining the major peanut allergens. *Immunologic 486 Research, 55*(1–3), 125–134. https://doi.org/10.1007/s12026-012-8355-x.