Università degli Studi di Napoli "Federico II"
Dipartimento di Agraria

DOTTORATO DI RICERCA IN SCIENZE AGRARIE E AGROALIMENTARI
XXXI ciclo

Intolerances and food allergies: assessment of the stability of allergenic proteins to gastrointestinal digestion

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2017/2018

A me stessa e a Dino,

sempre INSIEME nel raggiungimento dei traguardi più importanti della nostra vita.

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Overview of complete PhD training activities and list of publication

List of publication

Very related to PhD thesis

- **Di Stasio L**, Picariello G, Mongiello M, Nocerino R, Berni Canani R, Bavaro S, Monaci L, Ferranti P, Mamone G. Peanut digestome: Identification of digestion resistant IgE binding peptides. Food Chem Toxicol. 2017 Jun 17; 107(Pt A):88-98.

- Bavaro S.L, **Di Stasio L**, Mamone G, De Angelis E, Nocerino R, Berni Canani R, Logrieco AF, Montemurro N, Monaci L. Effect of thermal/pressure processing and simulated human digestion on the immunoreactivity of extractable peanut allergens, Food Res Int . July 2018 109:126-137.

- Mamone G, **Di Stasio L**, De Caro S, Picariello G, Nicolai MA, Ferranti P. Comprehensive analysis of the peanut allergome combining 2-DE gel-based and gel-free proteomics. https://doi.org/10.1016/j.foodres.2018.09.045

- Iacomino G, **Di Stasio L**, Fierro O, Picariello G, Venezia A, Gazza L, Ferranti P, Mamone G. Protective effects of ID331 Triticum monococcum gliadin on in vitro models of the intestinal epithelium. Food Chem. 2016 Dec 1; 212:537-42.

- Gianfrani C, Mamone G, la Gatta B, Camarca A, **Di Stasio L**, Maurano F, Picascia S, Capozzi V, Perna G, Picariello G, Di Luccia A. Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease. Food Chem Toxicol. 2017 Mar; 101:105-113.

- **Di Stasio L**, Tranquet O, Picariello G, Ferranti P, Denery-Papini S, Mamone G. Eliciting capacity of gastrointestinal digests from raw and roasted peanuts by Rat Basophil Leukemia cell-based assay. *Submitted 2018*

- **Di Stasio L**, Picariello G, Ferranti P, Mamone G. Assessment of the metabolic fate of tree nut allergens: the role of intestinal Brush Border Membrane digestive enzymes and food processing. *Submitted 2018*

Less related to PhD thesis

- **Di Stasio, L.,** 2019. Novel Foods: Allergens. In: Ferranti, P., Berry, E.M., Anderson, J.R. (Eds.), Encyclopedia of Food Security and Sustainability, vol. 1, pp. 393–398. Elsevier.

- **Di Stasio, L.,** 2019. New protein Sources: Novel Foods. In: Ferranti, P., Berry, E.M., Anderson, J.R. (Eds.), Encyclopedia of Food Security and Sustainability, vol. 1, pp. 276–279. Elsevier

- Arena S, D'Ambrosio C, Vitale M, Mazzeo F, Mamone G, **Di Stasio L**, Maccaferri M, Curci PL, Sonnante G, Zambrano N, Scaloni A. Differential representation of albumins and globulins during grain development in durum wheat and its possible functional consequences. J Proteomics. 2017 Jun 6; 162:86-98.

- Mazzeo MF, **Di Stasio L**, D'Ambrosio C, Arena S, Scaloni A, Corneti S, Ceriotti A, Tuberosa R, Siciliano RA, Picariello G, Mamone G. Identification of Early Represented Gluten Proteins during Durum Wheat Grain Development. J Agric Food Chem. 2017 Apr 19; 65(15):3242-3250.

- Picariello G, **Di Stasio L**, Mamone G, Iacomino G, Venezia A, Iannaccone N, Ferranti P, Coppola R, Addeo F. Identification of enzyme origin in dough improvers: DNA-based and proteomic approaches. Food Res Int. 2018 Mar; 105: 52-58.

- De Cicco M, Siano F, Iacomino G, Iannaccone N, **Di Stasio L**, Mamone G, Volpe MG, Ferranti P, Addeo F, Picariello G. Multianalytical Detection of Pig-Derived Ingredients in Bread. *Food Anal Methods* https://doi.org/10.1007/s12161-018-01410-6

- Giammarioli S, Boniglia C, Di Stasio L, Gargiulo R, Mosca M, Carratù B. Phytosterols in supplements containing Serenoa repens: an example of variability of active principles in commercial plant based products. Nat Prod Res. 2018 Oct 8:1-5. doi: 10.1080/14786419.2018.1490910

Participation in research project

- *Safe & Smart Nuove tecnologie abilitanti per la food safety e l'integrità delle filiere agroalimentari in uno scenario globale - National CL.AN Cluster agroalimentare nazionale programma area 2 (MIUR CTN01_00230_248064).*

- *Progetto Bandiera "InterOmics" Sviluppo di una piattaforma integrata per l'applicazione delle scienze "omiche" alla definizione dei biomarcatori e profili diagnostici, predittivi, e teranostici (MIR-CNR)*

Communications in congress

- **Luigia Di Stasio**. Intolerances and food allergies: assessment of the stability of allergenic proteins to gastrointestinal digestion. XXIII workshop on the developments in the Italian PhD Research on Food Science, Technology and Biotechnology. 19-21 September 2018, Oristano, Italy
- **Di Stasio L**., De Caro S, Picariello G, Nicolai MA.,Ferranti P., Mamone G. Resolving the complexity of peanut allergome using 2DE gel based and gel free proteomic analysis. ImpARAS 4rd International Conference, 19 – 21 June 2018, Portici (NA) Italy
- Oral Presentation of STSM project : Comparison of the digestibility and antigenicity of raw and roasted whole peanut allergens in the ImpARAS 3rd International Conference, 10-12 October 2017, Helsingør Denmark
- **Luigia Di Stasio**. Peanut digestome: identification of digestion resistant IgE binding peptides. XXII workshop on the developments in the Italian PhD research on Food Science, Technology and Biotechnology. 20-22 September 2017 Bolzano, Italy
- Oral Presentation of PhD project at IUBMB Advanced School "A molecular view of the food-health relationship, May 15-19 2017, Spetses (Greece)
- **Di Stasio L**., Ferranti P., Picariello G., Tranquet O., Pineau F., De Carvalho M., Denery-Papini S., Mamone G. Comparison of the digestibility and antigenicity of raw and roasted whole peanut allergens. INFOGEST 5° International Conference, 04-06 April 2017, Rennes France

Awards

- 1st position in the contest of "Best poster presented by PhD Student" at 5° International Conference of INFOGEST COST ACTION, 04-06 April 2017, Rennes France. Title: Comparison of the digestibility and antigenicity of raw and roasted whole peanut allergens.

- 3rd position in the contest of "What for" at XXIII workshop on the developments in the Italian PhD research on Food Science, Technology and Biotechnology; 19-21 September 2018 Oristano, Italy

Scientific mission

• *University of Manchester : Division of Infection, Immunity & Respiratory Medicine| School of Biological Sciences I Faculty of Biology, Medicine and Health, Manchester Institute of Biotechnology ,John Garside Building 131 Princess Street ,Manchester M1 7DN, England.*
Project: Defining effects of thermal processing on peanut allergens using mass spectrometry
Supervisor: Prof. Clare Mills
From 01/11/2017 to 30/04/2018

• *Institut National de la Recherche Agronomique (INRA), Unité 1268 BIA, Allergy team, Rue de la Geraudière, Nantes, France*
Project: RBL assay to evaluate the antigenicity of raw and roasted whole peanut allergens.
Supervisor: Drs. Sandra Denery-Papini
From 25/09/2016 to 14/10/2016

Chapter 1

Food allergy and intolerances: a general overview

Abbreviations

AGE	Advanced glycation products
BAT	Basophil activation test
BBM	Brush Border Membrane
CD	Celiac disease
ELISA	Enzyme-linked immunosorbent assay
FcƐRI	IgE receptor
HLA	Human Leukocyte Antigens
IgE	immunoglobulin E
IL	Interleukins
MHC	Major Histocompatibility Complex
MR	Maillard Reaction
MRPs	Maillard reaction products
RBL	Rat Basophilic Leukemia assay
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SPT	Skin Prick test
SSF	Simulated Salivary Fluid
TCR	T cell recptor
Th2	T helper 2 cells
tTGase	tissue transglutaminase
WAO	World Allergy Organization

1. Introduction

1.1 Food allergy and intolerance: a general overview

Food is indispensable to provide energy and nutrients for maintaining the body in an optimum state of health. However, some individuals can undergo adverse physiological and neurological reactions after ingestion of foods that are well-known to be safe for consumption by the majority of the population. The awareness that certain foods are considered as a cause of adverse reactions in sensitive individuals traces back to ancient times. Hippocrates (460—370 BC), for example, reported that cow's milk could cause gastric disorder and urticaria. Galen (131—210 BC) described a case of intolerance to goat's milk (Petruláková & Valík, 2015). Although there are several historical references regarding cases of food allergy and intolerances, in the past these were considered as minor health problems. Only in last decade of 20th century, scientific research has started to focus on food adverse reactions.

Recently, the European Academy of Allergy and Clinical Immunology task force published a revised nomenclature for adverse food reaction (Bruijnzeel-Koomen *et al.*, 1995) (***Figure 1***). F*ood hypersensitivities* take account of adverse reaction after the ingestion of a food and might be the outcome of food intolerances (non-allergic food hypersensitivities) or food allergy (allergic food hypersensitivity). The terms food allergy and food intolerance are often mixed and there is some confusion regarding as to how they diverge. Allergies are an immediate hypersensitivity reaction due to the exposure to specific proteins (allergens). They are commonly mediated by a specific class of antibodies, known as immunoglobulin E (IgE). In contrast, food intolerances are non-immune-mediated reactions and symptoms could take days to manifest themselves. They are adverse

responses caused by metabolic disability (e.g. lactase deficiency). They do not have an immunological origin although coeliac disease, defined as food intolerances triggered by gluten proteins, is mediated by the immune system (Mills & Breiteneder, 2005).

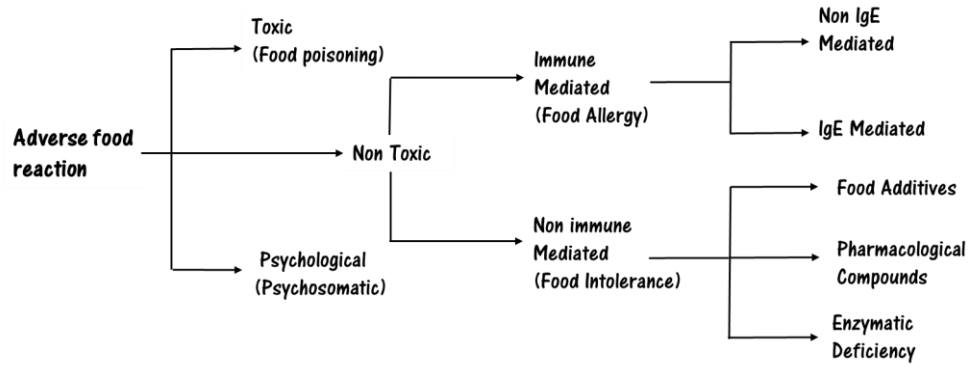

Figure 1*. Schematic workflow of adverse reactions to Food EAACI classification (Bruijnzeel-Koomen et al., 1995).*

For this reason, it is appropriate to explain and describe in a separate way in which some foods trigger, in susceptible individuals, different adverse reactions depending on whether it is food allergy or intolerance.

1.2. Food Allergy

As proposed from the World Allergy Organization (WAO) in 2003, allergy is defined as a hypersensitivity reaction with clinical symptoms triggered by immunological mechanisms, which is started by an IgE- or non-IgE-mediated response due to several environmental factors, normally well tolerated by healthy individuals (Mamone *et al.*, 2011). Referring to IgE-

mediated allergic reactions, the term allergen indicates an antigen triggering an allergic response by IgE binding. Two types of IgE-binding epitopes, linear and conformational, could occur in food allergens. Conformational (or incomplete) epitopes are a group of amino acid residues brought together by protein folding. In contrast, linear epitopes are constituted by a stretch of continuous amino acids, sufficient for the IgE binding (Forsstrom *et al.*, 2015). However, to induce an allergenic reaction an epitope should contain at least two high-affinity IgE binding sites in order to cross-link the IgE antibodies immobilized on the mast cell surface (Bannon, 2004)

Food allergens are classified as either type 1 o type 2 depending on their immunological and biochemical properties. Type 2 allergens are define as "partial allergens". Reactivity to these food allergens often is a consequence of a primary sensitization by aeroallergens (e.g., pollens). The successive ingestion of foods containing proteins homologous to the sensitizing allergen provokes an IgE-mediated reaction, often mild and restricted to the oral cavity. The major food allergens identified as Type 1 allergens are water-soluble (glyco)-proteins that are 10 to 70 kDa and stable to heat, acid and proteases (Sicherer, 2002; Sicherer & Sampson, 2006). Type 1 allergens are known as complete food allergens as they both sensitize the patient, through the gastrointestinal tract, and provoke allergic symptoms.

Over the last years, food allergy has gained an important global attention as the most prevalent disorder regarding the food-related diseases. The estimated prevalence of food allergy is about 8% in children and 5% in adults in the worldwide. Due to the increasing prevalence of food allergy, the development of more accurate treatment of prevention and diagnostic methods are constantly growing. The scientific community has highlighted that the extent to food allergy is associated with changing dietary habits and preferences, with food globalization, with the way in which food is prepared

and the introduction of new food (Lucas *et al.*, 2004). Previously, the most common allergenic foods, defined as the "big 8", were peanuts, tree nuts cows' milk, hens' eggs, soy, wheat, shellfish and fish . Recently, in Europe, that list has been changed in definition and incremented in number to 14: peanuts, tree nuts, soybeans, cereals containing gluten, crustaceans, molluscs, eggs, fish, milk, celery, mustard, sesame, lupin and sulphur dioxide (Kyprianou, 2006; Verhoeckx *et al.*, 2015)

1.3. Mechanism of IgE-mediated food allergy reaction

The development of IgE-mediated allergic reaction can be divided into two phases: sensitization phase and effector phase (Sampson, 2004). Both sensitization and development of allergenic reaction is caused by type 1 food allergens such as egg, peanut and cow's milk. Sensitization to food antigens can take place in the gastrointestinal tract, oral cavity, and skin and occasionally in the respiratory tract (Pekar et al., 2018). After ingestion, food allergens are broken down largely by digestive enzymes in the stomach and intestine. Following, the residual intact food proteins and peptides were adsorbed through the intestinal epithelium and access to the mucosa. In the intestinal mucosa, allergens are absorbed by antigen presenting cells (APCs), such as dendritic cells. The APCs, bound to the major histocompatibility complex (MHC) class II molecule, then present allergen to the T cell receptor (TCR) on native CD4+ allergen-specific cells (Huby *et al.*, 2000). These specific T cells are stimulated to transform mostly into T helper 2 (Th2) cells, which are able to release chemical mediators such as interleukins (IL) such as IL-4, IL-5 and IL-13. (Berin & Shreffler, 2008; Shreffler *et al.*, 2006). These chemical mediators stimulate the B cells to undergo immunoglobulin to synthesize large quantities of specific IgE

antibodies, which present a high affinity with IgE receptor (FcƐRI) on the surface of mast cells in the tissue or basophils in the blood (***Figure 2***) (Perry & Pesek, 2013; Ruiter & Shreffler, 2012; Sicherer & Sampson, 2014).

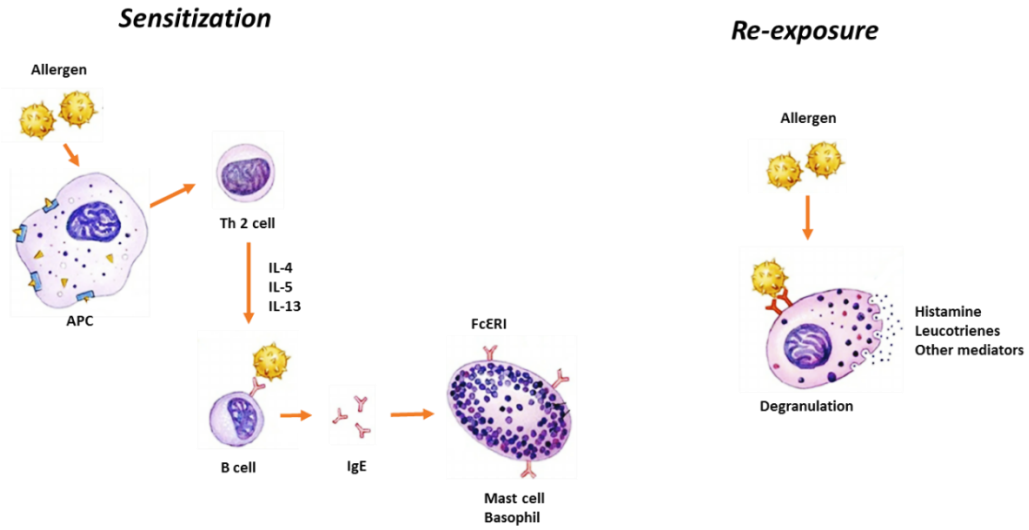

Figure 2*. Mechanism of IgE-mediated allergic reaction.*

The effector phase occurs during the second or subsequent exposure to the allergen, which re-enters the circulations and crosslink to the receptors present on the surface of mast cells or basophils. FcƐRI crosslinking triggers a cascade reaction that lead to the degranulation of mast cells and basophils and to the exocytosis of their granules that contain mediators of hypersensitivity, which include histamine, leukotrienes, prostaglandins and other inflammatory cytokines (Metzger, 1999; Renz *et al.*, 2018). The clinical symptoms can range from mild cutaneous (hives, itchiness, swelling, erythema) to severe respiratory (hoarseness, respiratory distress), cardiovascular (cardiac arrest) and gastrointestinal (nausea, vomiting and diarrhoea) (Sicherer & Sampson, 2014).

The relationship between proteins respect to their inherent allergenic potential or the properties that confer on proteins the ability to induce sensitisation are aspects that remain well not understand in detail. It is well-known, however, that protein stability (including resistance to proteolytic digestion), the impact of food processing and of the food matrix can affect both immunogenic and allergenic potential (Huby *et al.*, 2000).

1.4. Coeliac disease

Food intolerance is atypical non-immune response with allergy-like symptoms after the ingestion of food (Madsen, 1997; Ortolani & Pastorello, 2006). It results from enzyme deficiencies, pharmacological reactions, and response to toxic or irritant components present in food. Globally, it is estimated that 5-6 % of adults are affected by food intolerance, while the percentage in infants and young children is from 0.3 % to 20 %. Food intolerances may be immune mediated but also non-immune mediated. The most prevalent example of a clearly defined immune-mediated food intolerance is celiac disease (CD), a non IgE-immune-mediated enteropathy triggered by ingestion of gluten proteins in genetically susceptible individuals, which is assumed to affect approximately 1–2% of the population in Western countries (Mamone *et al.*, 2011). CD is the result of the interaction of a series of complex mechanisms involving genetic, immunological and environmental factors. Genetic factors play a basic role in CD development. In particular, several studies showed that the genetic association of CD with HLA-DQ2/8-resctricted CD4+ T lymphocytes in the adaptive immunity (Meresse *et al.*, 2012). These T lymphocytes specifically recognize gliadin peptides that survive gastrointestinal digestion and are deamidated (glutamine to glutamic acid modification) by the tissue-transglutaminase (tTGase). In addition, other studies indicate that specific gliadin peptides are able to activate the cells involved in innate immunity, such as macrophages, dendritic cells, and cytotoxic intraepithelial lymphocytes, or to induce a direct toxic effect on enterocytes (Gianfrani *et al.*, 2005; Sollid, 2000). Genetic aspects, however, are connected to environment factors, as this intolerance is elicited only upon the introduction of gluten present in some foodstuff in the diet of susceptible individuals. Gluten proteins, gliadins and glutenins, contain peptides that

can bind DQ2 or DQ8 and be recognized by intestinal T cells. T cells, in celiac patients, recognized mostly α-gliadins peptides, whereas γ-gliadins and glutenins are much less recognized (Arentz-Hansen *et al.*, 2000), as α-gliadin. This happens probably because α-gliadin contains a stable 33mer fragment that presents a cluster of epitopes (Arentz-Hansen *et al.*, 2002). Peptides arising from gluten proteins, after proteolytic degradation during gastrointestinal digestion, can stimulate either adaptive or innate immune responses and, in particular, this αG-33mer binds well to DQ2 after deamidation by tissue transglutaminase and it is recognized with high affinity by intestinal T-cell (Shan *et al.*, 2002). To date, a strictly gluten-free diet represents the only solution for CD patients. Moreover in recent years, there has been an increase in gluten-related disorders in western countries attributing the blame to the continuous exposure to toxic epitopes (de Lorgeril & Salen, 2014). Therefore, research is currently aimed to discover wheat cultivars with null or reduced toxicity that are potentially tolerated by most CD patients, conserving acceptable baking properties. Ancient wheats played an important role as a source of food for the early human civilizations, and among these, diploid *Triticum monococcum* wheat species are now considered suitable candidate because containing prolamins (gliadins and glutenins) exerting low-toxicity. In fact, *Triticum monococcum* lacks the D genome that encodes the immunodominant 33-mer fragment (Molberg *et al.*, 2005). It is well-know that ancient wheat is capable of triggering immunotoxicity in CD patients (De Vincenzi *et al.*, 1996; Gianfrani *et al.*, 2015; Gianfrani *et al.*, 2012; Pizzuti *et al.*, 2006; Suligoj *et al.*, 2013; Vaccino *et al.*, 2009) but some *Triticum monococcum* lines with a reduced toxicity have been identified, demonstrated both *in vitro* and in *ex vivo* (Gianfrani *et al.*, 2015; Gianfrani *et al.*, 2012)

1.5. Stability of allergens: a focus on the stability to gastrointestinal digestion

The ability of a protein to maintain its native and three-dimensional structure, after chemical, physical treatment or protease action, defines its stability (Mills & Breiteneder, 2005). The reason why certain proteins are (and others are not) allergenic is not yet understood. It is known however that the biochemical stability of the protein plays a fundamental role, an important characteristic that confers on protein the potential to trigger the immune system resulting an allergenic sensitization and allergic response. Allergens may access the immune system in different ways, i.e. via the skin or mucosal surfaces (Dunkin et al., 2011). Allergens able to enter in the organism via the skin are capable of crossing the epidermal barrier and for this reason, they tend to show hydrophobic properties or need binding to a lipophilic carrier due to the intercellular lipids present in the skin structure (De Benedetto *et al.*, 2012). Allergens that are inhaled such as pollen, house dust or spores, do not need specific stability to pH or to enzymes or to have any particular biochemical properties. Following inhalation, these allergens require to exhibit extensive solubility in the mucosal surfaces and to have a small size in order to not escape and to bind easier to the mucus (Pekar *et al.*, 2018).

The resistance to gastrointestinal digestion is a key characteristic of allergenic proteins. Digestion of protein starts with mastication in the oral cavity, in which food is broken into small particles enabling the action of gastrointestinal enzymes. First, in the oral cavity, α-amylase enzymes, secreted by the salivary glands, start enzymatic hydrolysis. This step is crucial considering the presence of glycoproteins in food. The amylases, in fact, reduce the 1,4-glycosidic bonds of carbohydrates and this may influence the allergen structure (Boehlke *et al.*, 2015). Following, proteins

pass in the stomach and denature due to the acidic environment. This low gastric pH, which optimum is from 1.8 to 4, is essential for pepsin activation (actual pH depends on the volume, meal content, presence or absence of antacids and the individual consumer). In acidic condition in fact, pepsinogen, the inactive enzyme, becomes active. The pepsin is an aspartic protease, which is able to hydrolyse proteins between hydrophobic and aromatic amino acid residues phenylalanine, tyrosine, tryptophan and leucine. Its important role in the proteolytic digestion of food is already documented in the scientific literature, even though the acidic environment, to which the capabilities of enzyme is closely correlated, has been the subject of many conflicting studies. Some studies affirmed that enzymatic digestion in the stomach is not indispensable for a complete protein digestion, as patients after gastric bypass surgery, why omits the main parts of the stomach from digestion, did not show signs of protein malabsorption (Bojsen-Moller *et al.*, 2015). Conversely,Shakeri-Leidenmuhler *et al.* (2015) affirm the important role of pepsin in the allergen digestion, highlighting an increased risk in developing allergies in patients gastric bypass. Therefore, the relationship between allergenic potential of protein and resistance to pepsin digestion is not clear and there is considerable evidence that is not absolutely predictive of allergenicity. Many food proteins that are stable to pepsin digestion, but non-allergenic were listed as lectins (Fu *et al.*, 2002). Even though they are not allergens, they are immunologically important. Therefore, in order to assess the stability of proteins and the relationship with allergenicity, it is not advisable to use pepsin digestion as an *in vitro* digestion model, but to exert a simulated human digestion model that mimic the *in vivo* environment more closely.

Proteins or peptides released after enzymatic hydrolysis in the stomach under the action of pepsin transit the duodenum where pancreatic proteases

act to further degrade the protein components. These enzymes are released in the small intestine from inactive precursors (trypsinogen, chymotrypsinogen, proelastase, and procarboxypeptidase) produced in the pancreas. Trypsinogen is transformed to an endopeptidase called trypsin by enterokinase secreted from the walls of the small intestine. Trypsin then activates the precursors of chymotrypsin, elastase, and carboxypeptidase. Peptides that are further hydrolysed by proteolytic enzymes, pass through the mucous layer (which contains additional eso- and endo-hydrolases) covering the epithelium before being absorbed. Beyond these luminal events, proteins and peptides, which are absorbed by the enterocyte, may undergo further intracellular degradation (Wickham *et al.*, 2009). Several studies in the scientific literature affirm that small peptides are unable to stimulate mast cell degranulation, unlike larger fragments or intact proteins (Poulsen & Hau, 1987). Nevertheless, it is important to consider, even though the bulk of food proteins are reduced into immunologically inactive fragments, it may possible that a small portions of foods evade digestion, maintaining immunological stimulatory properties.

In order to understand what is undergoing a protein in terms of allergenicity following gastrointestinal digestion, several *in vitro* digestion models have been proposed. Simulated digestion methods typically include the oral, gastric and duodenal phases, trying to simulate the *in vivo* physiological conditions using proteolytic enzymes and their correlated factor (concentrations, pH, digestion time, and salt concentrations). However, the majority of models reported in literature are static (Hur *et al.*, 2011); and many of these are derived from earlier reported methods in which there were significant discrepancy in the use of *in vitro* digestion parameters between them, impairing the ability to compare results across different research groups and to assume a common conclusion (Williams *et al.*, 2012a;

Williams *et al.*, 2012b). For example, results can be alter by using different pH value or kind of mineral. Differences in sources of enzymes (e.g. porcine, rabbit or human) can lead in different products due to changing in their activity. Other factors such as the presence of phospholipids and bile salts, gastric lipase and digestive emulsifiers and the proportion of food bolus to digestive fluids are also determinant in building a model of digestion as closely to *in vivo*. Moreover, digestive fluids are also a key consideration in establishing an *in vivo* model. For this, the COST Infogest network proposed a standardised and useful static digestion method based on relevant physiological conditions that can be applied for several food matrix (liquid or solid), which may be modified depending on experimental requirements (depending on food matrix composition) (Minekus *et al.*, 2014). The entire thesis work is based on the application of this model of gastrointestinal digestion, showed in the following workflow (***Figure 3***), in which conditions and composition of digestive fluids obtained a broad consensus in terms of physiological relevance.

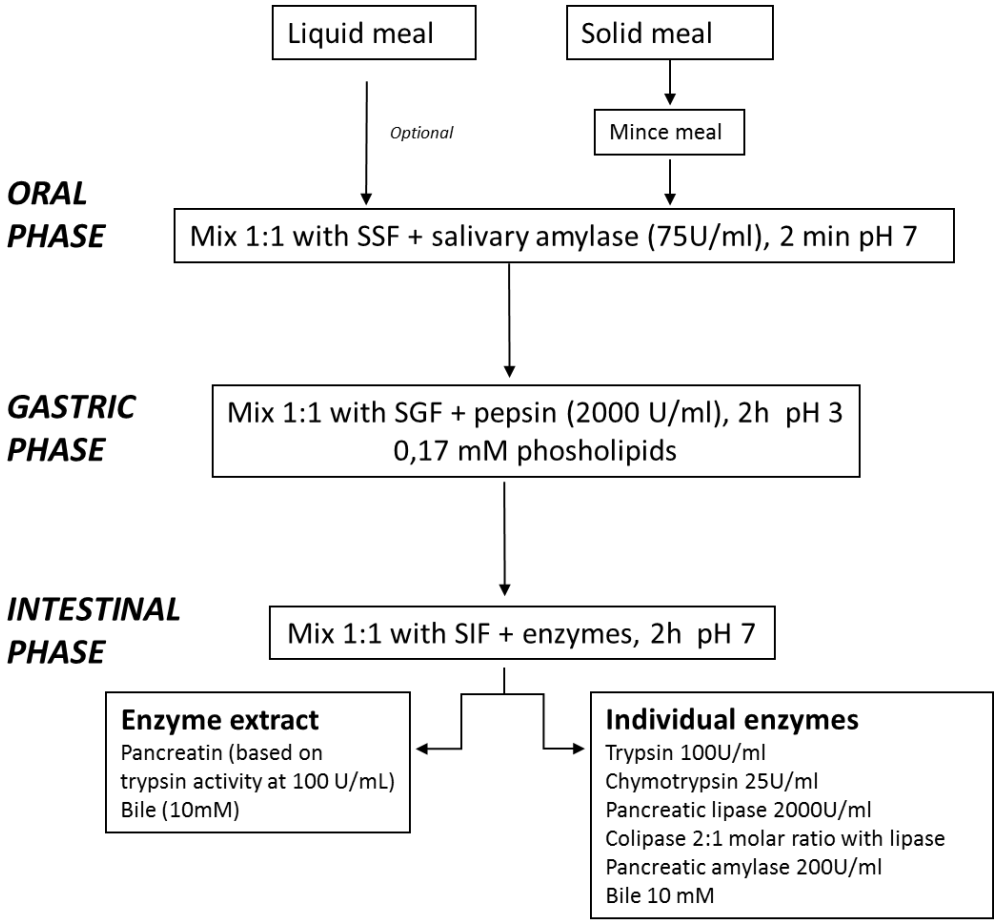

***Figure 3**. Workflow of simulated in vitro digestion method proposed by the COST Infogest network. SSF= Simulated Salivary Fluid, SGF= Simulated Gastric Fluid and SIF= Simulated Intestinal Fluid, respectively. Enzyme activities expressed in units per mL of final digestion mixture at each corresponding digestion step (Minekus et al., 2014).*

This protocol was integrated with the final intestinal degradation step using porcine jejunal brush border membrane (BBM) enzymes, located on the polarized apical surface of the duodenal and jejunal enterocytes. The use of BBM is an important step in evaluating intestinal stability because they contribute to deeply hydrolyse internally the "core" of (oligo)peptides. BBM, in fact, contains a series of endo- and exo-peptidases with different cleavage specificity, which reduce the surviving oral- gastric- and duoenal peptides into di-, tri- and oligopeptides as well a free amino acids (Mamone *et al.*, 2015; Picariello *et al.*, 2016).

1.6. Food processing and allergen stability

A simple fragment of few amino acid along the primary structure (linear epitope) or a three-dimensional motif of the protein structure (conformational epitopes) can cause an allergic reaction. Modification to these immunogenic epitopes, due to food processing, may influence protein allergenic properties. Foods undergo processing for several reason: to preserve them by extending the shelf-life and safety (e.g. heating, pasteurization), to transform their properties for the end use or to improve their sensory qualities (e.g. flavor, taste, texture and appearance). From a biochemical viewpoint, thermal processing can introduce chemical and physical changes to the food proteins that could affects protein conformation by destroying existing allergens or by promoting interactions of proteins with other components present in the food matrix generating new allergens (neoallergen) (Sathe *et al.*, 2005; Teodorowicz *et al.*, 2017). Several types of processing are implicated in influencing allergenic properties: thermal treatments (e.g. heating), enzymatic hydrolysis, fermentation, physical processing or combination of more of these (Mills &

Mackie, 2008; Thomas *et al.*, 2007). These methods can be classified into two processing types: thermal and non-thermal. Non-thermal processing methods include germination, fermentation, proteolysis, ultrafiltration, acid and enzymatic hydrolysis (Besler *et al.*, 2001; Sathe *et al.*, 2005). Thermal methods include all processes that use moist heat or dry heat. Heat treatment above 55 °C cause changes in protein structure, such as the loss of secondary and tertiary structure as well as cleavage of disulphide bonds at temperatures above 70–80 °C. Furthermore, protein denaturation may be irreversible and this can lead to a cross-linking reaction between protein and other components of food matrix (Teodorowicz *et al.*, 2017; van Boekel, 2001). For example, free amino groups of protein chain can be blocked by sugars due to Maillard reaction, affecting digestion and subsequently the absorption of proteins/peptides, as well as their recognition by immune system. (Hellwig & Henle, 2014). The Maillard reaction (MR) is a glycation event which starts with formation of covalent bonds between free amino groups of amino acids (mostly lysine and arginine) and the carbonyl groups of a reducing sugar, during food processing and meal preparation (cooking, baking and frying)(Liu *et al.*, 2012; Somoza, 2005). From this point, a cascade of chemical reaction as condensation, oxidation and hydration, contribute to the formation of Maillard reaction products (MRPs), which have the function to confer to food, appearance, smell, taste and texture. The formation of these products during the MR can affect the availability of enzymatic cleavage sites so modifying the susceptibility of proteins to gastrointestinal digestion due to unfolding, formation of new complex with other components of food matrix and heat-induced disulphide bond interchanges. Furthermore, conformational changes of proteins caused by MR, or more generally from any food processing, could lead to altered immunogenicity and consequently allergenicity, either masking existing epitopes or creating new complex that could promote the initiation of new

IgE-mediated allergies (Apostolovic *et al.*, 2016; Bogh & Madsen, 2016; Iwan *et al.*, 2011; Lehmann *et al.*, 2006).

It is important to consider that food processing can influence different aspects of the food so it is necessary to distinguish clearly between food and food. In milk, for example, denaturation and Maillard reaction of epitopes of both β-lactoglobulin and α-lactalbumin, after sterilization, results in decreasing of IgE and IgG binding capacity, probably due to the destruction or masking of epitopes (Bu *et al.*, 2009; Ehn *et al.*, 2004; Schoenfuss & Chandan, 2011; Taheri-Kafrani *et al.*, 2009; Zheng *et al.*, 2014). In the case of egg proteins, heating reduces the allergenicity while other treatment such as irradiation cause only a slight modulation in terms of reduced immunogenicity (Golias *et al.*, 2012; Lee *et al.*, 2007; Ma *et al.*, 2013; Shin *et al.*, 2013).

Wheat, a staple food for most of world's population and mainly used in baking and pasta products, is also cause of food allergy. Scientific studies affirm that thermal processing in baked products lead to allergenic protein aggregation hindering a complete proteolytic degradation. These large IgE-binding protein fragments that pass through the gastrointestinal tract, could elicit an allergic reaction (Pasini *et al.*, 2001; Simonato *et al.*, 2001). A very similar finding was found in pasta products: different conditions (time/temperature) of dry processing of pasta lead to a different stability to gastrointestinal digestion (Simonato *et al.*, 2004).

A decreased allergenicity was observed after roasting hazelnut in several studies in scientific literature. In particular, the eliciting dose for roasted hazelnut results higher compared to raw hazelnut with a consequence lower reactivity in the Skin prick test (SPT) and basophil activation test (BAT) (Hansen *et al.*, 2003; Worm *et al.*, 2009). About almond, thermal processing

as blanching and roasting did not have any influence the IgE-binding of the major allergen, Pru du 6 (de Leon *et al.*, 2003; Roux *et al.*, 2001; Venkatachalam *et al.*, 2002). In the same way, studies in scientific literature affirm that heating processing, as blanching, roasting and frying did not affect the IgG binding capacity of the major walnut allergens, Jug r 2 and Jug r 4 (Su *et al.*, 2004). Only a diminished IgE binding capacity resulted after autoclaving (Cabanillas *et al.*, 2014).

Changes in the allergenicity of food allergens following processing is also due to the interaction of them with different components of food matrix. During the MR, in fact, carbonyl compounds attack free primary amino groups, leading to the formation of stable advanced glycation products (AGE). These changes in proteins may influence antibodies' ability to bind to the modified protein, and in the case of IgE binding antibody, this may imply an altered capacity to elicit an allergic reaction (Maleki *et al.*, 2000). Chung & Champagne (2001) utilized antibodies specific for certain types of AGEs to demonstrate that Ara h 1 and Ara h 3 are more commonly modified than Ara h 2. Furthermore, the solubility of the target proteins and the extractability of these can be affected by thermal processing, and this is another drawback for the detectability of allergens in foodstuff. About roasting, scientific studies in literature show different results. Roasting of peanut is usually performed at 140 °C for 40 min. At this high temperature chemical modifications, like covalent links between lysine residues of the protein and other constituents of food matrix, may occur. The resulting in the formation of adducts may involve the formation of reactive complexes (Chung *et al.*, 2003). Several studies demonstrate that degranulation capacity is reduced by Ara h 2 and Ara h 6, purified from roasted peanuts, but significantly enhanced by Ara h 1(Vissers *et al.*, 2011). Others studies conversely, in which the ability of T-cell stimulation of Ara h1 and Ara h3

was compared, reported that Ara h 2 has higher IgE reactivity and T-cell stimulation property than Ara h 1 (Tordesillas *et al.*, 2014). More broadly, peanut allergens (Ara h 1, Ara h 2 and Ara h 3) from roasted peanuts extracts increase IgE binding by 90-fold compared with raw peanut extracts due to greater accessibility of IgE-binding epitopes in roasted peanuts. Several results in literature as well concern the effect of boiling processes on IgE binding capacity of peanut allergens. Some studies (Beyer *et al.*, 2001; Blanc *et al.*, 2011; Mondoulet *et al.*, 2005; Vissers *et al.*, 2011) demonstrated that boiling decreased the IgE- binding capacity than roasted peanuts, assessment lead by immunochemical assay like immunoblotting. Particularly, Turner *et al.* (2014) found that boiling for 6 hours lead to a loss of proteins, particularly the most immunogenic protein, Ara h 2 and Ara h 6, could be found in cooking water. Therefore, different from other technological process, boiling brings a decrease in allergenicity not associated with structural modifications but with a loss of low molecular weight proteins into the cooking water (Mondoulet *et al.*, 2005). Futhermore, Beyer *et al.* (2001) demonstrates like different methods of peanut preparation influence IgE-binding capacity. Particularly, frying (120 °C) and boiling (100 °C) reduced IgE binding of Ara h 1, Ara h2 and Ara h 3 compared with roasted preparations (150 °C-170 °C). In detail, the IgE binding to Ara h 2 and Ara h 3 was, in parallel with Ara h 1, significantly lower in boiled and fried peanuts in comparison with roasted preparations. This finding may explain the relationship that exists in lower prevalence of peanut allergy in China where the consumption of boiled peanuts is more widespread than in the United States where prevailing consumption of roasted peanuts. The autoclaving, also, was considered an important physical method able to decrease IgE-binding properties of roasted peanut promoting lost of most of the α-helical structure and then changing the structure of proteins. Both by *in vitro* experiments (Western blot, ELISA)

and *in vivo* experiments (SPT), IgE immunoreactivity of roasted peanut protein extract decreased significantly at extreme conditions of autoclaving (Cabanillas *et al.*, 2012).

The time, the temperature, the nature, the intensity and all conditions during food processing can affect the allergenicity of proteins either by destruction of epitopes or formation of new allergenic complexes. These factors associated with the effect of food matrix could explain why the effects of food processing are eliminated or attenuated for some whole food as compared with isolated pure allergens (Mondoulet *et al.*, 2005).

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2. PhD project aims

Resistance to proteolytic degradation in the gastrointestinal tract is considered a shared feature of major allergens able to trigger an immune response in predisposed individuals. In the case of food allergy and food intolerance, it is a crucial to investigate how the food matrix and food processing affect the digestion stability and subsequently the allergenic potential. To date, in the majority of studies, the digestion stability have been carried out on purified allergens neglecting, by this way, the relevant effect of both food matrix and food technologies. In this context, the aim of this PhD thesis was to assess the metabolic fate of food protein allergen directly in their natural food matrix, by using a harmonized *in vitro* digestion model, including the intestinal step with Brush Border Membrane (BBM) enzymes. The products of *in vitro* digestion were characterized by combined proteomics and immunological techniques. In particular, this PhD thesis aimed at investigating the stability of peanut and tree nuts (hazelnuts, walnuts and almonds) protein before and after roasting process. We also compare the behaviour of the gluten protein of ancient *Triticum monococcum* cultivars (ID331 and Monlis) from modern wheat *Triticum aestivum* cultivar (Sagittario cv). Notably, the experimental design of the great majority of studies assessing the cereal toxicity for celiac patients, including *Triticum monococcum*, consists of *in vitro* and *ex vivo* functional assays in which gliadin digested by pepsin-trypsin (PT), pepsin-chymotrypsin (PC), or chymotrypsin alone, have been used as stimulating triggers. However, in assessing digestion stability of the major nut allergens and in identifying new cereals with low or no immune toxicity for celiac patients, we cannot overlook their susceptibility to digestion in the gastrointestinal track, including a final degradation step by peptidases from the small intestinal brush border membrane (BBM). It has been reported

that the resistance to gastrointestinal digestion is an important constraint in determining the immune stimulatory and toxicity properties of allergenic peptides. The use of proteases located on enterocyte microvilli (BBM) is a fundamental step in assessing intestinal stability because they contain a series of endo- and exo-peptidases, which have negligible effects in gradually shortening peptides into di-, tri-, and oligopeptides as well as free amino acids, and constitutes a fundamental step for assessing the intestinal stability of large protein fragments produced upstream, during the gastric and duodenal phases.

The objectives of the present PhD project are illustrated in the workflow diagram show in ***Figure 4*** (in addition is reported a list of known allergens in the foods under study, ***Table 1***) and summarized as follow:

A1) evaluation of the digestion stability of major nut allergens (peanuts, hazelnuts, walnuts and almonds) as whole food, using an *in vitro* static model that simulates the gastrointestinal digestion process, including oral, gastric, duodenal and intestinal (brush border membrane enzymes) phases;

A2) determine the stability of nut allergens following gastrointestinal digestion of whole food using proteomic techniques (SDS-PAGE, RP-HPLC, LC- HR-MS/MS);

A3) assess the allergenic properties of nuts following gastrointestinal digestion of whole food using immunological methodology (ELISA, western-blot, dot-blot, RBL assay);

A4) investigate how *in vitro* gastro-intestinal digestion affects the immune toxic properties of gliadin from diploid (*Triticum monococcum*) compared to hexaploid (*Triticum aestivum*) wheat by an immunological and proteomic approach.

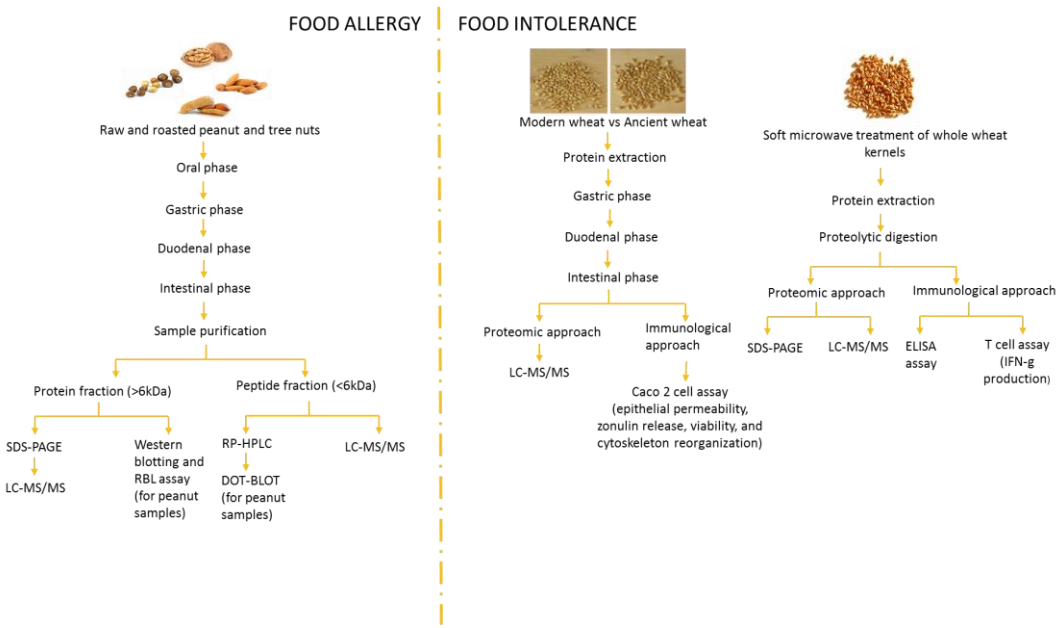

***Figure 4**. Schematic workflow of the experimental approach employed (immunoassay and MS-based analysis) for the PhD thesis.*

Table 1. Current repertoire of known allergens in the foods under PhD study
Sources: available at URL: http://www.allergome.org)
(Albillos et al., 2008; Geiselhart et al., 2018; Pastorello et al., 2004; Piersma et al., 2005; Rigby et al., 2008; Wieser, 2007).

GLUTEN PROTEINS		
		Molecular mass (SDS-PAGE)
Gliadins	ω5	49-55
	ω 1,2	39-44
	α/β	28-35
	γ	31-35
Glutenins	x-HMW-GS	83-88
	y- HMW- GS	67-74
	LMW-GS	32-39

PEANUT ***(Arachis hypogea)***		
Allergen	*Protein family*	*Molecular mass (SDS-PAGE)*
Ara h 1	Vicilin 7S globulin	64
Ara h 2	Conglutin 2S albumin	17, 19
Ara h 3/4	Legumin 11S globulin	13-45
Ara h 5	Profilin	15
Ara h 6	Conglutin 2S albumin	15
Ara h 7	Conglutin 2S albumin	15.8
Ara h 8	Bet v 1 homologous	16.8
Ara h 9	nsLTP	9.8
Ara h 10	Oleosin	16
Ara h 11	Oleosin	14
Ara h 12	Defensin	8
Ara h 13	Defensin	8

WALNUT ***(Juglans regia)***		
Allergen	*Protein family*	*Molecular mass (SDS-PAGE)*
Jug r 1	2S albumin	15-16
Jug r 2	Vicilin	44
Jug r 3	nsLTP	9
Jug r 4	Legumin	35-55
Jug r 5	Bet v-1like	48
Jug r 6	Vicilin	47
Jug r 7	Profilin	13

HAZELNUT ***(Corylus avellana)***		
Allergen	*Protein family*	*Molecular mass (SDS-PAGE)*
Cor a 1	Bet v 1-like (PR-10)	17
Cor a 2	Profilin	14
Cor a 8	nsLTP	9
Cor a 9	Legumin 11S globulin	31-35; 21-25
Cor a 11	Vicilin 7S globulin	48
Cor a 12	Oleosin	17
Cor a 13	Oleosin	14-16
Cor a 14	Conglutin 2S albumin	15-16; 17

ALMOND (*Prunus dulcis*)		
Allergen	*Protein family*	*Molecular mass (SDS-PAGE)*
Pru du 1	Pathogenesis-related protein	17
Pru du 2	Thaumatin	26
Pru du 3	nsLTP	9-10.4
Pru du 4	Profilin	13.9-14
Pru du 5	60s acid ribosomial protein	10-11.4
Pru du 6	Legumin	61-65; 55.9-65
Pru du 2S albumin	Conglutin 2S albumin	12
Pru du Conglutin	Conglutin	3

Statement of contribution to the papers

My contribution, to published and submitted papers related to my PhD project, is described in detail as follow:

1. Di Stasio L, Picariello G, Mongiello M, Nocerino R, Berni Canani R, Bavaro S, Monaci L, Ferranti P, Mamone G. Peanut digestome: Identification of digestion resistant IgE binding peptides. Food Chem Toxicol. 2017 Jun 17; 107(Pt A):88-98.
- *Experimental activities: in vitro gastroduodenal-BBM digestion of whole peanuts, purification of peanut digesta samples, SDS-PAGE analysis, Western blotting analysis, RP-HPLC and dot-blot analysis, characterization of IgE binding peptides, LC-high resolution (HR)-MS/MS analysis*
- *Data analysis and manuscript writing.*

2. Bavaro S.L, **Di Stasio L**, Mamone G, De Angelis E, Nocerino R, Berni Canani R, Logrieco AF, Montemurro N, Monaci L. Effect of thermal/pressure processing and simulated human digestion on the immunoreactivity of extractable peanut allergens, Food Res Int . July 2018 109:126-137.
- *Experimental activities: protein extraction and quantification, ELISA assay, in vitro gastroduodenal digestion of raw and treated peanuts, SDS-PAGE analysis, Western blotting analysis, LC-high resolution (HR)-MS/MS analysis*
- *Data analysis and manuscript writing.*

3. Mamone G, **Di Stasio L**, De Caro S, Picariello G, Nicolai MA, Ferranti P. Comprehensive analysis of the peanut allergome combining 2-DE gel-based and gel-free proteomics. https://doi.org/10.1016/j.foodres.2018.09.045
- *Experimental activities: sample preparation for shotgun proteomic analysis, SDS-PAGE analysis, LC-high resolution (HR)-MS/MS analysis*
- *Data analysis and manuscript writing.*

4. Iacomino G, **Di Stasio L**, Fierro O, Picariello G, Venezia A, Gazza L, Ferranti P, Mamone G. Protective effects of ID331 *Triticum monococcum* gliadin on *in vitro* models of the intestinal epithelium. Food Chem. 2016 Dec 1; 212:537-42.
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5. Gianfrani C, Mamone G, la Gatta B, Camarca A, **Di Stasio L**, Maurano F, Picascia S, Capozzi V, Perna G, Picariello G, Di Luccia A. Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease. Food Chem Toxicol. 2017 Mar; 101:105-113.
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6. **Di Stasio L**, Tranquet O, Picariello G, Ferranti P, Denery-Papini S, Mamone G. Eliciting capacity of gastrointestinal digests from raw and roasted peanuts by Rat Basophil Leukemia cell-based assay. *Submitted 2018.*
- *Experimental activities: In vitro gastroduodenal-BBM digestion of whole raw and roasted peanuts, purification of soluble and insoluble digesta samples, SDS-PAGE analysis, RP-HPLC, evaluation of allergenic capacity of peanut digests by humanized RBL cells, LC-high resolution (HR)-MS/MS analysis*
- Data analysis and manuscript writing.

7. **Di Stasio L**, Picariello G, Ferranti P, Mamone G. Assessment of the metabolic fate of tree nut allergens: the role of intestinal Brush Border Membrane digestive enzymes and food processing. *Submitted 2018.*
- *Experimental activities: In vitro gastroduodenal-BBM digestion of whole raw and roasted tree nuts, purification of soluble and insoluble digesta samples, SDS-PAGE analysis, RP-HPLC, LC-high resolution (HR)-MS/MS analysis*
- Data analysis and manuscript writing.

Chapter 2

PhD Publications

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Peanut digestome: Identification of digestion resistant IgE binding peptides

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ABSTRACT

Stability to proteolytic degradation in the digestive tract is considered a general feature shared by most food allergens. Current digestibility models exclusively utilize purified allergen proteins, neglecting the relevant effects of matrix that occur for foodstuff systems. In the present study, we investigated digestion stability of the major peanut allergens directly in the natural matrix using an in vitro static model that simulates the gastrointestinal digestion including the oral, gastric, duodenal and intestinal (brush border membrane enzymes) phases. Immunogenicity was evaluated by Western Blot using N=8 pooled sera of peanut allergic pediatric subjects. Immunoreactive, large-sized and fragments of Ara h 2, Ara h 6 and Ara h 3 survived hydrolysis as assessed by SDS-PAGE. Smaller resistant peptides mainly arising from Ara h 3 and also Ara h 1 were detected and further identified by LC-high resolution-MS/MS. RP-HPLC purification followed by dot-blot analysis and MS/MS-based identification demonstrated that stable IgE-binding peptides derived from Ara h 3. These results provide a more realistic picture of the potentially allergenic determinants of peanuts that survived the human digestion, taking into account the role of the food matrix, which may significantly affect gastrointestinal breakdown of peanut allergens.

1. Introduction

Peanut allergy is one of the most widespread and severe IgE-mediated food allergies, with an estimated prevalence of 1% in children and 0.6% in adults within the general population of developed countries (Ben-Shoshan et al., 2010; Sicherer and Wood, 2013). The complex allergome of peanut consists of several type I protein allergens triggering immune responses with different symptoms and prognosis, depending on characteristics of the offending protein (Vereda et al., 2011; Lauer et al., 2009; Nicolaou and Custovic, 2011; Mittag et al., 2004). Ara h 1, Ara h 2, and Ara h 3 are major allergens associated with primary sensitization to peanut (Mueller et al., 2014). Ara h 1 is a 63.5-kDa vicilin-type (7S) seed storage protein, representative of the cupin superfamily, which naturally occurs as up to 600–700 kDa non-covalent aggregates (van Boxtel et al., 2006). Ara h 3 is a glycinin-like protein (11S) consisting of a 60-kDa polypeptide post-translationally cleaved in acid and basic subunits which remain linked each other by a disulphide bond, similarly to the 11S plant seed storage protein signature (Boldt et al., 2005). Ara h 2 belongs to the conglutenin (2S albumin) superfamily and their folding resembles that of α-amylase/trypsin inhibitors from cereal kernels (Mueller et al., 2011). Ara h 2 comprises two isoforms, namely Ara h 2.01 (17-kDa) and Ara h 2.02 (19-kDa). Ara h 2.02 includes an insertion of 12 extra amino acid residues, constituting an additional IgE-binding epitope and is a more effective IgE cross-

Abbreviation: Ambic, ammonium bicarbonate; BBM, brush border member enzymes; DTT, dithiothreitol; IAA, iodoacetamide; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; TCA, trichloroacetic; TFA, trifluoroacetic; SSF, Simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

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linker than Ara h 2.01 (Chatel et al., 2003). Ara h 6 is a 14.5-kDa 2S albumin co-member, sharing 59% sequence identity, secondary and tertiary structure homology as well as immune cross-reactivity patterns with Ara h 2 (Koppelman et al., 2005; Lehmann et al., 2006). World Health Organization and International Union of Immunologic Societies Subcommittee (WHO/IUIS) catalogued several additional minor peanut allergens (www.allergen.org), including Ara h 5 (profilin), Ara h 8 (Bet v 1birch pollen homologue), Ara h 9 or lipid transfer protein (LTP), the latter being a relevant peanut allergen especially in the Mediterranean area (Krause et al., 2009). Ara h 10 (oleosin 1) and Ara h 11 (oroleosins) are not usually associated with severe allergic reactions (Zhuang and Dreskin, 2013).

Apart from geographical differences in the sensitization profiles (Sicherer and Wood, 2013), conglutenin Ara h 2 and Ara h 6 have been proven as the most harmful for peanut allergic subjects, in term of basophil activation, IgE-binding properties and skin prick test (Burks et al., 1991; Blanc et al., 2009). Both Ara h 2 and Ara h 6 are tightly coiled, heat-stable and resistant to gastrointestinal digestion (Suhr et al., 2004), which are structural features shared by a large number of common food allergens (Astwood et al., 1996). Early studies showed that digestion of Ara h 2 and Ara h 6 by pepsin and/or chymotrysin produce large stable fragments with unmodified immunological potential (Apostolovic et al., 2016). On the contrary, peanut allergen Ara h 1 and Ara h 3 have been described as highly susceptible to proteases (Koppelman et al., 2010). Nevertheless, peptides resulting from gastro-duodenal digestion of Ara h 1 retain T cell stimulatory, sensitizing capability and IgE-binding properties (Eiwegger et al., 2006; Bøgh et al., 2009), probably due to the formation of exceptionally stable non-covalent peptide aggregates (Bøgh et al., 2012).

Susceptibility of peanut food allergens to proteolysis has been typically assayed by using single purified proteins (Bøgh and Madsen, 2016), due to the drawbacks of analyzing the heterogeneous digestome of peanut as a whole food. Such an approach could suffer from scarce relevance, since the protein aggregation state, the interaction of allergens with non-protein components naturally occurring in whole foodstuff (e.g. polysaccharide, lipid), presence of protease inhibitors and the protein-protein interactions affect the accessibility of proteases to allergens, thereby contributing to the bioaccessibility and hence to the bioavailability of allergenic determinants (epitopes) (Teuber, 2002). Nowadays, the advances in "omic" sciences (*i.e.*, proteomics, metabolomics, lipidomics) have enabled the assessment of the food digestome as well as the identification of stable allergens and the monitoring of IgE-binding epitopes sequentially released upon digestion of complex matrices (Picariello et al., 2011, 2013).

Another relevant aspect, barely addressed so far, is the analysis of peptide fragments arising from the proteolysis process. In fact, the majority of the studies aimed at assessing the digestion stability of allergens only monitored the degradation of allergens by SDS-PAGE and Western Blot, neglecting the release of immunologically active proteolytic peptides, which escape the electrophoretic detection. Mapping the peanut resistant peptides harbouring IgE epitopes might improve the knowledge about the allergenic determinants and the pathogenic mechanism, paving the way to new immunotherapeutic approaches (Bannon et al., 2001; Li et al., 2003). These considerations prompted us to simulate the digestion of whole raw peanuts using an *in vitro* multicompartmental static digestion model with physiological relevance (Minekus et al., 2014), which includes the oral, gastric, duodenal and intestinal phases. IgE-binding (poly)peptides resulting from digestion were characterized by integrated proteomic/peptidomic and immunochemical assays.

2. Materials and methods

2.1. Chemicals

Raw peanuts (Virginia variety) were provided by Besana (Milano, Italy). HPLC-grade solvents were from Merck (Whitehouse Station, NJ, USA). Pepsin, trypsin, chymotrypsin, dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, urea, guanidine chloride, ammonium bicarbonate (Ambic), phospholipids, trichloroacetic (TCA), trifluoroacetic (TFA), *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) and the modified Lowry assay kit were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was from Lipid Products (Redhill, UK). Reagents for electrophoresis analysis were from Bio-Rad (Milan, Italy). Brush border membrane (BBM) vesicles were purified from porcine jejunum according to Cheeseman and O'Neill (2006), as previously detailed (Picariello et al., 2015). The aminopeptidase activity was determined by colorimetric assay using *p*-nitroaniline as the substrate, while the total activity of BBM peptidases was assayed by HPLC using angiotensin I as a substrate (Picariello et al., 2015).

2.2. Sera of peanut allergic patients

Sera were collected from peanut allergic subjects (N = 8, 80% male), all from Regione Campania (Southern Italy), according to the ethical requirements. The local Ethics Committee approved the study. The allergy symptoms ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table S1. Diagnosis of IgE-mediated allergy to peanut was confirmed by skin prick test (SPT) and oral food challenges. Either a SPT peanut extract or fresh peanut (prick-by-prick) was applied to the patients' volar forearm. Tests were performed using a 1-mm single peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/mL) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in millimeters) of the wheal and flare at 15 min. A SPT result was considered "positive" if the wheal was 3 mm or larger, without a reaction to the negative control.

The total serum IgE was quantified with the ImmunoCAP system (Phadia, Uppsala, Sweden). All the serum samples were stored at −20 °C before being used. Any sensitization was regarded as positive when the total IgE was greater than 0.35 kUA/L.

2.3. In vitro gastroduodenal-BBM digestion of whole peanuts

In vitro oral-gastro-duodenal digestion of peanuts was performed in triplicate, according to Minekus et al., (2014). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions. All digestion steps were carried out in a shaking incubator at 37 °C and 170 rpm. For the oral phase, peanuts were grossly minced using a coffee grinder and 100 mg of the resulting coarse powder was suspended in 207 μL of SSF (included of 1500 U/mL of human salivary amylase) and incubated for 2 min. Subsequently, oral digesta were mixed with 320 μL SGF containing 8 μL of previously sonicated phospholipids (10 mg/mL). The pH was adjusted to 2.7 and 40 μL of porcine pepsin (3000 U/mg) at a concentration of 12 mg/mL was added. Sample was incubated for 2 h at 37 °C. Pepsin hydrolysis was stopped by raising the pH to 7.0 with 1 M sodium bicarbonate. The duodenal digestion was carried out 2 h at 37 °C after incorporating 640 μL of SIF, bile salts (16 mg), porcine pancreatic lipase (1 mg), trypsin (0.7 mg, 100 U/mg as TAME activity), α-chymotrypsin (0.3 mg, 40 U/mg) and pancreatic α-amylase (1.1 mg, 10 U/mL). A final step of intestinal digestion was

performed with of BBM (13 μU/μL, final concentration) after adjusting pH to 7.2 with 1.0 M sodium bicarbonate. After 4 h at 37 °C, peptidases were inactivated by immersion in boiling water for 5 min. Digesta were then filtered by Millex GV 0.22 μm (Millipore, Billerica, MA, USA) and lyophilized.

2.4. Purification of peanut digesta samples

After digestion, sample was defatted through two 10 min extraction with diethyl ether under magnetic stirring, followed by centrifugation at 10,000*g* (10 min). Large-sized polypeptides were precipitated with TCA up to a final concentration of 30% (w/v). After centrifugation, pellet was four-fold washed with 1 mL of cold acetone, to remove residual TCA. The protein pellet was resuspended in 50 mM Tris-HCl, pH 7.0 and fractionated with Econo-pack 10-DG size exclusion chromatography (SEC) pre-packed columns (Bio-Rad), using 50 mM Tris as the eluent. Effluents were collected in 1 mL fractions and polypeptides monitored by the UV-absorbance at 280 nm (Ultrospec 160 2100 pro, Amersham Biosciences, Milan, Italy). Proteins (>6 kDa) and peptide (MW < 6 kDa) fractions were separately pooled. The low-sized peptide fraction was further desalted using Sep-Pak C18 pre-packed cartridges (Waters, Milford, MA, USA) washed with aqueous 0.1% TFA (v/v) and eluted with 70% ACN (v/v)/0.1% TFA (v/v). Proteins and peptides were concentrated in a speed-vac and finally lyophilized.

2.5. SDS–PAGE analysis

Digested protein fraction >6 kDa and urea-extracted proteins were loaded onto a precast 12% polyacrylamide gel (Bio-Rad) either under reduction or non-reduction conditions for SDS-PAGE analysis. The whole protein fraction of peanuts, extracted according to Koppelman et al., (2016), was used as a reference to monitor the proteolysis. Proteins were visualized with blue silver (G250) staining. The gel was imaged with a scanner and processed using the LABScan software 3.00 (Amersham Bioscience).

For proteomic analysis, protein bands were manually excised, destained with acetonitrile/25 mM Ambic (1/1, v/v) and dried under vacuum after dehydration in acetonitrile. Gel pieces were rehydrated with 20 μL of a 12 ng/μL trypsin solution in 50 mM Ambic for 45 min on an ice-cold bath. Afterward, the excess of trypsin solution was discarded and the protein bands were incubated overnight at 37 °C. The tryptic peptides were two-fold extracted in 40 μL of 50% acetonitrile containing 2.5% (v/v) formic acid and dried using a speed-vac.

2.6. Western blotting analysis

IgE binding peanut proteins were detected by immunoblot analysis, performed using a pool of sera from N = 8 children allergic to peanuts, as the source of specific IgE. SDS-PAGE resolved proteins

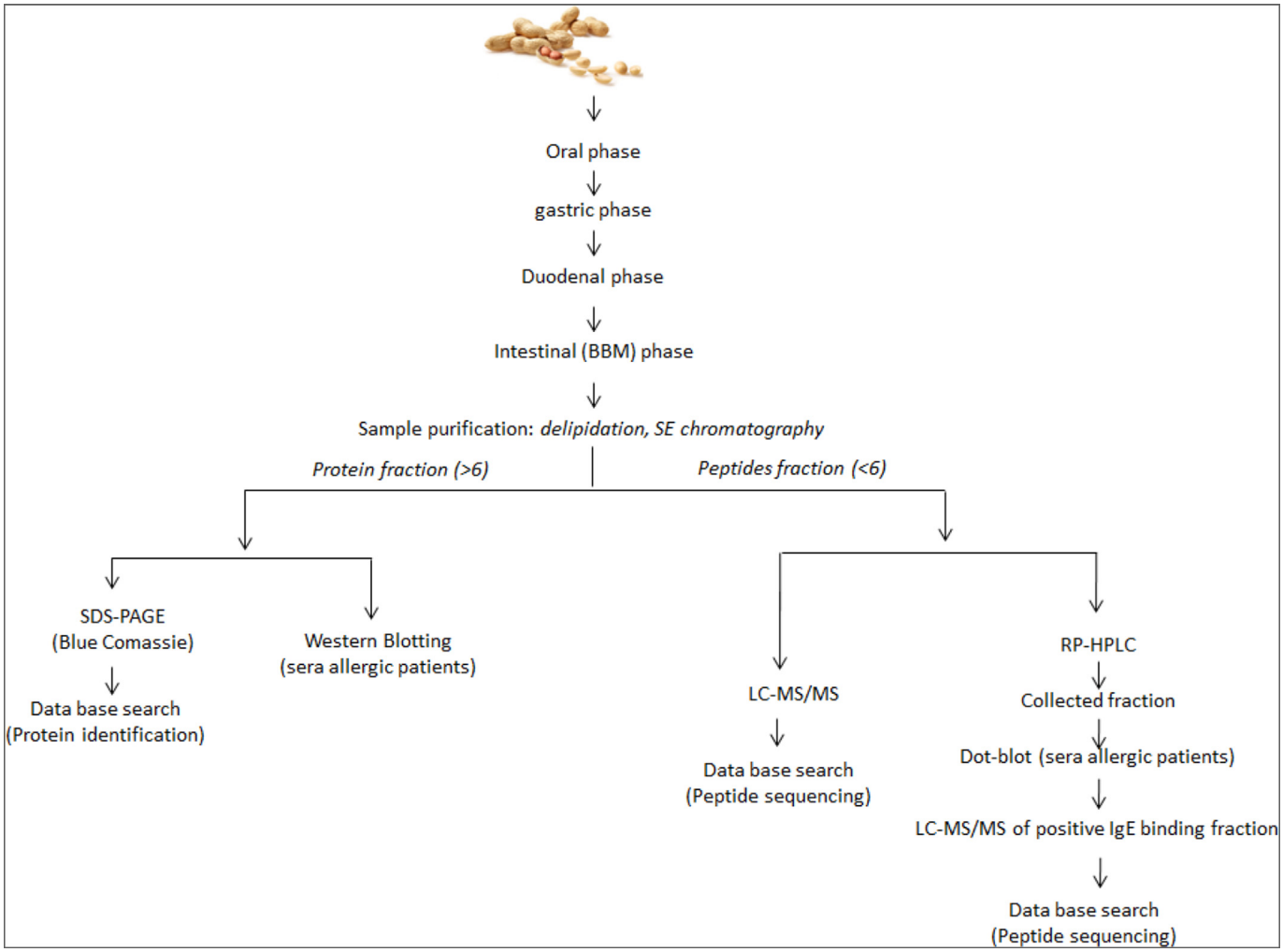

Fig. 1. Schematic workflow of the experimental approach employed (immunoassay and MS-based analysis) for the study and characterization of proteolytic digesta resulting from *in vitro* simulated digestion of whole peanuts.

were electroblotted onto nitrocellulose membrane using a Trans-blot cell (Bio-Rad) at 120 V for 60 min. The membrane was blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin.

(Sigma-Aldrich) in a Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C with the pooled sera diluted 1:50 in TBS-T. After extensive washing (3 × 10 min with TBS-T), the membrane was incubated 1 h with monoclonal peroxidase-conjugated anti-human IgE antibody developed in goat (Sigma, cod. A9667), diluted 1:10000 with TBS-T. The membrane was rinsed with TBS-T (3 × 10 min) and finally with TBS (1 × 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare. Milan Italy) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times (0.5–5 min range).

2.7. LC-high resolution (HR)-MS/MS analysis

Mass spectrometry analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were resuspended in 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 μm i.d. pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C18 column (2 μm, 15 cm × 75 μm) 3 μm 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 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 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were elaborated using the Proteome Discoverer 2.1 software (Thermo Scientific), restricting the search to *Arachis Hypogea* database extracted from the NCBI (downloaded on March 2017).

Database searching parameters for identification of SDS-PAGE protein bands were the following: Met oxidation and pyroglutamic for N-terminus Gln as variable protein modifications; carboxymethylcysteine as a constant modification; a 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. Database searching parameters for identification peptides is digests were the same described 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 four sequenced peptides.

2.8. RP-HPLC and DOT-BLOT analysis

Peanut digests were fractionated using a HP1100 modular system (Agilent, Palo Alto, CA, USA) equipped with a RP-HPLC a C18 (5μ, 4.6 mm i.d., 300A, 250 mm) reverse-phase column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min. Solvent A was 0.1% TFA (v/v) in water; solvent B was 0.1% TFA in acetonitrile. Separation of the peptides was effected with a 5–70% linear gradient of solvent B over 90 min, following 5 min of isocratic elution at 5% B. The column effluents were monitored at λ = 214 and 280 nm using a diode-array detector.

For dot-blot assay, the manually collected HPLC fractions were spotted onto a 0.22-μm trans-blot nitrocellulose membrane (Bio-Rad) and developed using the same protocol as the Western blot assay. The whole protein extract was used as the positive control.

3. Results

3.1. Gastrointestinal digestion of whole peanut

The stability of IgE binding proteins following gastrointestinal digestion of whole raw peanut, was determined by immunochemical and proteomic analysis, according to the workflow diagram shown in Fig. 1.

A widespread commercial variety of peanuts (Virginia *cv*) was used in this study (Koppelman et al., 2016). Whole peanuts were digested according to the harmonized *in vitro* static digestion

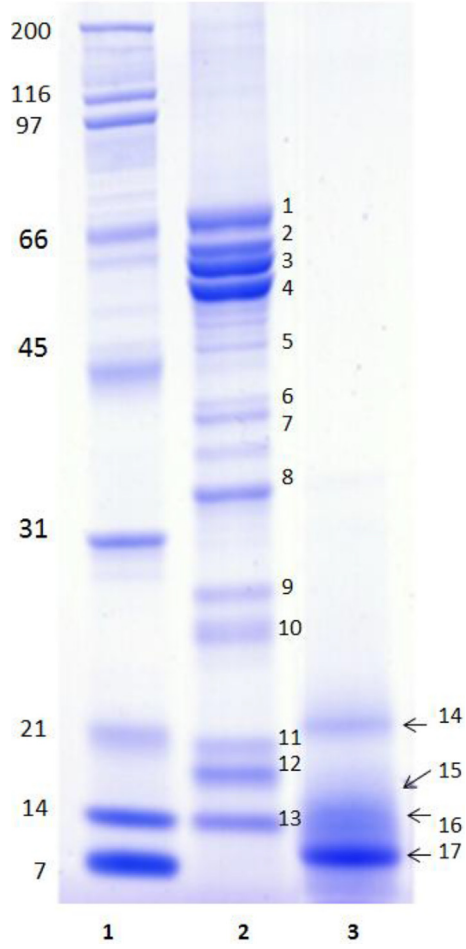

Fig. 2. SDS-PAGE comparison of undigested and digested peanut proteins under non-reducing conditions. Lane 1, molecular marker. Lane 2, whole peanut protein extract (urea extract). Lane 3, protein extract obtained from *in vitro* simulated gastrointestinal digestion of whole peanuts.

model (Minekus et al., 2014), based on the following phases: oral phase through mastication, gastric and duodenal phases with a sequential addition of digestive enzymes in physiological concentration ranges. The model was finally integrated with pig intestinal BBM mimicking the jejunal phase of peptide degradation. After *in vitro* digestion, samples were defatted and protein and peptide fractions were separated by SEC.

3.2. SDS-PAGE analysis digesta sample

The disappearance of peanut proteins after simulated digestion was monitored by SDS-PAGE (Fig. 2), compared with whole protein extract as the reference control of undigested proteins. Individual electrophoretic bands were identified by peptide sequencing using LC-MS/MS analysis, as reported in Table 1. Ara h 1 was identified at approximately 68.2 kDa (band 1) and as less abundant isoforms at 58.2 (band 2) and 33.4 kDa (band 8). Ara h 3 migrated in three different bands at 58.2 54.9 and 52.4 kDa (band 2, 3, and 4 respectively), close to Ara h 1. Low-abundance bands identified as Ara h 3 were detected between 52.4 and 17.2 kDa (bands 5–12). The high heterogeneity observed for Ara h 3 is a consequence of multiple post-translational proteolytic events, involving the N-terminal of the acidic subunit, which produces isoforms of various molecular size (Piersma et al., 2005). A similar processing has already been described for 11S glycinin storage proteins from other plant sources (Dickinson et al., 1989). The Ara h 2 migrated as a doublet at 17.8 (band 11) and 17.2 kDa (band 12) which were assigned to the Ara h 2.01 and Ara h 2.02 isoforms, respectively, according to migration (Koppelman et al., 2010). The Ara h 6 was identified as a well resolved band at 16.3 kDa (band 13). Minor allergens Ara h 7, Ara h 8 and Ara h 10 were also detected in band 12.

As expected, after gastrointestinal digestion of whole peanuts, the electrophoretic protein pattern radically changed, since large-sized proteins were no longer detectable, while the gel exhibited multiple polypeptide fragments at MW estimated between 7 and 23 kDa (Fig. 1). MS/MS-based analysis revealed the presence of Ara h 3 fragments in all these bands (bands 14–17), indicating that large domains of Ara h 3 survived proteolytic degradation. Ara h 6 fragments were detected in band 16 and 17, whilst practically intact residual Ara h 2 was revealed exclusively in band 15. No signal assigned to Ara h1 allergen was detected in the gel, confirming its susceptibility to digestion leading to low MW peptide products (Koppelman et al., 2010).

3.3. Western blotting of digesta sample

The IgE-reactivity of digested and undigested peanut proteins was assayed by immunoblotting using pooled sera from eight pediatric patients diagnosed with food allergy to peanut (Fig. 3). Before digestion, almost all the major peanut proteins appeared as IgE-reactive, mainly due to the individual heterogeneity in the recognition patterns of the allergic subjects. Consistently with SDS-PAGE, native allergens were no longer detectable in gastrointestinal digests, while neo-formed low MW bands were immunoreactive. The immunoreactive band at 23.0 kDa was identified from the corresponding Coomassie stained electrophoretic gel (Fig. 1, Table 1) as Ara h 3 fragments. The IgE-binding fragments in the immunoreactive bands between 20 and 7 kDa were not univocally assigned, due to co-migration of fragments arising from Ara h 2, Ara

Table 1
Identification of protein bands from SDS-PAGE through LC-HR-MS/MS of in gel produced tryptic peptides (Fig. 2).

	Band[a]	Accession	Description	Allergen name[b]	Coverage[c]	Peptides[d]	aa[e]	MW [kDa][f]	calc. p*I*[f]	Score Sequest HT[g]	Peptides SEQUEST HT[h]
Before digestion	1	N1NG13	Seed storage protein Ara h1	Ara h 1	65	64	626	71,302	7,06	689,59	64
	2	Q8LKN1	Allergen Arah3/Arah4	Ara h 3	73	32	538	61,7	5,72	489,49	32
		N1NG13	Seed storage protein Ara h1	Ara h 1	59	43	626	71,302	7,06	266,06	43
	3	Q647H3	Arachin Ahy-2	Ara h 3	56	31	537	61,494	5,73	499,05	31
	4	Q647H3	Arachin Ahy-2	Ara h 3	55	25	537	61,494	5,73	182,64	25
	5	Q647H3	Arachin Ahy-2	Ara h 3	50	23	537	61,494	5,73	205,92	23
	6	Q8LKN1	Allergen Arah3/Arah4	Ara h 3	47	18	538	61,7	5,72	116,78	18
	7	Q9FZ11	Gly1	Ara h 3	58	27	529	60,412	5,64	225,42	27
	8	A1DZF0	Arachin 6	Ara h 3	48	24	529	60,339	5,54	336,71	24
		N1NG13	Seed storage protein Ara h1	Ara h 1	43	26	626	71,302	7,06	137,80	26
	9	A1DZF0	Arachin 6	Ara h3	33	18	529	60,339	5,54	94,68	18
	10	A1DZF0	Arachin 6	Ara h 3	59	32	529	60,339	5,54	356,90	32
	11	Q6PSU2	Conglutin-7	Ara h 2	40	5	172	20,102	6,34	31,61	5
		Q647G5	Oleosin 1	Ara h 10	27	3	169	17,741	9,58	15,39	3
	12	A1DZF0	Arachin 6	Ara h 3	33	15	529	60,339	5,54	77,368	15
		Q6PSU2	Conglutin-7	Ara h 2	36	5	172	20,102	6,34	41,520	5
		Q647G5	Oleosin 1	Ara h 10	36	6	169	17,741	9,58	23,170	6
		B0YIU5	Ara h 8 allergen isoform	Ara h 8	38	3	153	16,402	5,2	16,86	3
		B4XID4	Ara h 7 allergen	Ara h 7	26	3	164	19,326	7,9	9,83	3
	13	A5Z1R0	Ara h 6	Ara h 6	61	9	145	16,909	6,52	60,31	9
After digestion	14	Q9FZ11	Gly1	Ara h 3	32	12	529	60,412	5,64	33,78	12
	15	Q5I6T2	Arachin Ahy-4	Ara h 3	32	14	531	60,699	5,48	56,624	14
		52001227	2S protein	Ara h 2	46	11	179	20,837	7,36	52,84	11
	16	Q9FZ11	Gly1	Ara h 3	34	15	529	60,412	5,64	64,41	15
		A5Z1R0	Ara h 6	Ara h 6	57	11	145	16,909	6,52	62,38	11
	17	Q9FZ11	Gly1	Ara h 3	18	8	529	60,412	5,64	42,81	8
		A5Z1R0	Ara h 6	Ara h 6	52	8	145	16,909	6,52	29,11	8

[a] Protein band from SDS-PAGE (Fig. 2).
[b] Allergen name according to WHO/IUIS database (www.allergen.org).
[c] Sequence coverage (%).
[d] Number of peptides identified.
[e] Number of amino acids (AA).
[f] Theoretical Mr and p*I* values.
[g] Sum of the scores of the individual peptides from the Sequest HT search.
[h] Numberof distinct peptide sequences in a protein group from the SEQUEST HT search.

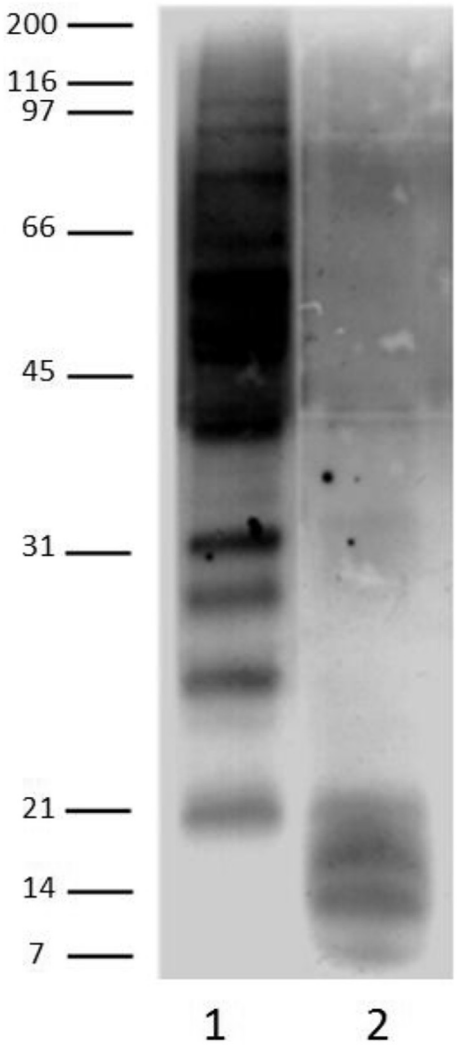

Fig. 3. Western Blotting analysis. Undigested (lane 1) and digested (lane 2) peanut extracts immunostained using specific IgE from pooled sera of (N = 8) pediatric allergic subjects.

h 3 and Ara h 6.

3.4. LC-MS/MS analysis of digesta sample

The peptide fraction of digested samples collected after SEC separation (see Fig. 1) was analyzed by LC-HR-MS/MS to identify the released peptides resistant to digestion. Table 2 lists the parent proteins identified through MS/MS based peptide sequencing. Details about peptide sequences are reported in Table S2. Fig. 4 highlights the sequence of peptides identified in the digesta belonging to Ara h 1, Ara h 3, Ara h 2 and Ara h 6 main isoforms. Notably, protein coverage data generated by peptide sequencing, might be not fully exhaustive, considering that database search is challenged by the non-tryptic nature of the peptides, the non predictable cleavage specificity as well as the extreme heterogeneity of the parent protein subset. Despite such drawbacks, LC-HR-MS/MS analysis revealed a heterogeneous mixtures of peptides (nearly 800 identified sequences) with molecular size up to 5000 kDa. The bulk of signals was assigned to fragments of Ara h 3 isoforms, which mapped 69% of the primary structure, indicating a significant stability of Ara h 3-derived peptides to gastrointestinal protease.

A lower number of peptides (MW between 400 and 2000 Da) matched with Ara h 1, showing a sequence coverage of 22%. Peptides arising from Ara h 2 and Ara h 6 digestion were detected as well. In particular, two and five peptides from Ara 6 and Ara h 2 were identified, respectively. Similarly, six peptides attributed to Ara h 8, occurred in the peptide mixture. Proteolytic fragments of actin, conarachin, 13-lipoxigenase were also detected, though with poor sequence coverage. Three peptides arising from the digestion of an olesin allergen, namely Ara h 10, were identified as well. As regarding these hydrophobic proteins, we can not exclude that large oleosin-derived peptides remained embedded in the lipid matrix and were removed during defatting process of digesta.

3.5. Characterization of IgE binding peptides

IgE binding capability of peptides (<6 kDa) was assessed by HPLC fractionation and Dot-blot analysis (Fig. 5a and b). Six out of sixteen spotted fractions (panel B) tested positive to IgE. Fractions 9, 10, 12, 13 and 16 exhibited the most intense response, indicating the presence of peptide(s) harboring IgE epitope(s). Fraction 5, 8 and 14 were also positive, although at a weaker intensity. IgE positive-HPLC fractions were sequenced by LC-HR-MS/MS (Table 3). Details about MS/MS based peptide sequencing are given in Table S3. The reference linear IgE-binding epitopes are taken from previous studies (Rabjohn et al., 1999; Rougé et al., 2009; Jin et al., 2009; Shinmoto et al., 2010). Overall, IgE-binding peptides were from Ara h 3, releasing three specific domains that appear particularly resistant to proteolysis (Fig. 4, boxed regions). In the HPLC peaks 12 and 13, "*chopped*" forms of a single peptide deriving from the protein region 260–274 (referred to amino acid sequences of Q5I6T2) (Fig. 4) harbored the epitope GNIFSGFTPEFLEQA (Rabjohn P. et al., 1999; Jin et al., 2009). The same epitope was also encrypted in peptides arising from Ara h 3 isoform (Q9FZ11), where an alanine is replaced by a glutamic acid residue (GNIFSGFTPEFLAQA) (Table 3). In contrast, the HPLC peaks 8, 9 and 10 contained peptides from Ara h 3 isoforms encrypting the IgE-binding epitope KNNNPFKFFVPP (Rougé et al., 2009). Partial sequences of a further Ara h 3 linear epitope (KKNIGRNRSPDIYNP), identified by Rougé et al., (2009), eluted in the HPLC peaks 14 and 16. Finally, the N-terminal fragment of Ara h 3 harboring the IgE-binding epitope IETWNPNNQEFECAG (Rabjohn et al., 1999) occurred in fraction 5. Noteworthy, exclusively Ara h 3 fragments were IgE-positive, whilst no immunoreactive peptide of Ara h 1 was identified.

4. Discussion

The mechanism by which dietary proteins sensitize and elicit an allergic reaction remains substantially unresolved. In particular it is still fervently debated whether gastrointestinal digestion stability could be an effective predictor of allergenicity. Anyway, it is widely accepted that many among the most common food allergens are digestion resistant proteins and probably induce sensitization at the level of the intestinal tract (Asero and Antonicelli, 2010). Unquestionably, the digestion stability increases the probability that a food protein (or its derived peptide) can sensitize an individual, because in addition to the skin, respiratory and oral mucosa ones, also the intestinal route of sensitization becomes accessible. In the last years, the scientific community has paid remarkable attention to the evaluation of the stability of food allergens along the human digestive tract, also providing information at the molecular level about the proteolytic fraction resulting from the digestion process (Huby et al., 2000). In a perspective of molecular resolved diagnostic and therapeutic approaches, these insights contribute to the precise identification of epitopic determinants able to reach the

Table 2
Identification of parent peanut proteins through LC-HR-MS/MS sequencing of peptide digests.

Accession	Description	Allergen name[a]	Coverage[b]	Peptides [c]	aa[d]	MW [kDa][e]	calc. pI[e]	Score SEQUEST HT[f]	Peptides SEQUEST HT[g]
Q5I6T2	Arachin Ahy-4	Ara h 3	69	403	531	60,69	5,48	6531	403
Q9FZ11	Gly1	Ara h 3	69	392	529	60,41	5,64	6523	392
B5TYU1	arachin Arah3 isoform	Ara h 3	69	346	530	60,58	5,55	5560	346
A1DZF0	arachin 6	Ara h 3	69	356	529	60,33	5,54	5925	356
Q0GM57	iso-Ara h3	Ara h 3	69	260	512	58,22	5,59	3464	260
P02872	peanut agglutinin precursor	Ara h agglutinin	35	25	273	29,30	5,66	224	25
B0YIU5	Ara h 8 allergen isoform	Ara h 8	69	6	153	16,40	5,2	41	6
A0A0A1EUV7	actin		25	15	323	35,86	5,97	168	15
N1NG13	Seed storage protein Ara h1	Ara h 1	22	41	626	71,30	7,06	630	41
B3IXL2	Main allergen Ara h1	Ara h 1	22	35	614	70,24	6,86	554	35
Q647H1	Conarachin		20	28	662	75,88	5,4	232	28
Q647H2	Arachin Ahy-3	Ara h 3	18	16	484	54,53	5,59	130	16
Q6PSU2	seed storage protein SSP1	Ara h 2	16	5	187	21,77	6,39	56	5
Q647G5	oleosin 17.8	Ara h 10	13	3	169	17,74	9,58	8	3
Q647G9	Conglutin	Ara h 6	7	2	145	16,91	6,01	36	2
A1DZE9	Conglutin 8 hypogaea	Ara h 6	7	3	145	16,82	6,54	20	3
Q4JME6	13-lipoxygenase		3	5	863	97,41	5,53	28	5

[a] Allergen name according to WHO/IUIS database (www.allergen.org).
[b] Sequence coverage (%).
[c] Number of peptides identified.
[d] Number of amino acids (AA).
[e] Theoretical MW and p*I* values.
[f] Sum of the scores of the individual peptides from the SEQUEST HT search.
[g] Number of distinct peptide sequences in a protein group from the SEQUEST HT search.

Fig. 4. Primary sequences of the major peanut allergens Ara h 1, Ara h 3, Ara h 2 and Ara h 6. The highlighted domains correspond to peptides arising from simulated digestion of whole peanut, identified by LC-HR-MS/MS. The underlined sequences belonging to Ara h 1 and Ara h 3 correspond to predicted IgE-binding regions. The boxed sequences are Ara h 3 IgE-binding peptides as assessed by Dot-blot and LC-HR-MS/MS.

intestinal mucosa in immunological active form, with relatively high likelihood to trigger immunological reactions in sensitised subjects. To this aim, more or less physiological-correspondent *in vitro* protocols have been developed to simulate human digestion. Early attempts evaluated the digestion stability of purified allergens by mimicking only the gastric phase of digestion (Astwood et al., 1996). Later on, digestion protocols were extended also to the pancreatic phase (Fu et al., 2002; Wickham et al., 2009). In the case of peanuts, nearly all model studies indicated that Ara h 1 and Ara h 3 are broken down into small peptide fragments within minutes, while Ara h 2 and Ara 6 remain almost unaffected by digestion (Koppelman et al., 2010). The digestion resistance might justify the predominant clinical relevance of Ara h 2 and Ara h 6 allergens (Flinterman et al., 2007). At the same time, these outcomes left partially unanswered the question why and how extremely proteolysis-labile proteins, such as Ara h 1 and Ara h 3, could be associated with primary sensitization.

The digestion and the immunogenic potential of allergenic proteins in a more realistic context, also taking into consideration the allergen containing food matrix, have remained poorly investigated so far. In this paper, we aimed at investigating the stability of peanut allergens throughout the digestive process and at identifying the potential antigenic determinants surviving the digestion. However, compared to the existing works, which are for the most tailored to the assessment of allergen stability using standard purified proteins, we herein introduced a further complexity factor represented by the whole peanut matrix to reproduce more realistically what happens after consumption of peanuts. To this purpose, we applied the harmonized *in vitro* digestion procedure, developed for mimicking protein degradation of unfractionated foodstuff (Minekus et al., 2014). This digestion model was also integrated with an additional step with porcine jejunal BBM enzymes. The jejunal phase of peptide degradation is a fundamental step for assessing the intestinal stability of large protein fragments produced upstream, during the gastric and duodenal phases (Mamone et al., 2009, Picariello et al., 2016).

In line with previous studies (Apostolovic et al., 2016), the outcomes of the digestion assays confirmed the substantial stability of Ara h 2 and Ara h 6, since these latter produced protein fragments with MW only slightly lower than their parent proteins, as detected by SDS-PAGE. Consistently, LC-HR-MS/MS of the small MW peptides revealed that Ara h 2 and Ara h 6 released a number of peptides relatively low.

The most striking result of the current study was the additional identification of digestion stable Ara h 3 large sized fragments (7–21 kDa). Such a finding contrasts with most of the previous literature, which claimed the almost complete susceptibility of Ara h 3 to gastrointestinal proteases (Koppelman et al., 2010). The partial resistance of Ara h 3 to digestion was also confirmed by LC-HR-MS/MS analysis of peptide digests, that contained several peptides with MW between 800 and 5000 Da mapping the Ara h 3 isoforms for 69% overall. A reasonable explanation of this finding may be the "*masking effect*" by the peanut matrix, delaying or impairing the protein degradation and altering the pattern of the peptide fragments released by proteolysis. Indeed, the peanut body includes a medium protein content and high level of lipids and polysaccharides, which may affect the proteolysis, sparing several immunological active polypeptides. It has been demonstrated that some type of polysaccharides decrease the digestibility of peanut allergens and increased the number of large-sized IgE-binding polypeptides (Mouécoucou et al., 2004). Similarly, reports assessing digestion stability of other food allergens (e.g. β-lactoglobulin, Act d 2 kiwi allergens, β-conglycinin) confirmed this trend (Mouécoucou et al., 2004; Polovic et al., 2007; De Angelis et al., 2017). The presence of lipids, like phospholipids naturally occurring in foods may greatly alter susceptibility of allergens to

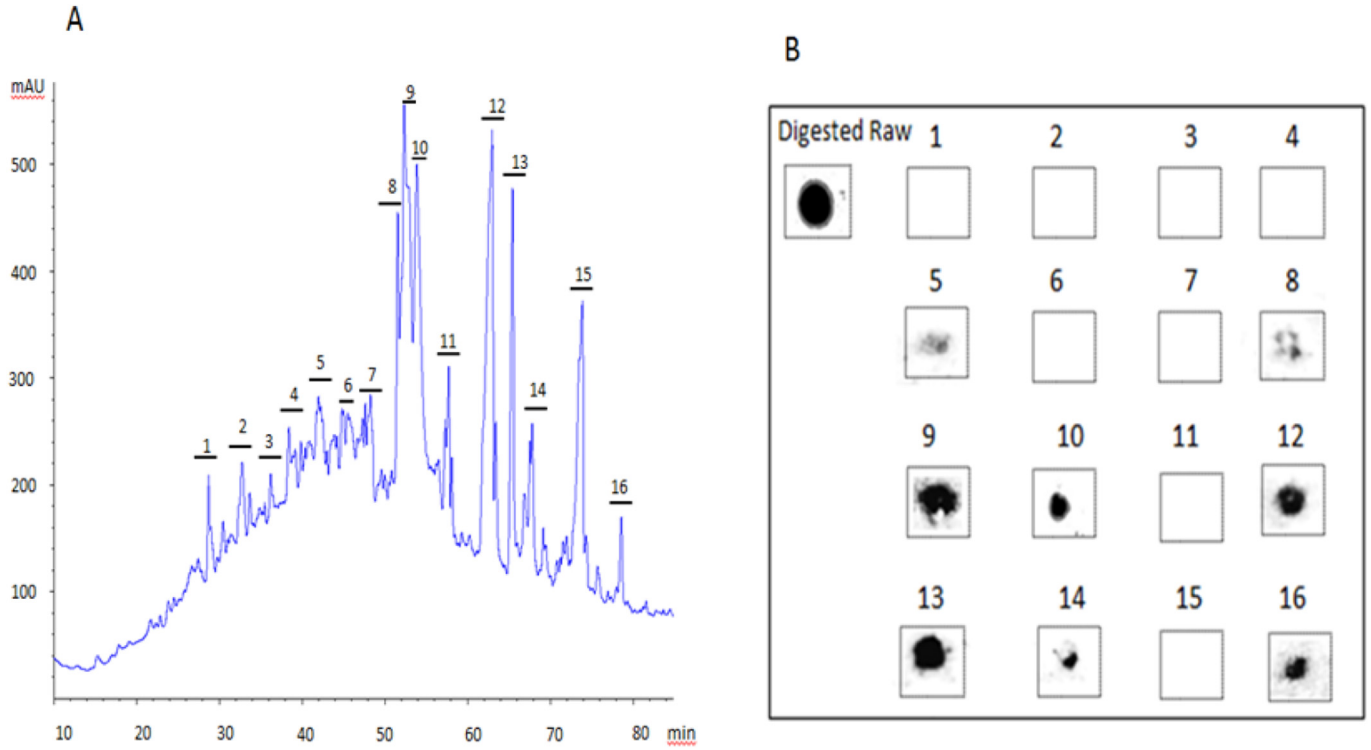

Fig. 5. HPLC fractionation (A) and dot-blot analysis of individual fractions (B) of low MW peptides (<6 kDa) arising from simulated digestion of whole raw peanut. IgE-immunoreactive peptides were identified by LC-HR-MS/MS.

Table 3
Sequences harbouring IgE binding peptides, identified in IgE-immunoreactive HPLC fractions (Fig. 5). Details about MS/MS based peptide sequencing are reported in Table S3.

Accession	Amino acid sequence	LC	MH^+(Da)
Q647H3; Q9FZ11	LNAQRPDNRLESEGGYIETWNPNN	#5	2787.30979
Q8LKN1; Q647H4; Q6IWG5; Q9SQH7; Q5I6T2.	LNAQRPDNRIESEGGYIETWNPNN	#5	2787.30979
Q647H4.	NRSPDIYNPQAGSLKTANELNLLILR	#8	2910.57749
Q9SQH7	QLKNNNPFKFFVPPFQQSPR	#8	2416.25553
	—KNNNPFKFFVPPFQQSPR		2192.13993
Q647H3;	NRSPDIYNPQAGSLKTANDLNLLIL—	#9	2740,46281
Q5I6T2	NRSPDIYNPQAGSLKTANELNLLILR		2910,57957
Q647H3;	QLKNNNPFKFF—	#10	1379,71064
Q5I6T2	—FKFFVPPFQQSP		1468,76234
	—NNPFKFFVPP—		1206,63060
Q9SQH7;	AGQEEENEGGNIFSGFTPEFLEQAFQVDDR—	#12	3360.50791
Q5I6T2	—EEENEGGNIFSGFTPEFLEQAFQVDDR—		3104.37456
	—EGGNIFSGFTPEFLEQAFQVDDRQIVQ		3071.47702
	—EENEGGNIFSGFTPEFLEQAFQVDDR—		2975.33585
	—ENEGGNIFSGFTPEFLEQAFQVDDR—		2846.28862
	—NEGGNIFSGFTPEFLEQAFQVDDR—		2717.23686
	—EGGNIFSGFTPEFLEQAFQVDDR—		2603.20561
Q8LKN1;	AGQEQENEGGNIFSGFTPEFLAQAFQVDDR—	#12	3301.50290
Q647H3; Q647H4	AGQEQENEGGNIFSGFTPEFLAQAF—		2688.22197
Q6IWG5;	QLKNNNPFKFFVPPFQQSPR	#12	2416.24857
Q9SQH7	QLKNNNPFKFFVPPF—		1819.95452
Q5I6T2;	—EEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE—	#13	4141.91421
Q9SQH7	—EENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE——		4012.89492
	EENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLR—		3826.81826
	EGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE—		3754.80979
	EGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE—		3640.76406
	EGGNIFSGFTPEFLEQAFQVDDRQIVQNLR—		3454.71054
	—EEDEGGNIFSGFTPEFLEQAFQVDDR—		2976.32573
	—EENEGGNIFSGFTPEFLEQAFQV—		2589.17803
	EGGNIFSGFTPEFLEQAFQVD—		2332.08745
Q8LKN1;	AGQEQENEGGNIFSGFTPEFLAQAFQVDDRQIL—	#13	3655.73288
Q647H3;	—EENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGEN—		4068.92885
Q647H4;	—EENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGE—		3954.88588
Q9FZ11.	—EENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLR—		3768.84463
	—EGGNIFSGFTPEFLAQAFQVDDRQILQ		3027.48223
	—QENEGGNIFSGFTPEFLAQAF—		2286.03105
	—EGGNIFSGFTPEFLAQAF—		1931.92278
Q647H4.	KKNIGRNRSPDIYNPQAGSLKTANELNLLILRWL	#13	3906.18349
	—KNIGRNRSPDIYNPQAGSLKTANELNLLILRWL		3778.09853
	—NIGRNRSPDIYNPQAGSLKTANDLNLLILRWL		3635.97622
	—RNRSPDIYNPQAGSLKTANDLNLLILRWL		3351.82325
	—NRSPDIYNPQAGSLKTANDLNLLILRWL—		3195.73061
Q6IWG5;	NRSPDIYNPQAGSLKTANELNLLILRWL	#14	3209.73337
Q647H4.	NRSPDIYNPQAGSLKTANELNLLIL—		2754.48044
	NRSPDIYNPQAGSLKTANELNL—		2415.22611
Q647H4;	NRSPDIYNPQAGSLKTANELNLLILRWLGLSAEYGNLYR	#16	4433.33387
Q647H3;	NRSPDIYNPQAGSLKTANDLNLLILRWLGLSAEYGNLYR		4419.32294
Q9FZ11;	NRSPDIYNPQAGSLKTANDLNLLILRWLGLSAEYGNLY—		4263.21840
Q5I6T2	–SPDIYNPQAGSLKTANDLNLLILRWLGLSAEYGNLYR		4149.17348
	NRSPDIYNPQAGSLKTANELNLLILRWLGLSAEYG—		3887.04555
	NRSPDIYNPQAGSLKTANDLNLLILRWLGL—		3365.84731
	NRSPDIYNPQAGSLKTANELNLLILRWL—		3209.74209
	NRSPDIYNPQAGSLKTANDLNLLILRWL—		3195.71914

digestion, as well. For example, phosphatidylcholine hinders the enzymatic degradation of β-lactoglobulin and α-lactalbumin (Moreno et al., 2005; Mandalari et al., 2009).

In addition to proteins, we evaluated the IgE-binding properties of peptides resulting from simulated digestion. By using a combined approach based on HPLC, dot-blot and LC-HR-MS/MS, for the first time, to the best of our knowledge, it was evaluated the IgE-binding property of peptide fragments produced as a consequence of the physiological digestion. Interestingly, linear epitopes GNIFSGFTPEFLEQA and IETWNPNN encrypted in digestion-stable Ara h 3 domains were sequenced. Their epitope nature had previously been assessed using overlapping synthetic peptides (Rabjohn et al., 1999) and their conformation within native Ara h 3 protein was determined by crystallographic methods (Jin et al., 2009). Notably, it has been reported that the side chain of GNIFSGFTPEFLEQA and IETWNPNN was nearly completely buried in the folded native allergen, thereby being not exposed to the IgE capture (Jin et al., 2009). However, as suggested by the same authors and confirmed in current study, the Ara h 3 peptides harboring these epitopes became available for interaction with immune system

effectors upon gastrointestinal release. These same authors also identified two additional linear Ara h 3 epitopes (DEDEYEYD and VTVRGGLRILSPDRK) that, on the contrary, are exposed on the surface of the allergen in the native folding and so immediately available for IgE-binding. Although peptides containing these epitopes occurred in the peanut digesta (Fig. 4, Table S1), none of them was IgE-immunoreactive in our conditions. This apparent incongruence is likely due to a poor specificity of these epitopes for the specific IgE in the pool of pediatric sera used in the current study. To this purpose, it has to be underlined that IgE-based immunochemical assays for the identification of allergenic determinants are in general affected by a high degree of individual variability.

Unlike Ara h 3, Ara h 1 was completely degraded and no derived peptide showed immunoreactivity. LC-HR-MS/MS analysis of digesta revealed Ara h 1 peptides with MW lower than 2 kDa, which mapped 22% of the whole protein. Although some of the Ara h 1 sequenced peptides included already described epitopes (Fig. 4, Table S3), these were not reactive against serum IgE in our conditions. Bøgh et al., 2009 demonstrated that gastro-duodenal digests from Ara h 1, containing peptides of size less than 2 kDa, could induce degranulation response with a similar magnitude as intact Ara h 1. These small peptides were shown to be aggregated in large complexes, which was hypothesized to be the reason for their eliciting capacity (Bøgh et al., 2009).

5. Conclusion

Our results point out the importance to investigate the digestion process of whole food, instead of purified allergen proteins, clearly increasing the correspondence of the model systems with human physiology. Remarkably, at the moment a digestion model assuring the complete *in vitro-in vivo* correspondence is not available, taken into account the large range of factors affecting human digestion and the complexity of an ordinary meal. Notwithstanding this, a static *in vitro* multi-compartmental model, recently developed in the framework of the EU Infogest Cost Action with the precise aim to harmonize digestion conditions based on human physiology, has been applied for the evaluation of allergen stability of food matrices (Picariello et al., 2015; Mamone et al., 2015), providing physiologically consistent outcomes (Egger et al., 2016). Along with the advancement of omic sciences, it is plausible to assume that all products arising from food digestion can be "*simply*" characterized in order to define with high accuracy the metabolic fate of either toxic or bioactive molecules. With regard to food allergens, improving the understanding of the spatio-temporal evolution of allergens in the gastrointestinal tract will facilitate the development of more sensitive and effective antibodies to detect food allergens, pushing to the edge the limit of allergen detection in complex matrices, and will support establishing threshold levels of sensitization/elicitation. In perspective, such an analysis could contribute to predict the allergenicity of proteins from novel and alternative foods.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.06.029.

Transparency document

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Effect of thermal/pressure processing and simulated human digestion on the immunoreactivity of extractable peanut allergens

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ABSTRACT

Peanut allergy is one of the most widespread types of food allergies especially affecting developed countries. To reduce the risk of triggering allergic reactions, several technological strategies have been devised to modify or remove allergens from foods. Herein we investigated the combination of high temperature and pressure on the modulation of peanuts immunoreactivity after simulated gastro-duodenal digestion. Extractable proteins of raw and autoclaved peanuts were separated on SDS-PAGE and immunogenicity was assessed by ELISA and Western Blot analyses. Proteins surviving the heat treatment and reacting towards allergic patients' sera were analysed and attributed to Ara h 3 and Ara h 1 proteins by untargeted LC-high resolution-MS/MS. A progressive reduction in the intensity of the major allergen proteins was also highlighted in the protein fraction extracted from autoclaved peanuts, with a total disappearance of the high molecular allergens when samples were preliminary exposed to 2 h hydration although the lower molecular weight fraction was not investigated in the present work. Furthermore, raw and processed peanuts underwent simulated digestion experiments and the IgE binding was assessed by using allergic patients' sera. The persistence of an immunoreactive band was displayed around 20 kDa. In conclusion, the synergistic effects of heat and pressure played a pivotal role in the disappearance of the major peanut allergens also contributing to the significant alteration of the final immunoreactivity. In addition, the surviving of allergenic determinants in peanuts after gastrointestinal breakdown provides more insights on the fate of allergenic proteins after autoclaving treatments.

1. Introduction

Food-induced allergy (FA) represents a public health problem affecting adults and children with a rising growth throughout the population especially in the developed countries. The current management of FA relies on the strict avoidance of the trigger food (Hebling, Ross, Callahan, & McFarland, 2012; Sicherer et al., 2010). Peanut allergy is one of the most widespread and life-threatening type of food allergy and is considered to be the major cause of anaphylactic shock (Al-Muhsen, Clarke, & Kagan, 2003; Pumphrey & Gowland, 2007).

Currently 16 peanut allergenic proteins have been registered by the IUIS Allergen Nomenclature Sub-Committee under the auspices of the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) in WHO/IUIS Allergen Nomenclature Database (http://www.allergen.org/). Among them, seed storage proteins Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are considered the most important allergens and predictive of allergic reactions (Koppelman, Hefle, Taylor, & de Jong, 2010). These proteins display different chemical, physical and structural characteristics. Ara h 1 is a glycoprotein of 65-kDa belonging to the cupin family. It is the most abundant allergen of peanuts and naturally occurs as a symmetrical non-covalent trimer with a 3-fold axis running between the monomers. Each monomer is comprised of two cupin domains (known as a bicupin) with small cavities flanked by α-helices (Mueller, Maleki, & Pedersen, 2014; Shin et al., 1998; Van Boxtel, Van Beers, Koppelman, Van Den Broek, & Gruppen, 2006).

Ara h 3 also belongs to the cupin family and shares 21% sequence identity with Ara h 1. Despite the low sequence identity, the crystal

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structure of Ara h 3 is very similar to that of Ara h 1. Ara h 3 forms a heat- stable hexameric structure consisting of two Ara h 1-like trimers stacked head to head (Adachi et al., 2003; Boldt et al., 2005; Dodo, Viquez, Maleki, & Konan, 2004; Guo, Liang, Chung, & Maleki, 2008; Liang, Luo, Holbrook, & Guo, 2006). This allergenic protein is post-translationally modified by a proteolytic cleavage that occurs between the two cupin domains on a flexible loop. The processed protein consists of a triplet at approximately 42–45 kDa, another distinct band at approximately 25 kDa, and some less abundant isoforms banding between 12 and 18 kDa (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Piersma, Gaspari, Hefle, & Koppelman, 2005).

On the contrary, 2S albumins Ara h 2 and Ara h 6 together with a third low abundant 2S albumin Ara h 7, have a single chain precursor, proteolytically cleaved in peanut seeds into two subunits linked by intramolecular disulphide bonds (Bernard et al., 2007; Shewry, 1995). All members of this superfamily share a characteristic cysteine skeleton with at least 8 conserved cysteine residues (Shewry, 1995) and a three-dimensional structure comprising 5 α-helices arranged in a right-handed super helix that give the stability to thermal processing and proteolysis (Barre, Borges, Culerrier, & Rougé, 2005; Lehmann et al., 2006; Marsh et al., 2008).

Several strategies have been developed over the years aimed to reduce or prevent peanuts allergenicity and representing potential alternatives to a strict peanuts-free diet. The most interesting ones are based on enzymatic hydrolysis, physical approach or genetic modification methods. Among the physical methods, there are heat-based treatments which involve chemical modification such as denaturation or covalent bound of protein allergen with other nutrients including lipids and carbohydrates (Maillard reaction) (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015). These modifications can produce an effect on the final allergenicity that might vary considerably depending on the temperature, type and duration of the treatment, the intrinsic characteristics of the protein and the physicochemical conditions of the food matrix under investigation (Nesbit et al., 2012; Sathe & Sharma, 2009; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). However, the effect of thermal treatments on peanuts has been questioned in the recent years. Whether roasting was reported to increase Ara h 1 and Ara h 2 allergenicity probably consequent to the formation of new epitopes, other hand treatments such as boiling or autoclaving were reported to effectively decrease peanut allergenicity (Blanc et al., 2011; Cabanillas et al., 2012). Herein we investigated the effect of autoclaving with or without preliminary hydration, performed at the temperature of 134 °C and the pressure of 2 atm, on peanut seeds in order to evaluate any alteration on the final immunoreactivity assessed on the soluble protein fraction by ELISA and western blot analysis by using allergic patients' sera. Furthermore, autoclaved peanuts were submitted to a standardized static *in vitro* digestion protocol in order to assess any change in allergen protein stability as a consequence of the technological process applied.

2. Material and methods

2.1. Chemicals

Trizma-base, sodium chloride, urea, ammonium bicarbonate (AMBIC), iodoacetamide (IAA), along with other chemicals for electrophoresis dithiothreitol (DTT), sodium dodecyl sulfate-SDS, glycine, glycerol, Coomassie brilliant blue-G 250 and methanol (HPLC grade) were purchased from Sigma-Aldrich (Milan, Italy). Bromophenol blue was provided by Carlo Erba Reagents (Cornaredo, Italia). Syringe filters in cellulose acetate (CA) from 1.2 µm were obtained from Labochem Science S.r.l. (Catania, Italy) whilst 0.45 µm filters in Polytetrafluoroethylene (PTFE) were purchased from Sartorius (Gottingem, Germania). Acetonitrile (Gold HPLC ultragradient), and trifluoroacetic acid (TFA) were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italia) and ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (MS grade) was provided by Fluka (Milan, Italy) while trypsin (proteomic grade) for in gel protein digestion was purchased from Promega (Milan, Italy). As for *in vitro* digestion model, pepsin, trypsin, chymotrypsin, Tris-HCl, urea, guanidine chloride, phospholipids and p-toluene-sulfonyl-L-arginine methyl ester (TAME) were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was purchased from Lipid Products (Redhill UK).

2.2. Sera of peanuts allergic patients

Sera were obtained from a total of 8 pediatric peanut allergic subjects with an age comprised between 3 and 8, according to the ethical requirements. The local Ethics Committee approved the study. The allergy symptoms in general ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table S1. Since 2 out of 8 patients deemed allergic to peanuts did not show a meaningful reactivity to the SPT (wheal lower than 0.3 cm) and the specific IgE content was lower than 0.35 kUA/l, only a total of 6 reactive sera were pooled together and used for further analysis. Diagnosis of IgE-mediated allergy to peanut was confirmed by skin prick test (SPT) and oral food challenges. Either a SPT peanut extract or fresh peanut (prick-by-prick) was applied to the patients' volar forearm. Tests were performed using a 1-mm single peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/ml) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in cm) of the wheal and flare at 15 min. A SPT result was considered “positive” if the wheal was 0.3 cm or larger, without a reaction to the negative control. The total serum IgE was quantified with the ImmunoCAP system (Phadia, Uppsala, Sweden) and was found to be ranging between 33 and 1836 kU/l. In particular, 6 out of the 8 patients enrolled in the study were found positive to the SPT and to the IgE assay with specific levels of IgE to peanuts higher than 0.35 kUA/l. All sera were stored at −20 °C before being used. Other details are reported in the manuscript from Di Stasio et al. (2017).

2.3. Autoclaving based treatments

Raw peanut seeds (*Arachis hypogaea* var. *Virginia*) analysed in the present study were provided from Besana s.p.a. (San GennaroVesuviano, NA, Italy). A total of 8 seeds (corresponding to approximately 10 g) were placed into a centrifuge tube and submitted to autoclaving treatments. Two processing schemes were applied including or not a preliminary hydration of the peanuts. The hydration of whole peanut seeds were performed for 2 h at room temperature in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany) with ultrapure water, before autoclaving. Autoclave settings were: temperature at 134 °C at the pressure of 2 atm for 10 min and 20 min, respectively. The system took about 40 min to reach the final temperature of 134 °C.

2.4. Protein extraction and quantification

Ten gram of raw and thermally processed peanut seeds were milled by using an electric miller (Mulinex, Milan, Italy) and an aliquot was extracted by 7 M Urea (pH 8) containing TBS (50 mM Tris-HCl, 150 mM NaCl) buffer. Briefly, 10 ml of extraction buffer were added to 0,4 g of sample and left shaking for 1 h at room temperature in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany). Afterwards, samples were centrifuged for 15 min at 1734*g* at 18 °C, the upper phase was discarded and the supernatant was carefully collected and filtered through 1.2 µm CA syringe filters. Protein concentration of raw and thermally processed peanuts was calculated as mg/albumin equivalent by Bradford assay (Quick Start™ Bradford Protein Assay). Samples were stored at −20 °C until use and filtered through 0.45 µm PTFE filters just before electrophoretic analysis.

2.5. ELISA assay

The decrease in the level of peanut allergens was evaluated by using a commercially available peanut ELISA kit (RidaScreen Fast, R-Biopharm, Germany), according to the instructions provided by the manufacturer. The R-Biopharm kit was directed to detect raw and roasted peanut proteins, although the antibodies immobilized in the kit were mainly raised against Ara h 1 and Ara h 2 proteins (as reported by the instructions). Samples were assayed at 1:10,000 dilution, to obtain test values within the standard calibration curve and analysed in three replicates. Plates were read at the wavelength 450 nm using a microplates reader (BioTek Instruments Inc. USA). Three extracts were analysed for each treatment under investigation and final results underwent statistical analysis according to the Tukey-Kramer test for multiple mean comparison.

2.6. SDS-PAGE analysis

Ten microgram of protein extracts from raw and treated peanuts, along with *in vitro* digested proteins, were separated, under reducing condition, by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8–16% polyacrylamide pre-cast gels (8.6 cm × 6.7 cm × 1 mm) using a Mini-Protean Tetra Cell equipment (Bio-rad Laboratories, Segrate, MI, Italy). Samples were dissolved in a Laemmli buffer (62.5 mM TrisHCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, in the presence or not of 100 mM DTT) (1:1 ratio) and denatured for 5 min at 100 °C. Running buffer was TGS (25 mM Tris, 192 mM Glycine, 0.1% SDS). Electrophoretic separation was performed at 100 V until the end. Gels were stained by using a Coomassie Brilliant Blue G-250 solution and the bands were detected on a Gel Doc EZ Imager system (Bio-Rad Laboratories, Segrate, MI, Italy). Precision Plus Protein™ all blue standards (10–250 kDa, Bio-Rad Laboratories) was used as protein molecular weight referencing.

2.7. In-gel protein digestion

Selected protein bands were cut from the polyacrylamide gel and destained by repeated washing (45 min, 37 °C) with 100 mM AMBIC/acetonitrile (1/1, v/v). Gel slices were further dehydratated in 100 µl of acetonitrile (5 min at room temperature) and dried in a "speed Vac" centrifuge (Christ RVC 2-18, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 10–15 min (room temperature). After drying, proteins were reduced by adding 10 mM DTT (prepared in 25 mM AMBIC) for 1 h at 60 °C and alkylated for 30 min (room temperature) with 55 mM iodoacetamide (prepared in 25 mM AMBIC). Digestion was carried out overnight at 37 °C with proteomic grade trypsin solution (0.1 µg/µl, enzyme: protein ratio 1:50) in 25 mM AMBIC. Successively, gel slices were incubated with 150 µl of MilliQ water for 10 min, with frequent vortex mixing. Then the liquid was removed and transferred into a new microcentrifuge tube. Peptides extraction from gel was accomplished by incubation with 50% acetonitrile/5% trifluoroacetic acid/ (1/1, v/v) for 60 min. This step was repeated twice. Peptide mixtures obtained from each extraction step were then pooled together and dried. Finally each sample was re-suspended in 70 µl of H_2O/ACN, 95/5 + 0.1% formic acid (*v*/v) and 20 µl were further injected into LC/MS apparatus.

2.8. Protein identification by untargeted HR MS/MS analysis

Protein bands were analysed by using a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a UHPLC pump systems (Thermo Fisher Scientific). Peptides mixture was separated on a reversed phase Aeris peptide analytical column (internal diameter 2.1 mm, length 150 mm, particle size 3.6 µm, porosity 100 Å, Phenomenex, Torrance, CA, US) at a flow rate of 200 µl/ml, using the following elution gradient: from: 0–55 min solvent B increased from 5% to 60%, 55–56 min further increase from 60% to 80%, then kept constant for 10 min, 66–85 min at a constant 5% for column conditioning before next injection. MS Spectra were acquired in positive ion mode. The HESI ion source setting are here reported: spray voltage at 3.4 kV, capillary temperature at 320 °C, sheath gas and auxiliary gas flow rates at 25 and 15 arbitrary units, respectively, S-lens at 55. MS analysis was carried out in data-dependent MS^2 acquisition mode (dd-MS^2). Up to 10 most intense ions in MS^1 were selected for subsequent fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), a microscan of 1, an automatic gain control (AGC) target of 1 e6 and a maximum injection time (IT) of 30 ms were set to generate precursor spectra into the scan range 200–2000 m/z (full MS analysis). The parameters for MS^2 fragmentation experiments were set as following: resolving power 17,500 FWHM, microscan of 1, AGC target 1 e^5, maximum IT 60 ms, loop count 10, MSX count 1, isolation window of 2 m/z, isolation offset 0.4 m/z and normalized collision energy (NCE) at 27 eV; as for dd-setting maximum AGC target was set at 5.00 e^2, dynamic exclusion at 20 s, peptide match set to preferred and exclude isotopes enabled. All ions with charge equal to 1 and higher than 4 were excluded.

Raw data were processed *via* the commercial software Proteome Discoverer™ version 2.0 (Thermo-Fisher-Scientific, San Josè, US) and protein identification was achieved by Sequest^{HT} search against a peanut customized database extracted by Swiss Prot DB basing on the taxonomy code of *Arachis hypogaea* (ID: 3818) and containing about 1250 sequences. The identification of tryptic peptides originated by in gel digestion experiments was accomplished by setting at 5 ppm and 0.05 Da, respectively, the mass tolerance on the precursor and fragment ions. Only trustful peptide-spectrum matches were accepted and in particular a minimum of three peptides or higher were the minimum criteria for protein identification by selecting a high confidence (FDR < 1%).

2.9. Immunoblot for IgE-binding assay

SDS-PAGE of peanut protein extracts (corresponding to 5 µg of proteins loaded of both raw and treated peanuts) and SDS-PAGE of *in vitro* digested proteins (approximately 6 µg loaded onto the gel), under reducing and non-reducing conditions, were electroblotted onto nitrocellulose paper using a Trans-Blot Cell from BioRad (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V and 4 °C for 1 h. Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma) in TBS containing 0.05% of Tween 20 (TBS-T). The membranes were incubated overnight at 4 °C with a pool of sera of young allergic patients (3–8 age) and healthy individuals were chosen as negative controls, by 1/20 dilution in TBS-T. After washing with TBS-T, monoclonal peroxidase-conjugated mouse anti-human IgE antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 1 h at room temperature. The membrane was extensively rinsed with TBS-T (3 × 10 min) and finally with TBS (1 × 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times ranging from 0.5 to 10 min.

2.10. In vitro gastroduodenal digestion of raw and treated peanuts

Peanut seeds autoclaved for 10 min with and without pre-hydration, were submitted to *in vitro* gastro-duodenal digestion according to the protocol by Minekus et al. (2014). Raw peanuts were instead used as control. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions. All digestion steps were carried out in a shaking incubator at 37 °C, at 170 rpm. For the oral phase, peanuts were grossly minced using a coffee grinder and 100 mg of the resulting coarse powder was suspended in 207 µl of SSF (included of 1500 U/ml of

human salivary amylase) and incubated for 2 min. Subsequently, the oral digest was mixed with 320 µl SGF containing 8 µl of phospholipids (10 mg/ml). The pH was adjusted to 2.7 with HCl 3 M and 40 µl of porcine pepsin (3000 U/mg) at a concentration of 12 mg/ml was added. Samples were then incubated for 2 h at 37 °C. Pepsin hydrolysis was stopped by raising the pH to 7.0 with 1 M sodium bicarbonate. The duodenal digestion was carried out for 2 h at 37 °C after incorporating 640 µl of SIF, bile salts (16 mg), porcine pancreatic lipase (1 mg), trypsin (0.7 mg, 100 U/mg as TAME activity), α-chymotrypsin (0.3 mg, 40 U/mg) and pancreatic α-amylase (1.1 mg, 10 U/ml). A final step of acidification with HCl was performed to stop the enzymatic reaction. After digestion, samples were subjected to a defatting step with diethyl ether under magnetic stirring (two steps of agitation for 10 min), followed by centrifugation at 10,000*g* (10 min). Large-sized polypeptides were precipitated with TCA up to a final concentration of 30% (w/v). After centrifugation, pellet was four-fold washed with 1 ml of cold acetone, to remove the residual TCA. The final digest was re-suspended in 50 µl of sample buffer before loading it onto the electrophoresis gel.

3. Results and discussion

3.1. Effect of autoclaving on the extractable peanut proteins/allergens as assessed by Bradford and ELISA assays

Raw and autoclaved peanuts were extracted with a 7 M urea extraction buffer, the extract was preliminary quantified by Bradford assay and subsequently analysed by SDS-PAGE. According to the result of the Bradford assay, a decrease in the protein levels was recorded in the extractable protein fraction, which extent varied in dependence of the processing conditions applied. In particular, a protein reduction down to 40 and 25% was pointed out in autoclaved peanuts strictly related to the extension of the autoclaving applied (10 or 20 min), as shown in Fig. 1. Our findings are in agreement with data obtained by Fu et al. that also found a decrease in protein content down to 38% according to the results of the BCA assay performed on peanut flour autoclaved for 10 min (Fue & Macs, 2013). This trend was even more remarked when seeds were hydrated (for 2 h) before autoclaving at 134 °C and 2 atm. In this case, a dramatic reduction in the extractable proteins down to nearly 15% in hydrated and autoclaved samples was highlighted, as pictured in Fig. 1. In order to investigate on the reduction of the main allergenic proteins recognized by the most common antibody-based kits, samples were analysed by ELISA kits. A general decrease in the IgG reactivity was observed after autoclaving. In particular, Fig. 2 reports in histograms the results of the ELISA tests carried out on raw and autoclaved peanuts with and without pre-incubation in water. Compared to the raw material, where a very high reactivity was recorded, in peanuts undergoing the autoclaving treatments preceded or not by hydration, a modulation of the immunoreactivity was observed.

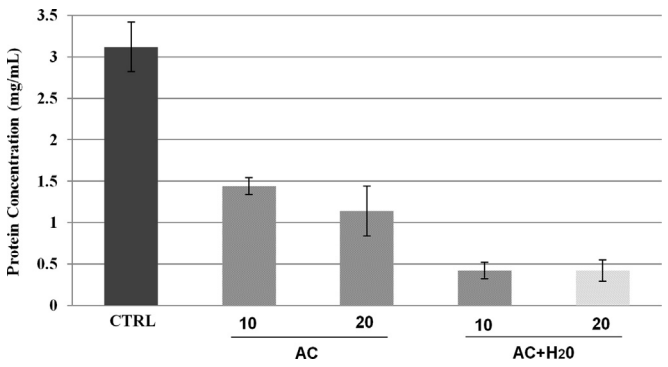

Fig. 1. Protein content in peanut extracts referred to raw (CTR), autoclaved (AC) samples for 10 and 20 min and pre-hydrated and autoclaved samples for 10 and 20 min at 134 °C.

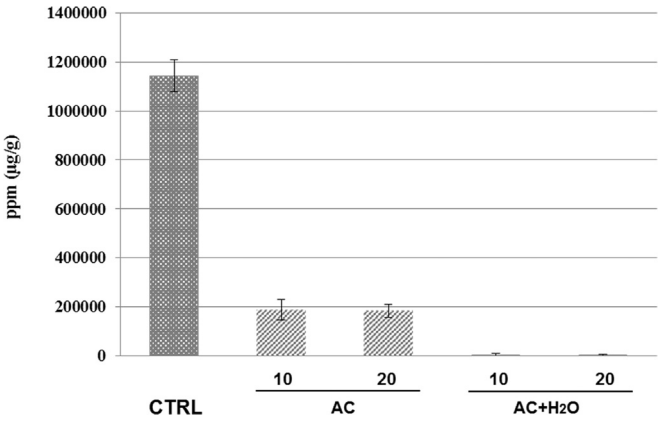

Fig. 2. Immunoreactivity of peanut proteins measured by ELISA referred to raw (CTRL), autoclaved (AC) samples for 10 and 20 min and pre-hydrated and autoclaved samples (AC + H_2O) for 10 and 20 min at 134 °C.

Similar results were also reported by Fu et al. indicating that despite protein quantification by BCA assay performed, the heat treatments resulted in a lower level of peanuts detected by using two different ELISA test kits (Fue & Macs, 2013). The degree of underestimation differed depending on the extent and type of heating applied and the specific test kit employed in the study (Fue & Macs, 2013).

According to Fig. 2, the reduction in immunoreactivity, in peanuts autoclaved for 10 and 20 min, was calculated to be approximately 78% compared to the control. By contrast, when a hydration step preceded the heating, a total absence of antibody reactivity was observed at both investigated times. All results underwent statistical Tukey-Kramer tests for multiple mean comparison and a statistically significant difference in the final immunoreactivity was found. In general, the efficacy of the treatment appeared to be enhanced when a preliminary incubation of the seeds in water was introduced along the procedure. It is worth noting that according to manufacturer instructions, the antibodies immobilized on the ELISA micro wells were directed towards Ara h 1 and Ara h 2; consequently, this kit can assess reactivity towards these only proteins.

3.2. Results of SDS PAGE and MS/MS analysis in peanuts subject to different processing

Peanut extracts were further subjected to SDS-PAGE analysis as shown in the Fig. 3. In addition, in order to deepen the knowledge on the stability of peanut allergens undergoing autoclaving treatments, in-gel tryptic digestions were carried out on selected bands detected in lanes 1–2–3-4 and labelled from *a* to *q* (see Fig. 3) and the resulting peptide pool was further analysed by HPLC and untargeted HR MS detection.

Protein identification was accomplished by means of a commercial software using HR MS and MS/MS spectra obtained for each individual protein band analysed and by putatively assigning each peptide detected to the corresponding peanut protein and/or subunit.

As pictured in the gel (Fig. 3), proteins 1 and 2 detected along lane 1 appeared unresolved, therefore they were pooled together, marked as band *b*, and further processed as a single spot. Table 1 summarizes the results retrieved by the software for each spot analysed. For more info on the list of peptides detected, please see Supplementary material Table S2. Due to the low resolution of the SDS-PAGE technique, several proteins were identified in the same band. As shown in Fig. 3, protein bands referred to raw peanuts (Fig. 3, lane 1) with molecular weight comprised between 60 and 150 kDa (Fig. 3, lane 1, band *a* and *b*) were mainly attributed to Ara h 1, while bands between *c* and *i*, with MW in the range 25–50 kDa were assigned to Ara h 3. Moreover, *c* and *g* bands also contained fragments of Ara h 1. Finally, protein bands at lower MW

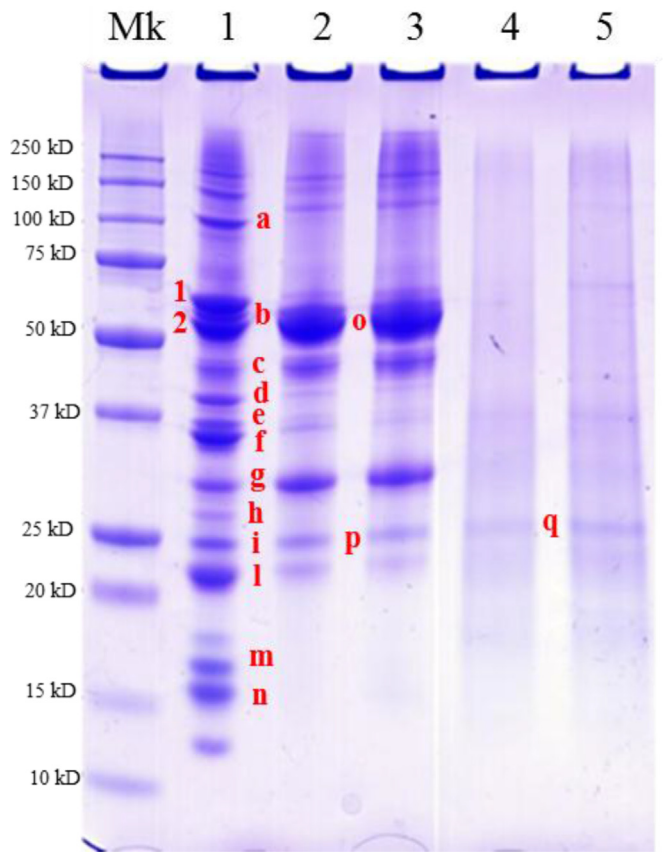

Fig. 3. SDS-PAGE of peanuts submitted to the different treatments: raw (lane 1), autoclaved at 134 °C, 2 atm for 10 min (lane 2) and for 20 min (lane 3), pre-hydrated and autoclaved at 134 °C, 2 atm for 10 min (lane 4) and for 20 min (lane 5).

(below 25 kDa, Fig. 3, lane 1, bands *l*, *m* and *n*) were mainly attributed to Ara h 2, Ara h 6 and Ara h 7, along with some subunits belonging to Ara h 3 group.

By comparing sample protein profiles of raw (Fig. 3, lane 1) and autoclaved (Fig. 3, lanes 2–3, 4–5) peanuts employing or not a pre-hydration, a significant difference in the electrophoretic bands was displayed. Protein bands detected in autoclaved samples at MW above 75 kDa (Fig. 3, band *a*, lane 1) in raw peanuts, showed a marked reduction when samples were autoclaved for 10 and 20 min at 134 °C (Fig. 3, lanes 2 and 3). Proteins banding around 65–60 kDa appeared to be composed by two close bands (*1* and *2* in lane 1): the higher (band 1) attributed to Ara h 1 disappeared after the treatment, whereas the lower showed to be resistant to the autoclaving (Fig. 3, bands *b* and *o* in the lanes 2 and 3). A previous study had already shown that the level of soluble Ara h 1 was greatly reduced in the boiled/autoclaved samples while remained unaffected in samples dry-heated at temperatures up to 176 °C (Fue & Macs, 2013). According to their results, authors demonstrated that the higher temperatures and pressure applied during autoclaving resulted in a similar decrease in protein yield and changes in the intensities of certain protein/peptide SDS-PAGE bands of what we found in our work. Dry-heat treatments also resulted in a decrease in protein solubility, although the decrease occurred at a much higher temperature (≥ 176 °C), suggesting that peanut proteins are more resistant to thermal denaturation under dry-heat conditions (Fue & Macs, 2013). In stark contrast with the bands at higher MWs, the band labelled as 1 in raw peanuts (at nearly 20 kDa) exhibited a much weaker intensity after autoclaving. Basing on bioinformatics tools for protein attribution, band *a* was assigned to Ara h 1, while band *b* (Fig. 3, lane 1, merge of band 1 and 2) and *o* were both assigned to Ara h 1 and to Ara h 3 proteins. The remarked decrease of band intensity after autoclaving, suggests a partial degradation or a rearrangement of the original protein. A thermal resistance, was observed also for bands *c* (with MW slightly below 50 kDa) and *g* (MW approximately 30 kDa), where the intensity was found nearly unaltered both in 10 min and 20 min autoclaved samples (Fig. 3, lanes 2 and 3) compared to raw peanuts. These protein bands were instead attributed both to Ara h 1 and Ara h 3 allergen proteins. By contrast, protein bands around 37 kDa (Fig. 3, lane 1, bands *d*, *e* and *f*) and protein banding at 26 kDa (Fig. 3, lane 1, band *h*), all assigned to Ara h 3 in untreated peanut, were not evidenced in autoclaved samples suggesting a susceptible behaviour of these proteins to the proposed treatment. Similar results were also displayed for proteins banding below 20 kDa (Fig. 3, lane 1, bands *m* and *n*), mainly assigned to Ara h 3. A different behaviour was observed for protein bands with MW between 20 and 25 kDa (Fig. 3, lane 1, bands *i* and *l*), where a partial reduction of intensity was displayed after autoclaving (Fig. 3, lane 2–3, bands *p*). Interestingly, while in the raw sample the protein bands *i* and *l* were mainly attributed to Ara h 2 and Ara h 7 along with Ara h 3 allergens, the corresponding band visualized in autoclaved samples (Fig. 3, lanes 2 and 3, band *p*) was putatively attributed only to Ara h 3; this suggests that Ara h 2 and Ara h 7 allergens are likely affected by thermal/pressure treatment. Concerning pre-hydrated and autoclaved peanuts at 134 °C for 10 and 20 min, a drastic reduction in the protein content of the extracts was highlighted by SDS-gel and it is worth noting that no protein bands were detectable in the corresponding electrophoretic patterns (Fig. 3, lanes 4 and 5 respectively), with the exception of a faint band displayed at approximately 25 kDa and putatively assigned to Ara h 3 group. Previous studies, already reported the resistance of some peanut proteins to the heating (Cabanillas et al., 2015; Kopper et al., 2005; Maleki & Hurlburt, 2004). Like other cupins, Ara h 1 is a thermostable protein and undergoes irreversible denaturation after heating at the temperatures above 80 °C causing a loss in the secondary and tertiary structures and an extensive aggregation (Koppelman, Bruijnzeel-Koomen, Hessing, & De Jongh, 1999). On this regard, the extreme heating like roasting at the temperatures higher than 140 °C was reported to produce an enhancement of IgE binding capacity of Ara h 1 (Mondoulet et al., 2011).

In this work, we found an extensive reduction of Ara h 1 when peanuts underwent hydration (for 2 h) followed by autoclaving at 134 °C for 10 or 20 min, as depicted in Fig. 3 (Fig. 3, lane 4 and 5). Such results are in agreement with what reported by Cabanillas et al. (2015), although the technological treatment used by those authors slightly differed from that herein described. Specifically, in that paper authors investigated the influence of thermal/pressure processing on the IgE binding properties of raw, fried and roasted peanuts inferring that autoclaving samples at 138 °C and at 2.56 atm for 15 or 30 min in the presence of water produced a dramatic reduction of Ara h 1 levels. The same authors found a decrease in Ara h 1 content also when peanuts were subjected to mild thermal/pressure treatment, in contrast with our results instead showing a certain resistance of these proteins to autoclaving (134 °C, 2 atm, 10 or 20 min). These different results could be likely ascribed to the different treatment conditions applied to peanuts (presence/absence of water during autoclaving). Moreover, a drastic reduction of Ara h 3 proteins (proteins banding around 37 kDa in Fig. 3 lane 2–3 and 4–5) was observed in autoclaved peanuts submitted or not to preliminary hydration. The susceptible behaviour of this allergen group to thermal/pressure treatment was in line with what previously described by Cabanillas et al. (2015).

A similar trend was recorded for proteins banding in the range 20–25 kDa (mainly attributed to Ara h 2, Ara h 3 and Ara h 7) with a progressive disappearance of bands below 20 kDa in pre-hydrated autoclaved samples (Fig. 3, lanes 2–3 and 4–5). On the other hand, proteins banding below 20 kDa (attributed to Ara h 2, Ara h 3, Ara h 6 and Ara h 7) disappeared from the gel already after 10 min of autoclaving (Fig. 3, lanes 2, 3, 4, 5) demonstrating a high susceptibility of these proteins to the heating. On this regard, Johnson et al. have recently reported that Ara h 2/Ara h 6 exposition at the temperatures higher

Table 1
Identification of protein bands excised from the SDS gel and analysed by LC-HR-MS/MS through detection of the proteotypic peptides.

Sample	Band	Accession number	Allergenic protein	Coverage	Score	Filtered peptides (unique)
Raw peanut	*a*	Q6PSU4	Ara h 1	51,60	6,44	7(0)
		B3IXL2	Ara h 1	50,00	13,12	9(0)
		N1NG13	Ara h 1	48,10	10,22	8 (0)
	b	Q6PSU4	Ara h 1	69,16	131,64	23 (0)
		B3IXL2	Ara h 1	67,30	155,19	36 (0)
		Q6PSU6	Ara h 1	65,35	84,80	14 (0)
		E5G076	Ara h 1	50,56	93,38	27 (0)
		Q5I6T2	Ara h 3	33,14	2,76	5 (1)
	c	B5TYU1	Ara h 3	70,75	89,97	20 (0)
		Q5I6T2	Ara h 3	68,17	87,21	19 (1)
		Q9FZ11	Ara h 3	66,54	92,99	19 (1)
		Q8LKN1	Ara h 3	65,61	66,65	16 (0)
		Q6PSU4	Ara h 1	65,18	153,66	27 (0)
		Q647H3	Ara h 3	64,62	87,56	17 (1)
		A1DZF0	Ara h 3	61,06	81,39	17 (0)
		Q0GM57	Ara h 3	43,55	17,37	5 (0)
		B3IXL2	Ara h 1	59,93	148,54	33 (0)
		Q6PSU6	Ara h 1	58,75	85,21	14 (0)
		E5G076	Ara h 1	47,98	85,65	25 (0)
	d	Q647H3	Ara h 3	71,88	171,03	19 (0)
		Q8LKN1	Ara h 3	75,28	161,74	17 (0)
		Q9FZ11	Ara h 3	68,81	160,57	19 (1)
		B5TYU1	Ara h 3	65,09	150,06	17 (1)
		Q5I6T2	Ara h 3	68,74	142,35	19 (2)
		A1DZF0	Ara h 3	66,73	138,28	15 (1)
		Q9SQH7	Ara h 3	43,02	120,87	10 (0)
		O82580	Ara h 3	51,48	110,60	12 (0)
	e	Q9FZ11	Ara h 3	68,24	97,93	14 (0)
		Q5I6T2	Ara h 3	68,17	90,21	14 (2)
		B5TYU1	Ara h 3	65,28	92,08	12 (0)
		Q8LKN1	Ara h 3	60,04	78,33	9 (0)
		A1DZF0	Ara h 3	58,41	78,96	8 (0)
		Q9SQH7	Ara h 3	47,36	70,29	6 (0)
		E5G077	Ara h 3	40,63	13,65	6 (0)
	f	Q9FZ11	Ara h 3	50,47	23,28	8 (1)
		Q5I6T2	Ara h 3	49,53	23,20	8 (1)
		Q8LKN1	Ara h 3	41,64	17,07	6 (0)
		A1DZF0	Ara h 3	41,02	18,72	6 (0)
	g	A1DZF0	Ara h 3	48, 00	336,71	24 (2)
		N1NG13	Ara h 1	43,00	137,8	26 (1)
	h	Q9FZ11	Ara h 3	33,84	5,07	4 (0)
	i	A1DZF0	Ara h 3	37,62	3,91	4 (0)
		Q5I6T2	Ara h 3	34,65	1,83	3 (0)
	l	Q6PSU2	Ara h 2	72,09	59,52	15 (15)
		B4XID4	Ara h 7	53,05	9,84	7 (4)
		Q647H4	Ara h 3	46,83	242,25	20 (0)
		A1DZF0	Ara h 3	45,37	256,11	19 (0)
		Q647H3	Ara h 3	43,58	256,57	17 (0)
		B5TYU1	Ara h 3	43,40	249,89	18 (0)
		Q5I6T2	Arah 3	38,04	255,99	17 (0)
		Q9FZ11	Ara h 3	37,24	272,49	18 (1)
		O82580	Ara h 3	33,33	155,38	15 (0)
		Q0GM57	Ara h 3	30,08	60,24	7 (0)
		N1NG13	Ara h 1	28,91	0,00	3 (0)
		Q647H1	Ara h 1	24,92	24,30	7 (3)
	m	A5Z1R0	Ara h 6	64,83	17,76	6 (3)
		Q6PSU2	Ara h 2	55,81	11,40	6 (6)
		B4XID4	Ara h 7	46,95	13,07	9 (4)
		Q647G8	Ara h 7	46,20	9,62	5 (0)
		A1DZF0	Ara h 3	44,23	63,14	13 (0)
		A1DZE9	Ara h 6	42,07	17,76	3 (0)
		B5TYU1	Ara h 3	41,32	63,35	13 (0)
		Q8LKN1	Ara h 3	41,08	44,58	11 (0)
		Q647H3	Ara h 3	38,73	60,71	12 (1)
		Q9FZ11	Ara h 3	35,16	60,71	12 (1)
		Q0GM57	Ara h 3	28,52	8,05	4 (0)
	n	A5Z1R0	Ara h 6	72,41	28,08	12 (8)
		A1DZF0	Ara h 3	45,18	45,55	5 (0)
		Q9FZ11	Ara h 3	41,21	51,05	6 (0)
		A1DZE9	Ara h 6	40,00	20,64	4 (0)
		O82580	Ara h 3	36,49	51,05	6 (0)
		Q6PSU2	Ara h 2	34,88	0,00	4 (4)
		Q8LKN1	Ara h 3	33,83	47,15	5 (0)

(*continued on next page*)

Table 1 (*continued*)

Sample	Band	Accession number	Allergenic protein	Coverage	Score	Filtered peptides (unique)
		B4XID4	Ara h 7	28,05	0,00	5 (3)
Autoclaved 10'	*o*	Q9FZ11	Ara h 3	32,14	5,33	4 (0)
		Q5I6T2	Ara h 3	30,51	5,33	4 (0)
		N1NG13	Ara h 1	43,61	21,36	10 (0)
		Q6PSU3	Ara h 1	52,07	21,36	8 (0)
	p	B5TYU1	Ara h 3	41,89	83,46	12 (0)
		Q8LKN1	Ara h 3	41,64	68,84	11 (0)
		A1DZF0	Ara h 3	41,02	84,14	13 (0)
		Q647H3	Ara h 3	39,29	95,04	11 (1)
		Q9FZ11	Ara h 3	35,73	95,17	13 (1)
		Q0GM57	Ara h 3	27,34	34,46	6 (0)
Autoclaved 10' + H_2O	*q*	Q5I6T2	Ara h 3	25,99	4,21	3 (1)

than 110 °C induced a change in their secondary structure with a consequent transition from α-helix to random coil and, as a results, the formation of dimeric (MW 26–29 kDa) and tetrameric (MW 60–65 kDa) structures (Cabanillas et al., 2015; Johnson et al., 2010).

In addition, it has been demonstrated that like in the case of high pressure treatments applied up to 180 MPa, Ara h 2 unfolding can occur with consequent exposition of hydrophobic residues (Hu et al., 2011). Similar results were obtained by Cabanillas et al. (2015) that ascertained reduced levels of Ara h 2/Ara h 6 allergens in autoclaved raw, roasted and fried peanuts.

It can be speculated that exposition of peanuts to water before autoclaving could alter protein stability also inducing extensive protein denaturation. Water absorption by seeds might on one hand facilitate heat propagation in the inner part of the seed and on the other hand exert a mechanical effect while autoclaving at the higher pressure thus causing protein disgregation and a decrease in spot intensity. Several reasons might account for such behaviour *e.g.* conformational changes in the protein, formation of intra and/or inter-molecular covalent and non-covalent interactions, *etc.* Some authors hypothesized that, in general, structural changes caused by heating can alter protein solubility consequently lowering the extraction efficiency of the containing proteins or in other cases promote protein aggregation thus preventing the protein complex from entering the polyacrylamide gel (Comstock, Maleki, & Teuber, 2016).

In this study our investigation was only addressed to the extractable proteins with TBS also containing 7 M urea. However, taking into account the solubility issues, targeted analyses on the insoluble fraction of raw and treated peanuts were carried out (data not shown). Specifically, a sequential extraction procedure was followed based on a first step with TBS buffer also containing 7 M Urea, and a subsequent extraction on the remaining pellet with harsher conditions using the SDS-PAGE sample buffer. Protein pools sequentially extracted, presumably composed by most soluble (first fraction) and partly insoluble proteins (second fraction), were analysed by SDS-PAGE. Electrophoretic patterns showed that the profiles of pellets undergoing a harsher extraction were similar to those obtained by the first extraction (data not shown). Similar results were also described by Sanchiz et al. (2018), the did not report any difference in the electrophoretic pattern nor in the IgE reactivity of cashew and pistachio extracts subjected to heat and pressure treatments, applying strong conditions for protein solubilization (flours directly solubilized in SDS-PAGE sample buffer) compared to the first extraction.

In conclusion, our results demonstrated a certain decrease for certain allergenic proteins after autoclaving peanut seeds, according to the results provided by the Bradford assay and ELISA analysis. In addition, the remarked reduction in the intensity of the protein bands along the individual lanes after a prolonged heating, could be attributed either to a reduced extraction yield from the processed food material (Walczyk, Smith, Tovey, & Roberts, 2017) or to protein fragmentation caused by the thermal treatment applied, that could finally lead to a lower IgE binding.

3.3. Immunoblot analysis to assess IgE binding reduction in the extractable fraction of autoclaved peanuts

In order to compare the efficacy of autoclaving for different time extents including or not an incubation step in water, the change in the final immunoreactivity was assessed by Immunoblot analysis using a pool of sera from allergic young patients (3–8 age). A picture reporting the western blot analysis, under reducing conditions, performed with sera of patients allergic to peanuts is shown in Fig. 4.

As for autoclaved peanuts, while band at 120 kDa MW (Fig. 4, lane 1, band *a*, assigned to Ara h 1) lose its immunoreactivity after treatment, bands with MW of approximately 60 kDa (Fig. 4, lanes 2 and 3 corresponded to band *o*, experimentally assigned to Ara h 1 and Ara h 3), confirmed the reduction of their IgE binding after autoclaving (Fig. 4, lane 2, 3). By contrast, no difference in IgE reactivity was observed for protein bands with MW of approximately 50 kDa (band *c* in untreated sample), that dd-MS^2 experiments putatively attributed to Ara h 3 and Ara h 1, and of band at 25 kDa (Fig. 4, lane 2 and 3, band *p*) belonging to Ara h 3 (Table 1). On the contrary, the general IgE reactivity of these proteins appeared to be drastically reduced when peanuts were hydrated for 2 h prior to autoclaving (10 or 20 min). In this case, only proteins banding at 25 kDa (previously attributed to Ara h 3 in raw samples) still displayed a IgE binding and assigned to Ara h 3 subunit. On the other hand some other bands displayed in lane 4 and 5 at the lower MW around 25 kDa, might appear also more reactive compared to the control giving rise to infer that a change in the immunoreactivity (reduction for certain proteins and enhancement for others) may occur under specific processing conditions applied (Guillon, Bernard, Drumare, Hazebrouck, & Adel-Patient, 2016).

It is well known that food processing can induce conformational changes on the allergenic protein, influencing its allergenicity by disruption of conformational or linear epitopes spread along the moiety. As a result, conformational epitopes can be exposed or hidden by unfolding or aggregation of proteins (Rahaman, Vasiljevic, & Ramchandran, 2016). On this regard, a recent study have reported that thermal processing of peanuts induced a major decrease in Ara h 1 immunoreactivity compared to Ara h 2. This different behaviour could be due to a higher degree of denaturation and/or aggregation of Ara h 1 (Montserrat et al., 2015). Blanc et al. (2011) showed that after boiling, Ara h 1 formed branched rod-shaped aggregates with a loss of some secondary structures and consequently a reduction of IgE binding ability. In general, the loss or change in the conformational or linear

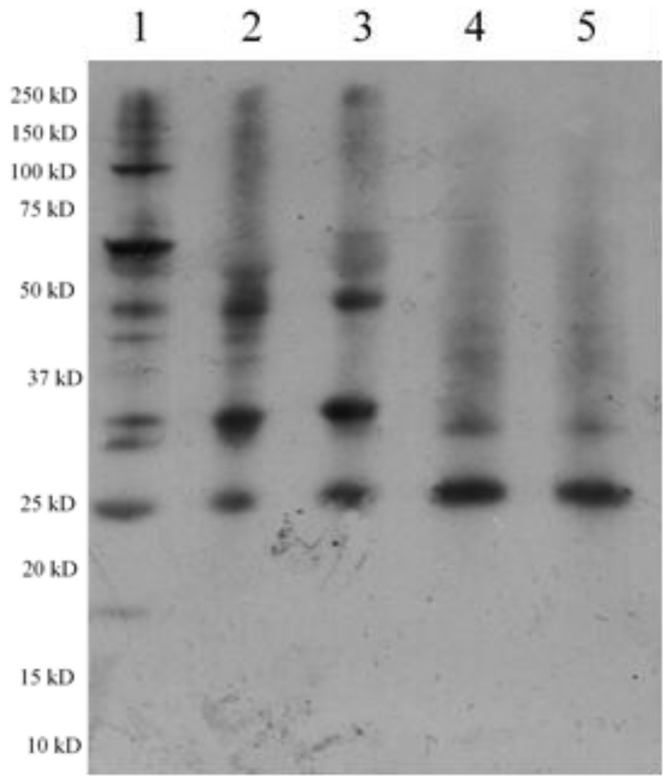

Fig. 4. Immunoblot of peanuts under reducing conditions referred to raw (lane 1), autoclaved for 10 min (lane 2) and for 20 min (lane 3); pre-hydrated and autoclaved for 10 min (lane 4), pre-hydrated and autoclaved for 20 min (lane 5) at 134 °C and 2 atm. The immunoblot was carried out on a pool of sera of young patients (3–8 age) with a clinical allergy to peanuts.

epitopes, can play an important role in modulating the allergenic potential of a food by altering the IgE binding capacity, the activation of basophils and mast cells that causes a reduction in histamine release (Nesbit et al., 2012). In addition, it has been shown that an extensive protein fragmentation can lead to a reduced allergenicity (Fue, 2002). As found in this work, when autoclaving was preceded by a hydration step, a strong reduction in the final immunoreactivity was observed. According to our findings, only a protein banding at MW 25 kDa showed to retain some reactivity, giving rise to exclude any aggregation phenomena occurring or an eventual decrease in protein solubility; instead, a probable fragmentation of proteins might have occurred as a consequence of the prolonged exposition to water. In support of this, Cabanillas et al. (2015) reported that both autoclaving and boiling caused protein fragmentation. In the same work, they also reported that after solubilising peanut flour directly in the sample buffer, proteins showed a high extent of fragmentation, that also reflected a decreased capacity to bind IgE. In addition, another study demonstrated that boiling peanut seeds in closed vessels resulted in a loss of Ara h 2, Ara h 6 and Ara h 7 proteins due to a probable leaching of these allergens into the cooking water (Turner et al., 2014). However, those data demonstrated that boiling reduced IgE reactivity but did not vanish the capacity to stimulate antigen-specific T cells, as shown by activation and proliferation tests (Tao et al., 2016). On the other hand, study on structural alterations induced by heating Ara h 2 and carried out by CD spectroscopy revealed that Ara h 2 did not refold upon temperature decrease but remained in this partially unfolded state with a significantly increased of protein oligomers (Starkl et al., 2011).

The global reduction of peanuts allergenic potential recorded in the present study proved that the implementation of wet heat and high-pressure treatments is essential to significantly decrease the IgE response. However, it is worthy to be underlined that this does not confirm the total abolishment of allergenicity and an antibody reactivity cannot be excluded. According to what described in other studies a persistent allergenicity can be displayed after heat treatments applied depending on the type of nut under study or the specific cultivar/variety (Downs et al., 2016; Noorbakhsh et al., 2010). In addition, the effect of these treatments cannot be uniquely associated with structural modifications of proteins, but also with the generation of protein fragments as also confirmed by other studies (Cabanillas et al., 2012, 2014; Cabanillas et al., 2015).

3.4. Digestibility and IgE binding capacity of autoclaved peanuts after in vitro gastro-intestinal digestion

Food allergens display the typical characteristics to resist to the proteolytic activity of the enzymes occurring along the gastrointestinal tract (GI), being able to reach the intestinal mucosa such as large immunologically active fragments and capable of inducing sensitization after their absorption. Several studies demonstrated that digestion of Ara h 2 and Ara h 6 by pepsin and/or trypsin/chymotrypsin can originate large residual peptides (Koppelman et al., 2010) endowed with unmodified immunological potential (Apostolovic et al., 2016). In addition, these proteins also proved to be resistant to gastro-intestinal digestion even after heating (Koppelman et al., 2010; Maleki & Hurlburt, 2004; Sen et al., 2002; Suhr, Wicklein, Lepp, & Becker, 2004). In stark contrast with that, Ara h 1 and Ara h 3 were rapidly hydrolyzed by pepsin (Koppelman et al., 2010). Nevertheless, peptides obtained after gastro-duodenal digestion of Ara h 1 still retained sensitizing capability and IgE-binding properties. The limits of these studies lied in the fact that they were carried out on single purified proteins (Bøgh & Madsen, 2016) or by employing very simple digestion fluids (Apostolovic et al., 2016) not taking into account the overall complexity of the real physiological conditions. In a recent study, a standardized *in vitro* digestion protocol has been utilised to assess stability of the major peanut allergens by simulating human digestion, also evaluating the residual immunoreactivity of the generated peptide mixture (Di Stasio et al., 2017). In order to assess the effect of the digestion on the major peanut allergens, autoclaved peanuts were subjected to *in vitro* digestion experiments and the residual immunoreactivity of the digests was finally assessed. To this purpose, in this work peanuts autoclaved for 10 min, including or not a pre-hydration phase, underwent a standardized *in vitro* digestion protocol (Minekus et al., 2014) where chew, gastric and duodenal phases simulated physiological conditions (as for enzymes and fluids composition).

Digestibility of raw peanut proteins, undergoing or not digestion, is shown in the SDS-PAGE gel obtained under reducing conditions, pictured in Fig. 5A. As appearing in the figure, the prominent band with MW at approximately 60 kDa detected in undigested raw peanuts was lost after simulated digestion (Fig. 5A, lane 2).

When autoclaved peanuts underwent digestion, an additional protein banding between 37 and 50 kDa were displayed along the gel (Fig. 5A, lane 3), despite its absence in digested raw peanuts (Fig. 5A, lane 2) with special regard to a defined spot nearly 42 kDa. On the contrary, the high intense band detected between 20 and 25 kDa in undigested raw samples proved to be resistant throughout digestion (Fig. 5A, lanes 1, 2). New smeared bands in the lower MW range below 15 kDa were also highlighted in all digests (Fig. 5A, lane 2, 3). On the base of the MS/MS identification accomplished in undigested and digested raw peanuts, polypeptides banding at 50 kDa in the raw digests were mainly attributed to Ara h 1 digestion, whereas the protein at 37 kDa attributed to Ara h 3 appeared completely degraded. Moreover, the protein banding at 22 kDa, showed to persist throughout digestion, suggesting a good resistance of this protein towards digestive enzymes. Notably, smeared bands below 15 kDa might represent some digest products of several. Ara h proteins. However, further investigations will be directed to give more insights on these polypeptides and on the

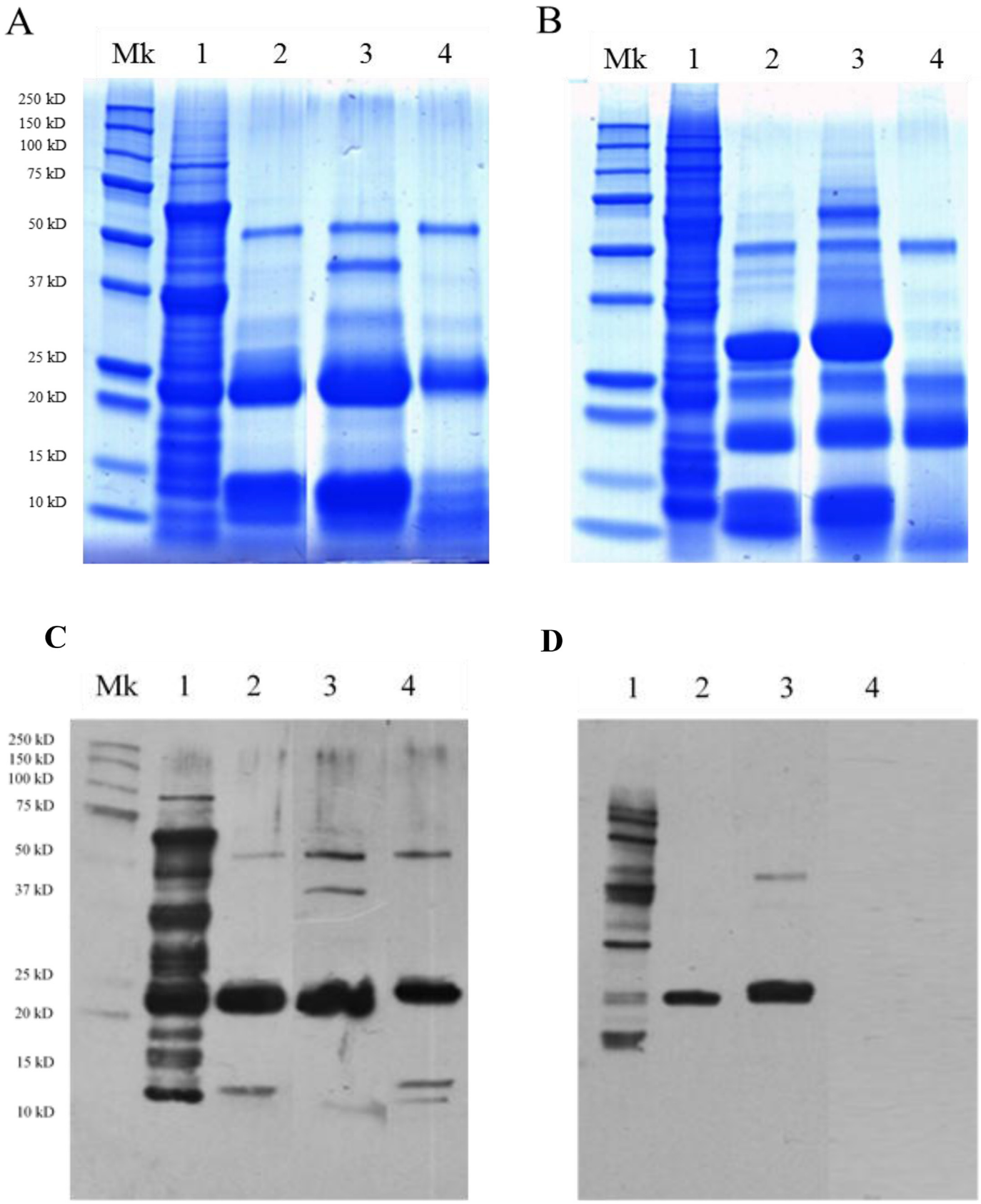

Fig. 5. SDS-PAGE analysis under reducing **(A)** and non-reducing **(B)** conditions of peptides mixture generated from untreated and treated peanuts submitted to simulated gastro-duodenal digestion. Undigested raw peanuts (lane 1), digested raw peanuts (lane 2), autoclaved (at 134 °C, 2 atm for 10 min) and digested peanuts (lane 3), pre-hydrated, autoclaved (at 134 °C and 2 atm for 10 min) and digested peanuts (lane 4). In the lower panel are shown immunoblot with a pool of 6 sera of young patients allergic to peanuts, under reducing **(C)** and non-reducing **(D)** conditions relative to peptides mixture generated from untreated and treated peanuts submitted to simulated gastro-duodenal digestion along with undigested control. Undigested peanut (lane 1), digested raw peanut (lane 2), autoclaved at 134 °C, 2 atm for 10 min (lane 3), pre-hydrated and autoclaved at 134 °C and 2 atm for 10 min (lane 4).

fraction lower than 10 kDa. By inspecting the protein pattern of autoclaved and digested peanuts (Fig. 5A, lanes 3, 4), slight differences were found compared to digested raw samples. Specifically, the electrophoretic profiles of digested autoclaved peanuts without pre-hydration (Fig. 5A, lane 3) showed the appearance of an additional band at approximately 42 kDa. This new emerging band could be the result of an incomplete digestion of Ara h 1, likely due to conformational modifications occurring during the treatment that contributed to hide enzyme cleavage sites. Whereas, the digestion of hydrated and autoclaved peanuts produced an electrophoretic profile (Fig. 5A, lane 4) with a general decrease of protein bands intensity especially noticed in the MW ranging from 15 and 45 kDa.

Different results were obtained for peanut digests analysed under non-reducing conditions as shown in Fig. 5B. Again a change in the protein pattern was noticed in the autoclaved samples (see Fig. 5B, lane 3), where a protein banding at approximately 60 kDa was detected By contrast, a high resistance of proteins comprised in the range from 37 to 20 kDa was highlighted in the lanes 2, 3 of Fig. 5 B with special regard to band at 25 kDa.

Conversely, in hydrated and autoclaved peanuts digests (Fig. 5B, lane 4), a protein banding at 50 kDa was highlighted with a concomitant disappearance of bands between 50 and 37 kDa. Moreover, a resistance of bands with MW in the range 25–15 kDa, along the electrophoretic profile of raw and autoclaved digested samples, was also observed, with a significant reduction in the intensity of bands below 15 kDa.

These results, in both cases, point out that pre-hydrating peanuts before autoclaving is likely to extensively promote digestion of peanut allergens thus facilitating proteolysis of the major protein allergens. Nonetheless, some protein bands showed to persist throughout digestion.

Finally, due to the scarce knowledge of the residual immunoreactivity still retained from autoclaved peanuts after human simulated digestion, digests obtained from raw and autoclaved peanuts were submitted to final immunoblot analysis, under reducing and non-reducing conditions, by using sera of allergic patients.

Under reducing conditions (Fig. 5C), only peptide fragments banding at approximately 20 kDa determined an IgE binding in both raw and autoclaved samples, while the two signals at approximately 50 and 15 kDa were weakly detected in untreated and pre-hydrated autoclaved samples. In addition, a high intense signal was observed for the proteins at 20 kDa in untreated and treated digested peanuts.

On the contrary, under non-reducing condition (Fig. 5D), raw and autoclaved samples showed IgE reactivity of proteins at approximately 20 kDa, despite hydrated and autoclaved samples were none antibody response was recorded.

The loss of IgE response under non-reducing condition, would suggest that the combination of heat and pressure coupled with a preliminary hydration step might account for a structural change in peanut proteins, hiding the allergen structure with a probable aggregation phenomenon. These new protein structures could inhibit the action of trypsin as well as other digestive enzymes, leading to a decrease in protein digestibility, and consequently masking some IgE reactive determinants. Only the action of a strong reducing agent such as DTT or β-mercaptoethanol (β-ME) might induce reduction of aggregates and the consequent exposure of epitopes to IgE binding.

To the best of our knowledge, this is the first time that the stability and the residual immunoreactivity of peanut allergens submitted to autoclave process and simulated gastro-intestinal *in vitro* digestion was studied. Compared to the existing literature, and in order to have more realistic data about the digestibility of processed peanut proteins, the whole peanut commodity was submitted to digestion accounting then for the influence of the matrix on the proteolytic degradation of the contained allergens. A similar approach has been recently described by Di Stasio et al. (2017). The authors investigated the digestion stability of the major peanut allergens directly in the natural matrix using the standardized Infogest digestion protocol and the residual immunoreactivity of the resulting digest fragments was finally assessed. It was found that only large sized fragments of Ara h 2, Ara h 6 and Ara h 3 survived the hydrolysis. In the same paper authors finally identified by LC-MS/MS analyses smaller resistant peptides mainly arising from Ara h 3 and Ara h 1. Concerning untreated peanuts, our results are in agreement with what described by Di Stasio and co-workers (2017). We also found a thermostable and an immunoreactive protein at approximately 22 kDa in the SDS-gel of gastro-duodenal digest of unprocessed peanuts that was attributed to Ara h 3 in line with the MS/MS analysis of the corresponding band done by Di Stasio et al.

Concerning samples submitted to autoclaving treatment without pre-hydration, we observed that protein fragments/subunits arising from gastro-duodenal digestion still retained their allergenic potential because no significant differences were highlighted in Western-blotting profiles of raw and autoclaved peanuts samples under reducing and non-reducing condition.

By contrary, immunoblot analysis carried out on gastro-duodenal digests of pre-hydrated and autoclaved samples (Fig. 5D, lane 4) demonstrated that the allergenic potential was lost after digestion by working under non reducing condition.

However, it is worthy to be said that in this paper we did not investigate peptides lower than 6 kDa that escaped the electrophoretic detection. Work is in progress to identify the reactive band visualized around 20 kDa as well as to characterize the lower MW fraction.

Our findings confirm that when a more drastic processing was applied (*e.g.* hydration followed by autoclaving), a different result in the final immunoreactivity was displayed strictly depending on the operative conditions adopted during SDS PAGE experiments (under reducing or non reducing conditions).

4. Conclusions

In the light of our results, thermal/pressure treatment has demonstrated to modulate peanuts immunoreactivity. In particular, hydration prior to autoclaving proved to increase the efficacy of the thermal treatment contributing to the disappearance of the main allergenic protein bands and altering significantly the final immunoreactivity as assessed by immunoblot experiments. Furthermore, attention was placed on the residual immunoreactivity detected after gastro-intestinal digestion, thus demonstrating that the combination of hydration and autoclaving may induce a drastic reduction of peanuts immunoreactivity especially displayed when working under non reducing conditions.

However, further studies will be necessary to better investigate the decrease in IgE crosslinking capacity of heat/pressure treated samples in *in vivo* models.

Understanding the fate of allergenic proteins subjected to novel processing techniques can help to develop useful strategies for food tolerance induction and/or to establish threshold levels of sensitization/elicitation for hypoallergenic foods. In order to confirm these results, a deeper investigation should be undertaken by using individual sera of allergic patients and designing a food oral challenge test study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.04.021.

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Comprehensive analysis of the peanut allergome combining 2-DE gel-based and gel-free proteomics

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ABSTRACT

In this work, we explored the "deep" seed peanut proteome by using both two dimensional electrophoresis (2-DE)-based analysis run under reducing and non-reducing condition (protein-centric) and LC-MS/MS gel-free proteomic (peptide-centric). The former approach allowed to identify high molecular weight disulfide-linked Ara h 1 and Ara h 3 heteroligomers and Ara h 1 homoligomers linked through covalent bonds other than disulfides. The occurrence of these protein complexes revealed natural interactions between Ara(s) subunits with a possible involvement in the allergenic potential of peanut.

The second approach, also referred to as shot-gun proteomics, allowed the identification of 149 gene products, including low-abundance proteins escaped the 2-DE detection. Interestingly, we identified 60 proteins never catalogued previously. The complementary exploitation of two proteomic approaches enabled the access to new relevant information about the complexity of the peanut proteome, with special emphasis to the complement of allergens (allergome).

1. Introduction

Peanuts, the seeds of the legume plant *Arachis hypogaea*, are responsible of one among the most severe and life-long persistent food allergies, affecting nearly 1% of children and 0.6% of adults within the general population of Westernized countries (Sicherer & Wood, 2013). Overall peanut proteins account for approximately 24% in mature seed. World Health Organization and International Union of Immunologic Societies Subcommittee (WHO/IUIS) have catalogued seventeen protein allergens in peanut seeds (www.allergen.org), which have been classified into six different allergen superfamilies, including the cupin (Ara h 1 and Ara h 3/4), prolamin (Ara h 2, Ara h 6, Ara h 7, and Ara h 9), profilin (Ara h 5), Bet v 1 (Ara h 8), oleosin (Ara h 10, Ara h 11, Ara h 14, Ara h 15) and defensin (Ara h 12, Ara h 13, Ara h 16, Ara h 17) superfamilies (Bublin & Breiteneder, 2014). Each of these allergens might trigger immune response in susceptible subjects, with a variety of symptoms and prognosis depending on both individuals and offending agent(s). In general, Ara h 1, Ara h 3, Ara h 2 have been associated with primary sensitization, while Ara h 2 and Ara h 6 are commonly considered the most clinically relevant in terms of basophile activation, IgE binding properties and skin prick test (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Mueller, Maleki, & Pedersen, 2014).

Because peanut is largely used as a food ingredient and it is responsible of persistent food allergic reactions, the molecular structure of peanut proteins has been the subject of fervent investigation over the last decades. The knowledge about the composition of peanut allergens has been significantly advanced by the latest progresses of proteomic methods (Mamone, Picariello, Addeo, & Ferranti, 2011). So far, most of the reports have exploited the SDS-PAGE-based proteomic, providing deep insights in the peanut proteome and contributing to determine the serum IgE-binding properties as well as the stability of allergens to proteolytic digestion (Chruszcz et al., 2011; Di Stasio et al., 2017; Koppelman, Hefle, Taylor, & de Jong, 2010). Recently, a more detailed characterization about composition of peanut proteins and their isoforms has been achieved through two dimensional electrophoresis (2-DE) (Grishina, Bardina, & Grishin, 2017). The "classical" 2-DE/mass spectrometry (MS) proteomic approach has been used to determine the protein profile of mature seeds (Schmidt et al., 2009), to monitor protein accumulation and spatial-temporal expression during seed development (Kang, Srivastava, Ozias-Akins, & Gallo, 2007), to associate cultivar to allergenic potential (Chassaigne et al., 2009; Kottapalli et al., 2008; Schmidt et al., 2009) and to evaluate IgE-binding affinity of

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specific allergen types (Guo, Liang, Chung, Holbrook, & Maleki, 2008; Porterfield et al., 2009). Similar to other *Leguminosae* seeds, major storage proteins of peanut tend to form large molecular weight (MW) multimeric complexes, including both homo- and hetero-oligomeric aggregates. For instance, vicilin-like Ara h 1 is reported to form non-covalent protein aggregates up to 600–700 kDa (Shin et al., 1998). Likewise, the two sub-units (acid and basic) of Ara h 3, covalently linked by an intermolecular disulfide bridge, are further associated into non-covalent hexameric aggregates (Jung, Scott, Nam, & Beaman, 1998). To improve the quality of protein separation, 2-DE analysis is commonly performed under denaturing and reducing conditions (Chevalier, Hirtz, Sommerer, & Kelly, 2009). In these conditions the information about the quaternary organization of proteins goes lost, hampering the structural elucidation of protein aggregates via disulfides and the composition of interacting polypeptides. To the best of our knowledge, there is no comprehensive 2-DE investigation on peanut proteins considering the formation of complexes and aggregates, which may provide significant additional insights if compared to the canonical 2-DE-based proteomic exploration.

In spite of the ability of 2-DE to preserve the information about the intact proteins and occurrence of possible isoforms, shotgun proteomics has emerged as a powerful technique overcoming many of the 2-DE pitfalls (Rogowska-Wrzesinska, Le Bihan, Thaysen-Andersen, & Roepstorff, 2013). According to the shotgun proteomic workflow, the tryptic digests of an unfractionated protein extract is resolved by liquid chromatography-high resolution tandem mass spectrometry (LC-MS/MS). Proteolysis dramatically increases the complexity of the analytical system. However, the method takes advantage from the much higher sensitivity in the analysis of peptides compared to proteins and from the possibility of sequencing at least one peptide arising from each parent protein. A software-assisted inference process allows recovering back the identity of the proteome components, but the information about the original intact polypeptides and possible isoforms is lost at the digestion stage (Nesvizhskii & Aebersold, 2005).

Although preliminary attempts to characterize the peanut proteome using the shotgun "peptide-centric" workflow have been reported (Kottapalli et al., 2013; White et al., 2013), they lack a direct correlation with the 2-DE-based analysis.

Herein, the peanut proteome was investigated by using complementary 2DE-based and shotgun proteomic approaches. In particular, the former was applied under both reducing and nonreducing conditions, in order to investigate the covalent protein complexes of peanut, while the latter had the specific purpose to enhance the proteome coverage, expanding the dynamic range of the electrophoresis-based methods.

2. Material and methods

2.1. Chemicals

Raw peanuts (Virginia, Zambia and China variety) were provided by Besana (Milano, Italy). Dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, urea, ammonium bicarbonate (AMBIC), acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcium chloride ($CaCl_2$) was provided from J.T: Baker (Rutland, Austin, USA). Trifluoroacetic acid (TFA) was from Fluka. Proteomic grade modified trypsin Gold® was provided by Promega (Madison, WI, USA). Reagents for electrophoresis analysis were from Bio-Rad (Milan, Italy).

2.2. Preparation of peanut protein for electrophoresis analysis

Raw peanut flour from individual varieties (100 mg) was defatted by stirring thrice for 10 min in five volumes (w/v) of diethyl ether. Proteins were extracted from the flour pellet in 1 mL of urea 7 M, TBS buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) with 20 mM DTT (reduced sample) or without DTT (unreduced sample). After 1 h stirring at room temperature, the suspension was centrifuged at 10,000*g* for 30 min and supernatant was collected. Proteins were alkylated by incubation with IAA (55 mM final concentration) at room temperature for 30 min. Protein concentration was determined with the Bradford assay (Kit from Bio-Rad). Finally, samples were desalted against 25 mM AMBIC using Econo-Pac 10 DG columns (Bio-Rad), lyophilized and stored at −20 °C.

2.3. Sample preparation for shotgun proteomic analysis

Peanut flour (100 mg) was extracted with 1 mL of urea 7 M, TBS containing 20 mM DTT for 1 h at 50 °C. In this case, the defatting step was omitted in order to minimize the possible loss of hydrophobic proteins. Sample was centrifuged at 5000*g* for 30 min and 55 mM IAA was incorporated into the supernatant, incubating for 30 min in the dark. To quench the alkylation reaction, DTT was added up 30 mM final concentration and incubated for additional 15 min at room temperature. Protein concentration was determined with the modified Lowry assay (kit from Sigma-Aldrich). The peanut protein extract (100 μg) was diluted with ten volumes of 50 mM AMBIC, pH 8.0, containing 1 mM $CaCl_2$ and incubated overnight at 37 °C with modified proteomic grade trypsin (Promega, Madison, WI, USA) using a 1:20 (w/w) trypsin-to-protein ratio. Resulting peptides were desalted using Sep-Pak® C18 cartridges (Waters, Milford, MA, USA) and finally dried in a "speed-vac" centrifuge, prior to LC-MS/MS analysis.

2.4. SDS − PAGE analysis

Peanut proteins were analysed by monodimensional (1-DE) SDS − PAGE (Bio-Rad, Mini-Protean) on precast 12% acrylamide gel. Protein aliquots were dissolved in Laemli buffer (0.125 M Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) β-mercaptoethanol (βMe), 0.02% bromophenol blue) at a 2.0 μg/μL concentration and denatured in a boiling water bath for 5 min. A final volume of 10 μL was loaded into the SDS-PAGE wells. Analysis under nonreducing condition was carried out in the same way of reducing SDS-PAGE, but omitting βMe. Electrophoresis was performed at constant voltage (100 V) at room temperature. After migration, the gels were stained with Coomassie Brilliant Blue G-250 and finally imaged with a LABScan scanner (Amersham Bioscience/GE Healthcare, Uppsala, Sweden).

2.5. 2-DE analysis

Peanut proteins were dissolved in immobilized pH gradient (IPG) strip rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 2% (v/v) Pharmalytes pH 4.0–7.0 (namely, Pharmalytes 4–6.5 and 5–7 in the 1/1 ratio), traces of bromophenol blue, with 20 mM DTT (reduced sample) or without DTT (unreduced sample). Notably, the 2DE gels were slightly overloaded (1.0 mg/0.500 mL), aiming to improve the visualization of the low-abundance proteins, especially the high molecular weight aggregates. Immobiline Dry Strips (pH 4–7 linear gradient, 11 cm, Bio-Rad Laboratories, Inc., Hercules, CA) were rehydrated overnight in the Immobiline Dry-Strip Reswelling Tray (Amersham Pharmacia). Isoelectrofocusing (IEF) was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). The proteins were focused up to 15,000 Vh at a maximum voltage of 6000 V at 20 °C. After focusing, proteins were reduced for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 2% DTT), and alkylated for 15 min with 2.5% IAA. In the case of the nonreducing conditions, the equilibration buffer was deprived of DTT or IAA. SDS-PAGE in the second dimension was performed on a 12% polyacrylamide gel using a Protean II system (BioRad Laboratories, California). The run was performed at 220 V constant voltage, and each gel was stained in a water solution containing 0.05% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 0.7% (v/v) acetic acid, and destained in water solution containing 10% (v/v) acetic acid and 40% (v/v) methanol.

2.6. Protein in-gel digestion

Protein bands/spots (1-DE/2-DE, respectively) were repeatedly washed with 25 mM AMBIC/acetonitrile (1/1, v/v) up to complete destaining. The proteins were reduced for 1 h at 56 °C with 10 mM DTT in 25 mM AMBIC and alkylated for 1 h at room temperature with 55 mM IAA in 25 mM AMBIC. Proteins were in-gel digested overnight at 37 °C using proteomic grade trypsin (12.5 ng/μL) in 25 mM AMBIC. Peptides were extracted twice in 5% formic acid/acetonitrile (1/1, v/v), dried in a "speed-vac" centrifuge and finally reconstituted in 50 μL of 0.1% TFA for MS analysis.

2.7. LC-high resolution (HR)-MS/MS analysis

Tryptic digests were separated using an Ultimate 3000 ultra-high performance liquid chromatography instrument (Dionex/Thermo Scientific, San Jose, CA, USA), coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Peptide mixtures, re-suspended in 0.1% (*v*/v) TFA solution, were loaded through a 5 mm long, 300 μm i.d. pre-column (LC Packings, USA) using a Famos autosampler (Thermo) and separated with an EASY-Spray™ PepMap C18 column (2 μm, 15 cm × 75 μm) 3 μm 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 separation was carried out at a flow rate of 300 nL/min with a linear gradient from 4% to 40% of solution B over 60 min for 1-DE/2-DE in-gel tryptic digests or over 240 min for the gel-free shotgun analysis. Mass spectra, generated by Xcalibur Software 3.1 version (Thermo Scientific), were elaborated using the Proteome Discoverer 2.1 software (Thermo Scientific). Matching entries from both 2-DE protein spots and shotgun analysis were filtered according to the following parameters: two tryptic missed cleavage, 5 ppm tolerance for precursor ions and 0.01 Da for MS/MS fragments, carbamidomethylation of cysteines as a fixed modification, oxidized Met and N-terminus Gln as pyroglutamic acid as variable peptide modifications. The identification of protein entries was validated using the Target Decoy PSM Validator (Proteome Discoverer software), restricting the search at 0.01 False Discovery Rate (FDR).

3. Results and discussion

3.1. Electrophoresis of peanut extract of varieties

Proteins from three commercial peanut cultivars (Virginia, China and Zambia), extracted by an optimized urea-based protein recovery method, were analysed by SDS-PAGE (12% gradient) under both reduction and nonreducing conditions (Fig. 1). Preliminarily, the major allergens Ara h 1, Ara h 3, Ara h 2 and Ara h 6 h were assigned according to the electrophoretic mobility as suggested by previous investigations (Apostolovic et al., 2013; Di Stasio et al., 2017; Koppelman et al., 2010; Koppelman et al., 2016). Among the cultivars, the electrophoretic profiles were similar except for Ara h 3 isoforms which appeared as a triplet band in Virginia and China samples and doublet band in Zambia (Koppelman et al., 2016). Interestingly, SDS-PAGE revealed that under nonreducing conditions all the cultivars contained significant amounts of high MW (97 up to > 250 kDa) aggregates, which for the most disappeared after reduction of disulfide bonds (dashed boxes in Fig. 1). When analysed by LC-MS/MS, these protein bands contained signals of tryptic peptides from both Ara h 1 and Ara h 3. At the high MW protein range a single band at ca. 110 kDa was not affected by disulfide reduction. By LC-MS/MS analysis of in-gel tryptic digests, this protein band was identified as Ara h 1 (not shown), in line with Maleki et al., (Maleki et al., 2000), suggesting that intermolecular cross-links can be established through covalent bonds other than disulfide linkages.

3.2. 2-DE analysis

Proteins from peanut varieties were separated by 2-DE under both reducing and nonreducing conditions (Fig. 2). In all cases, the 2-DE maps evidenced a significant heterogeneity within the protein families due to the presence of multiple proteoforms, also including components differing by post-translational modifications. In agreement with the SDS-PAGE analysis, three main spot groups at high MW (estimated 240, 210, 180 and kDa) occurred in the 2-DE map of nonreduced protein extracts, which for the most disappeared upon disulfide reduction, confirming their nature of disulfide-linked protein aggregates (Fig. 2).

A detailed 2-DE proteomic investigation was carried out on Virginia peanut, chosen as a model cultivar (Koppelman et al., 2010; Kottapalli et al., 2008; Schmidt et al., 2009) (Fig. 3). The 2-DE isolated spots were characterized by LC-MS/MS analysis of tryptic peptides. Proteins are inventoried in Table S1 and Table S2, relevant to the 2-DE maps generated in the absence (Fig. 3A) or in the presence (Fig. 3B) of DTT, respectively, as the reducing agent. The main allergen families are individually examined in the following sections.

3.2.1. Ara h 1

The protein spots of Ara h 1 (7S vicilin-like) belonged to two co-migrating isoforms, namely P14B and clone-P17, which differ each other by a series of punctiform substitutions and share 94.6% homology. Under nonreducing conditions (Fig. 3A; Table S1) the expected full-length Ara h 1 occurred in the poorly resolved spots 21–25 (ca. 68 kDa, pI range 6–6.5). Because of N-glycosylation micro-heterogeneity, the Ara h 1 spots partially overlapped those of Ara h 3 isoforms, so that the two protein families co-migrated in spots 24 and 25. In contrast, after reduction (Fig. 3B; Table S2), as a consequence of a splitting of the Ara h 3 subunits, Ara h 1 was clearly resolved into multiple spots, namely spots no. 12–19 and 37, at estimated MW 68 kDa, covering a relatively large p*I* range (p*I* 6.0–6.5), due to N-glycosylations (Chassaigne et al., 2009).

Additional shorter Ara h 1 forms, with experimental MW lower than theoretical one, were detected in the 2-DE maps of both nonreduced (Spot 48, 47; Fig. 3A) (Table S1) and reduced (spots 21–24, 34–36, 40, 47, 48, 66, 67; Fig. 3B; Table S2) protein extracts. Low MW isoforms of Ara h 1 probably were proteolytic fragments produced by endogenous proteases (Chassaigne et al., 2009).

Covalent polymeric aggregates of Ara h 1 were identified in the high MW region of nonreduced 2-DE gel (Fig. 3A; Table S1). Almost all the high MW spots disappeared under reducing conditions (Fig. 3B; Table S2) due to the disulfide nature of the inter-chain linkages. The LC-MS/MS analysis of high MW aggregates at 240 kDa (spot 1), 210 kDa (spots 2–3), 180 kDa (spots 4–7) and 90–110 kDa (spots 8–11 and 14–20) contained tryptic peptides matching both Ara h 1 and Ara h 3 (Fig. 3A). This finding suggested that Ara h 1 and Ara h 3 might co-migrate even as aggregates due to the poor resolution of nonreducing 2-DE or, most likely, it is possible that additional disulfide bonds are established between Ara h 1 and Ara h 3 chains, producing heteropolymeric aggregates, which naturally occur into mature peanut seeds. The capability of Ara h 1 to establish disulfide bonds, generating both hetero- and homo-polymeric aggregates, contrasts with previous observations which excluded the involvement of Ara h 1 in disulfide cross-links (Shewry, Napier, & Tatham, 1995).

Actually, the mature protein chains of both the Ara h 1 isoforms contain seven Cys residues, at least one of which is expected to be susceptible of inter-molecular pairing, supporting on a structural basis the possibility it plays a role in the formation of disulfide-mediated aggregates (Khan, Di, Patel, & Nanda, 2013). Interestingly, the Ara h 1 tryptic peptide containing the consensus triplet for the N-glycosylation escaped the MS detection in all the Ara h 1-containing spots, confirming its effective glycosylation. The specific monitoring of the glycopeptide (s) and the characterization of the glycoforms would require a dedicated analysis.

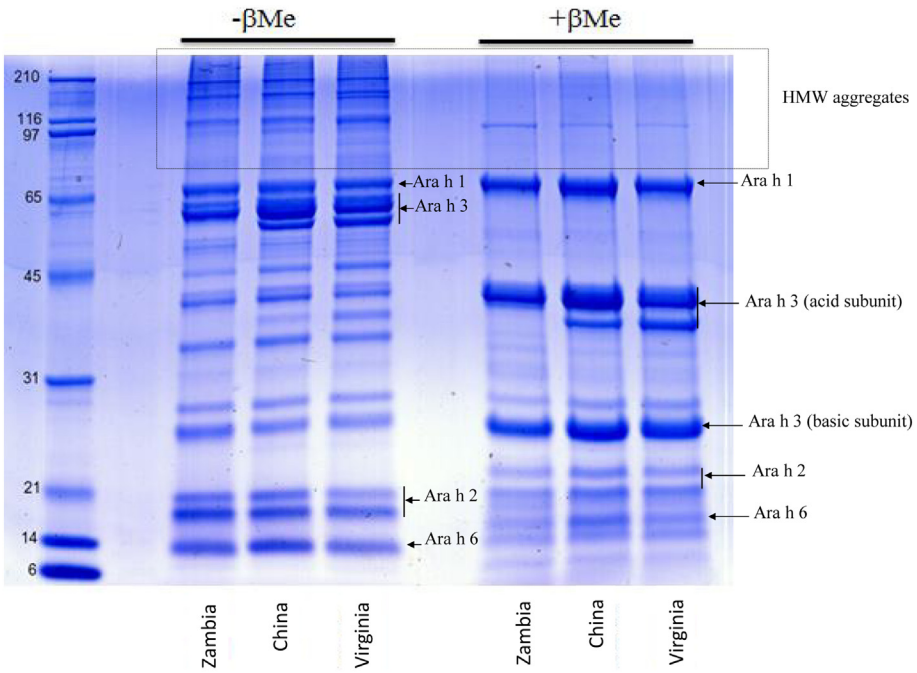

Fig. 1. SDS-PAGE comparison of Virgina, Zambia and China peanut proteins under nonreducing (−βMe) and reducing (+βMe) conditions. The major allergens were assigned in line with previous investigations (Di Stasio et al., 2017; Koppelman et al., 2010; Koppelman et al., 2016). Inserts delimited by dashed lines indicate the HMW aggregate.

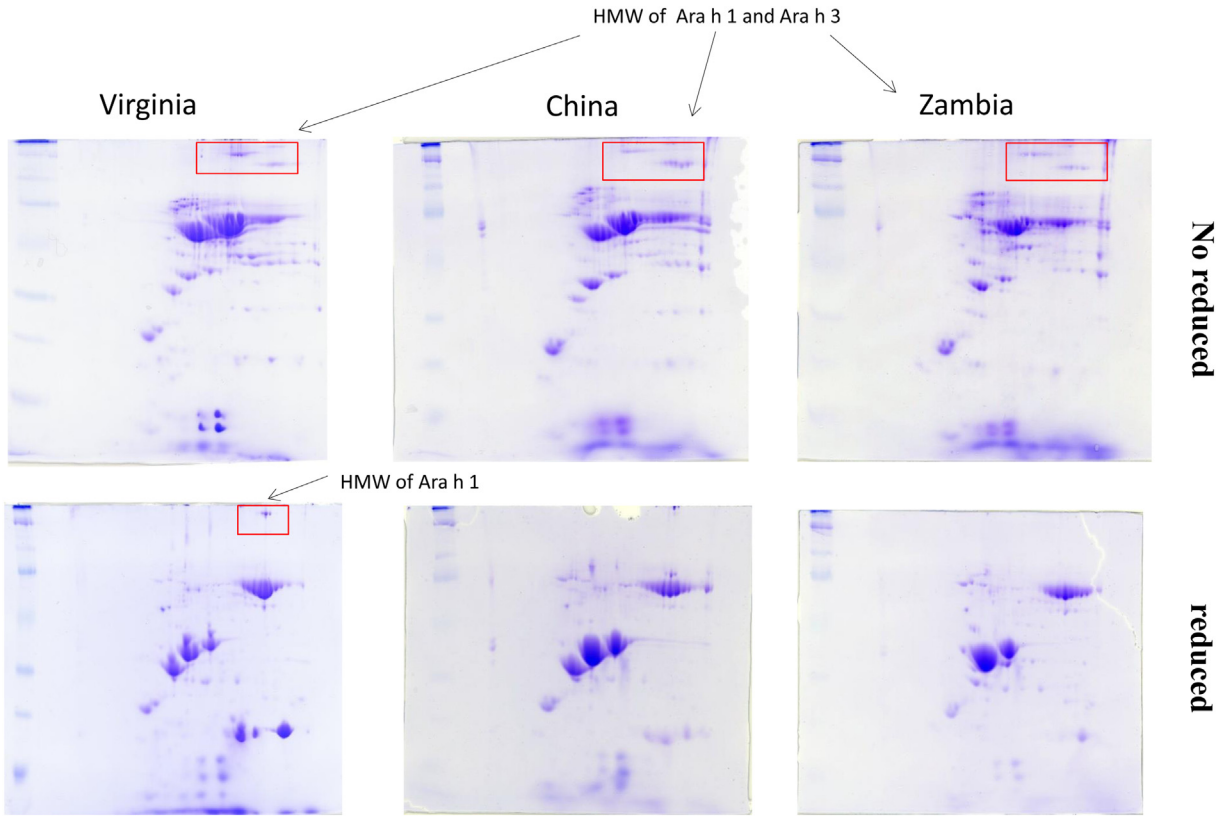

Fig. 2. 2-DE comparison of peanut proteins of Zambia, China and Virginia cultivars, under reducing and nonreducing conditions. HMW regions are delimited by lines.

In spite of the DTT-induced disappearance of aggregates with estimated MW 210 and 180 kDa, the reduction did not affected the spot train at mass line 240 kDa (spots no. 1–3)- detected only as a faint band in the 1D SDS-PAGE (Fig. 1) - and the isolated spots at ~ 110 kDa (spots no. 4 and 5) (Fig. 3B), which were univocally identified by LC-MS/MS as homopolymers of Ara h 1 cross-linked via covalent bonds other than disulfides (Table S2). This finding confirmed those obtained by 1-D SDS-PAGE-MS analysis by other authors (Koppelman et al., 2016; Maleki et al., 2000) and by us in this work. However, the additional aggregates at 240 kDa are compatible with the occurrence of stable tetrameric Ara h 1 homopolymers with non-disulfide covalent cross-linkages, which have been never reported previously. Several kinds of covalent bonds could be involved in the spontaneous formation of protein aggregates (Gerrard, 2002).In the case of Ara h 1, glycans might

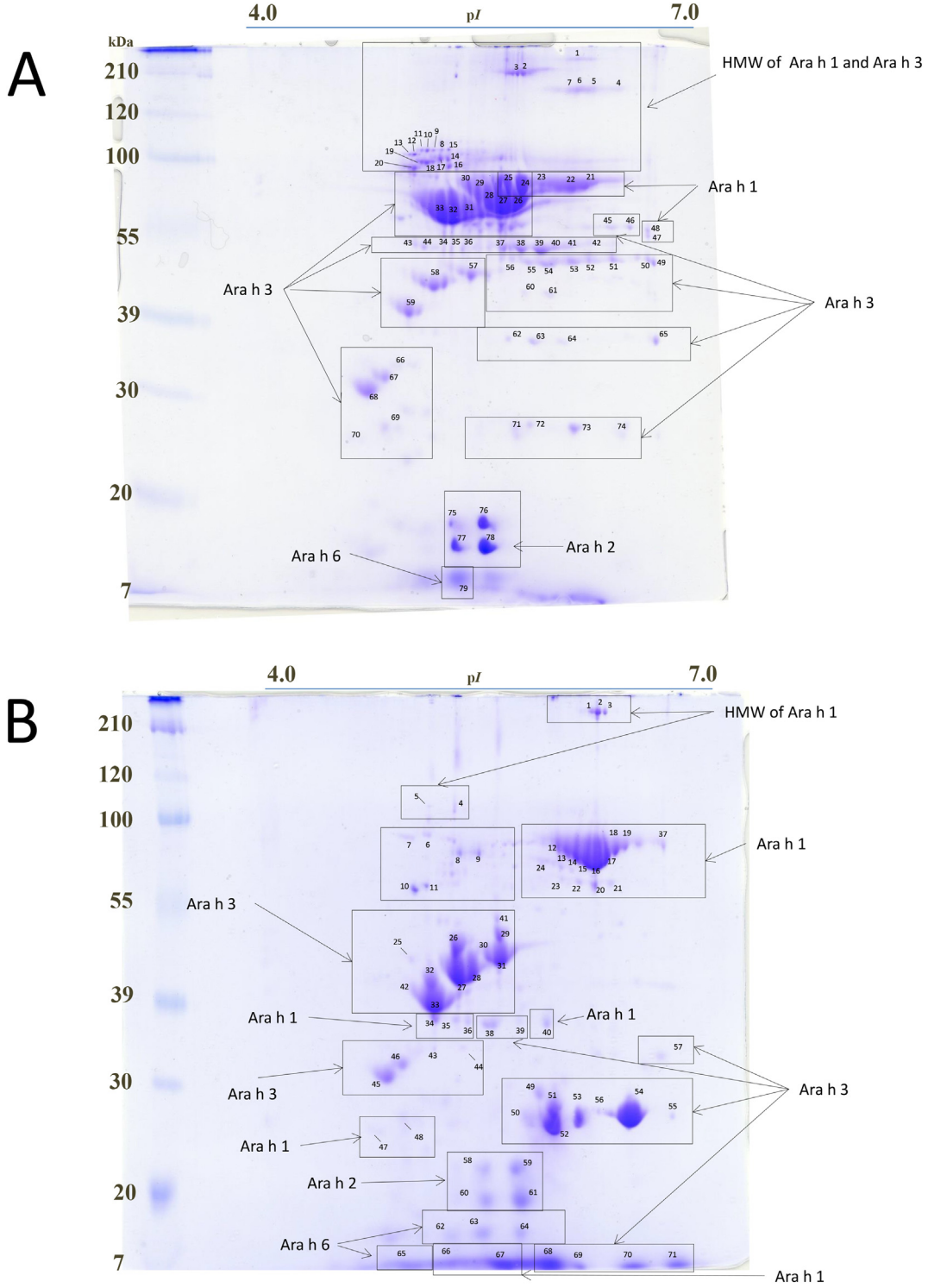

Fig. 3. 2-DE of peanut proteins (Virginia cultivar) separated under unreducing (panel A) and reducing (panel B) conditions. Spots as indicated by numbers were submitted to LC-MS/MS base analysis (Table S1 and Table SII). HMW regions and individual protein isoforms are annotated and delimited by dashed lines.

be responsible of the polypeptide inter-linkage as well. However, the specific nature of the covalent bonds and their potential involvement in the intrinsic allergenicity of peanut proteins should be elucidated with dedicate investigations.

3.2.2. *Ara h 3*

Ara h 3 dominated the 2-DE maps of peanut proteins and demonstrated a significant heterogeneity due to several simultaneous factors, including: i) occurrence of multiple isoforms, ii) post-synthesis processing, which involves the splitting in an acidic and a basic subunits held together by a disulfide, analogously to the 11S globulins from other *Leguminosae* (e.g. soybean) or non-*Leguminosae* tree nuts (e.g. walnut and hazelnut), iii) progressive N-terminal trimming of the basic subunit, iv) possible glycosylation (Piersma, Gaspari, Hefle, & Koppelman, 2005). Prior to reduction, Ara h 3 migrated as three full-length polypeptides at estimated mass lines 60 (spot 24, 25, 29–30), 58 (spot 26–28) and 56 kDa (spot 31–33), covering the 5.5–6 *pI* range. Peptides arising from Ara h 3 were also found in a series of minor spots

spread out at ca. 52–56 kDa (spot 42–46) at the acidic and basic side, as well as in low abundance spots at ca. 34-43 kDa (spot 49–65) and 24–25 kDa (spot 69–74), at the acidic and basic side of the gel, respectively (Fig. 2, Table S1). These latter spots of Ara h 3 are probably due to a partial proteolysis affecting the full length polypeptides. Additional Ara h 3 forms, with experimental MW higher than theoretical one, were detected in spot 12 and 13 in the 2-DE maps of nonreduced gel (Fig. 3A, Table S1). Following reduction, the expected acidic (p*I* 5–6) and basic (p*I* 6–8) Ara h 3 subunits were detected in spots 25–33, 41–42 and 49–56, respectively (Fig. 3B; Table S2) (Piersma et al., 2005). Ara h 3 fragments were also found in spot 38–39, 43–46, and 57. Several additional low MW Ara h 3 fragments (ca. 7 kDa, spot no. 68–71) were generated by the DTT-mediated release of proteolyzed subunits.

Interestingly, low-abundance isoforms of Ara h 3 did not completely disappear under reducing conditions and were still detected in the 56–60 kDa mass range (spots no. 6–11; Fig. 3B). Since LC-MS/MS analysis matched the sequences in both acidic and basic subunits, we deduce that these spots corresponded to proteolytic isoforms of Ara h 3 of different origin than post-translational processing (Dam et al., 2009).

Notably, whereas Ara h 3 was engaged with Ara h 1 in the formation of disulfide-linked heteropolymeric aggregates, no evidence of Ara h 3-containing aggregates persisted under reducing conditions.

3.2.3. Ara h 2 and Ara h 6

Ara h 2 and Ara h 6, both members of the 2S albumin family, were the only proteins detected in the 2-DE maps in addition to Ara h 1 and Ara h 3. In line with previous characterizations (Chatel, Bernard, & Orson, 2003), Ara h 2 occurred as two isoforms (2.01 and 2.02), which included multiple spots each, differing by p*I*. They were spots no. 75–78 in nonreduced (Fig. 3A; Table S1) and spots no. 58–61 in reduced 2-DE gels (Fig. 3B; Table S2).

Under nonreducing conditions, Ara h 6 (expected 14.6 kDa) was detected as a single polypeptide chain (spot no. 79; Fig. 3A; Table S1), which did not evidence the presence of multiple proteoforms (Bernard et al., 2007). Analogously to Ara h 3, Ara h 6 occurs as a heterodimer produced by *endo*-proteolytic cleavage of a single polypeptide precursor, whose subunits remain linked via a disulfide bond. The disulfide reduction split Ara h 6 into multiple spots, namely 62–64 at ~9 kDa and 65 at ~7 kDa (Fig. 3B; Table S2), probably arising from different post-translational proteolytic processing of the 9 kDa subunit, in agreement with the typical electrophoretic pattern of Ara h 6 previously observed (Suhr, Wicklein, Lepp, & Becker, 2004).

3.3. Shotgun proteomic analysis

> 100 protein spots were resolved by 2-DE gels, providing a clear picture of the major storage protein families and possible isoallergens, with an estimation of their p*I*/MW and information about the effects of proteolytic events. On the other hand, Ara h 2 and Ara h 6 allergens along Ara h 1 and Ara h 3, which together account for about 87% of storage peanut protein, were clearly individuated in 2-DE maps.

The pitfalls of 2-DE to detect low-abundance proteins as well as to separate hydrophobic and extremely acidic or basic proteins have been largely reported (Rogowska-Wrzesinska et al., 2013). Overall, these limitations hampered the detection of many among the expected gene products occurring in peanut seeds and already catalogued among the peanut allergens, such as oleosins (Ara h 10/11), defensins (Ara h 12/13), lipid transfer protein (LTP, Ara h 9), besides enzymes involved metabolic processes, not described as allergens yet. The shotgun proteomics enabled accessing to groups of low-abundance proteins (Zhang, Fonslow, Shan, Baek, & Yates, 2013). Using the shotgun proteomic approach, 149 gene product entries were identified with high confidence (Table S3), including the main storage proteins Ara h 3 (ten identified isoforms), Ara h 1 (isoform P17 and P418), as well as Ara h 6 and two isoforms of Ara h 2 (Ara h 2.0.1 and 2.0.2), in line with the 2-DE based proteomic analysis.

Differently from the 2-DE, shotgun proteomics allowed the identification of minor allergens and metabolic enzymes in addition to the dominant storage protein components. Two isoallergens of the Ara h 9 protein, namely LTP isoallergen 1 (ABX56711) and LTP isoallergen 2 (ABX75045) were detected and identified. In analogy to the homologous protein from other plant sources, LTP is considered a clinically relevant peanut food allergen, especially in the Mediterranean area (Krause et al., 2009). Ara h 7, belonging to the conglutin 2S albumin protein family occurred as two isoforms. This finding was in agreement with previous ones, reporting the missed detection of Ara h 7 by 2-DE and the identification of two isoallergens sharing high sequence homology (Schmidt et al., 2009). Ara h 8, homologous to Bet v 1 allergen, which may justify cross-sensitization of some peanut allergic subjects to birch pollen (Mittag et al., 2004), was identified as well with 64% of the protein sequence coverage.

Among the identified oleosins, which consist of several gene products tightly associated to the seed oil bodies, oleosin Ara h 10 (oleosin 17.8), Ara h 11.0101 (oleosin 1), Ara h 11.0102 (oleosin 2), Ara h 14 (oleosin variant A) and Ara h 15.0101 (oleosin 3) are clinically relevant peanut allergens and most likely associated with severe allergic symptoms (Schwager et al., 2017). Minor oil body proteins such as steroleosin B-type and A-type, whose allergenic properties have not been proven so far, were also identified. In the current investigation, the identification of oleosins was successful because the crude protein extracts were trypsinized before defatting the peanut seeds, according to the alternative protocol of sample preparation described above. Actually, due to their peculiar properties, the purification of peanut oleosins requires specific protocols of extraction making use of detergents or denaturing and delipidating agents (Schwager et al., 2015).

Low-abundance Ara h 12 (defensin 1) and Ara h 13 (defensin 3), with still uncertain allergenic properties (Petersen et al., 2015), were confidently identified. Many additional minor protein entries identified by shotgun proteomics were enzymes involved in metabolism of starch, proteins expressed in response to stress and involved in defence mechanisms. The allergenic potential of these latter proteins still awaits to be investigated. Overall, our analysis allowed to catalogue nearly 60 low abundant peanut seed gene products more than the unique previous attempt carried out with shotgun proteomics, probably because of the better performances of the Q-Exactive Orbitrap instrument (White et al., 2013).

4. Conclusions

The results of this investigation pointed out the complexity of peanuts allergome. 2-DE analysis run under reducing and nonreducing conditions highlighted the natural occurrence of HMW disulfide-linked multimers of Ara h 1 and Ara h 3. Due to poor resolution of 2-DE deprived of DTT, further dedicated studies would be necessary to definitely assess if Ara h 1 and Ara h 3 form exclusively homopolymers or also heteropolymers. Another relevant insight achieved was the identification of HMW aggregates of Ara h 1 stable under reducing condition. The observation of all these complexes may reveal natural interactions between Ara(s) subunits with a potential involvement in their allergenic potential, still unknown. Herein results also confirmed the complementarity of the “protein-centric” and “peptide-centric” proteomic methodologies, when the purpose of the study is a “discovery-driven” characterization of the proteome. In fact, 2-DE analysis practically allowed only the detection of the major storage proteins, including the main four allergen families (Ara h 1, Ara h 3, Ara h 2 and Ara h 6), while it failed to disclose the “deep” peanut proteome. In contrast, the shotgun analysis enabled accessing to a more comprehensive coverage of the peanut allergome, but losing information about the quaternary structure of the proteins and the possible occurrence of isoallergens (Rogowska-Wrzesinska et al., 2013).

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.09.045.

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Protective effects of ID331 *Triticum monococcum* gliadin on *in vitro* models of the intestinal epithelium

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ABSTRACT

A growing interest in developing new strategies for preventing coeliac disease has motivated efforts to identify cereals with null or reduced toxicity. In the current study, we investigate the biological effects of ID331 *Triticum monococcum* gliadin-derived peptides in human Caco-2 intestinal epithelial cells. *Triticum aestivum* gliadin derived peptides were employed as a positive control. The effects on epithelial permeability, zonulin release, viability, and cytoskeleton reorganization were investigated. Our findings confirmed that ID331 gliadin did not enhance permeability and did not induce zonulin release, cytotoxicity or cytoskeleton reorganization of Caco-2 cell monolayers. We also demonstrated that ID331 ω-gliadin and its derived peptide ω(105–123) exerted a protective action, mitigating the injury of *Triticum aestivum* gliadin on cell viability and cytoskeleton reorganization. These results may represent a new opportunity for the future development of innovative strategies to reduce gluten toxicity in the diet of patients with gluten intolerance.

1. Introduction

Coeliac disease (CD) is the most common wheat intolerance worldwide. It is triggered by the ingestion of gluten-based food in genetically predisposed individuals (Sollid, 2000). Its extraordinary concentration of proline amino acid makes gluten protein highly resistant to gastric, pancreatic and intestinal enzymes (Hausch, Shan, Santiago, Gray, & Khosla, 2002; Mamone, Picariello, Addeo & Ferranti, 2011). Gluten-derived peptides cross the intestinal barrier, triggering the adaptive and innate immune reactions, which are responsible for mucosal destruction (Gianfrani, Auricchio, & Troncone, 2005). Gliadin peptides also affect intestinal epithelial permeability by triggering the release of zonulin. Zonulin release induces protein kinase C-(PKC) mediated polymerization of intracellular actin filaments, which are directly connected to the structural proteins of tight junctions (TJs) regulating the epithelial permeability (Visser, Rozing, Sapone, Lammers, & Fasano, 2009).

Because the only current therapy for CD is a strict gluten-free diet, researchers are seeking cereals or pseudo-cereals with low or null toxicity. *Triticum* (*T.*) *monococcum* wheat has been considered a suitable candidate because it lacks the D genome encoding the immunodominant 33-mer fragment (Molberg et al., 2005). The capability of *T. monococcum* to trigger innate and adaptive immunity in CD patients was investigated by several studies (De Vincenzi, Lucchetti, Giovannini, & Pogna, 1996; Pizzuti et al., 2006; Vincentini et al., 2007; Vaccino, Becker, Brandolini, Salamini, Kilian, 2009; Gianfrani et al., 2012, 2015; Šuligoj, Gregorini, Colomba, Ellis, & Ciclitira, 2013). In particular, two ancient *T. monococcum* cultivars, named ID331 and Monlis, have received the bulk of attention (Gianfrani et al., 2012, 2015). *Ex-vivo* experiments have demonstrated that both cultivars induce a slight adaptive immune response in CD patients (Gianfrani et al., 2015). Conversely, "*innate immune*" responses were triggered only by Monlis and not by ID331 (Gianfrani et al., 2012). We recently demonstrated that the gliadin expression profiles of ID331 and Monlis are highly comparable, differing only in the lack of ω-gliadin in Monlis (Gianfrani et al., 2015). Therefore, it was tempting to speculate that the expression of ω-gliadin by ID331 would protect against the *toxicity* induced by common wheat gliadin.

In the current study, we investigated the biological effects of ID331 gliadin-derived peptides on the human intestinal epithelial cell line Caco-2 (human colon adenocarcinoma cell line). Subsequently, we assessed the potential of ID331 ω-gliadin and its

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gastrointestinal-resistant peptide ω(105–123) to protect against the toxicity induced by *T. aestivum* gliadin in Caco-2 cells.

2. Material and methods

2.1. Materials

All reagents and solvents were of the highest purity available from Sigma-Aldrich (Milan Italy). *T. monococcum* and *T. aestivum* wheat flours were provided by Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA-QCE) (Rome, Italy). Gliadin proteins were extracted from wheat flour according to Mamone, Ferranti, Chianese, Scafuri, & Addeo, 2000.

2.2. ID331 ω-gliadin purification

Proteins were purified with an RP-HPLC Agilent 1100 modular system with an integrated diode array detector (Palo Alto, CA) using a semi-preparative C18 reverse-phase column (5 μ, 10 × 250 mm, 300A) (Phenomenex, Bologna, Italy) with a flow rate of 3 ml/min. Solvent A was 0.1% trifluoroacetic acid (v/v) in water, and solvent B was 0.1% TFA in acetonitrile. The column was equilibrated at 25% solvent B. The gliadin extracts were separated with a gradient of 25–55% solvent B over 100 min. The chromatographic separation was performed at 55 °C using a thermostatic column holder. Peaks were collected manually, and protein concentration was determined with a modified Lowry kit (Sigma-Aldrich).

2.3. In vitro simulated gastrointestinal digestion

Gliadin proteins extracted from wheat flour and ID331 ω-gliadin were digested by gastric, duodenal (pepsin-trypsin-chymo trypsin-carboxypeptidase-elastase) and porcine brush border membrane enzymes (BBM) as described elsewhere (Gianfrani et al., 2015). Digested samples were stored at −80 °C until further analysis. Whole digested gliadins will hereafter be abbreviated as WDG.

2.4. Preparation of synthetic ω-gliadin peptide

The ω(105–123) peptide was obtained by Fmoc solid-phase synthesis using a Pioneer Peptide Synthesis System 9050 (PE-Biosystems, Framingham, MA). The peptide was purified by HPLC, and its identity was assessed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), using a Voyager DE-PRO instrument (Perseptive BioSystems, Framingham, MA, USA).

2.5. Cell culture conditions and in vitro studies

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA). Cells were cultured in Dulbecco's modified (high-glucose) medium supplemented with 20% heat-inactivated fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Caco-2 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells used in this study were at passage 20−30. Subculture was performed at 80% of confluence. The medium was changed every other day. For transepithelial electrical resistance (TEER) studies, cells were seeded at 450,000 cells/cm^2 in cell culture inserts with PET (polyethylene terephthalate) membranes (0.4 μm pore size, 23.1 mm diameter, 4.2 cm^2 growth surface area; BD Falcon, Italy). Cell attachment was improved by first covering the membrane with bovine collagen type I (Gibco, Invitrogen, Italy) (Iacomino et al., 2013). Cells were allowed to differentiate for 21 days before experiments. Cell differentiation was evaluated by the alkaline phosphatase activity assays as previously described (Iacomino, Tecce, Grimaldi, Tosto, & Russo, 2001), using p-nitrophenolphosphate (Sigma-Aldrich) as a substrate. TEER was assessed using an epithelial voltammeter (Millicell ERS-2, Millipore, Italy). TEER values were calculated as Ohms per square centimeter. Only Caco-2 monolayers showing TEER values higher than 800 Ω/cm^2 were selected for subsequent experiments.

Caco-2 cells were incubated in the presence or absence of digested gliadins for the assessment of zonulin release, epithelial permeability, viability and cytoskeleton reorganization. The optimal concentrations and incubation times were experimentally determined by preliminary assays (data not shown).

2.6. Determination of zonulin release

Zonulin release into the cell culture media was quantified using the human zonulin ELISA Kit (EMELCA Biosciences, Breda, Netherlands) according to the manufacturer's instructions.

2.7. Cytotoxicity

Cytotoxicity was determined by measuring cellular ATP levels with a high sensitivity cell proliferation/cytotoxicity kit (Vialight Plus, Cambrex Bio Sci Rockland Inc., Rockland, ME). Briefly, cells were plated in 96-well arrays at a density of 2×10^4 per well and differentiated for 21 days before being exposed to digested gliadins in a reduced serum medium (5% FCS). The optimal concentrations and incubation times were experimentally determined by preliminary assays (data not shown). After 24 h incubation time, nucleotide-(ATP) releasing reagent (50 μl) was added to each well and the plate was incubated for 10 min at room temperature. Cell lysates (100 μl) were transferred to a luminescence-compatible plate. The 96-well plates were read with a 1 s integration time using a TopCount-NXT™ luminometer (Packard, USA). ATP levels in cells were expressed as relative light units (RLUs). The results represent the mean of five measurements ± SD.

2.8. Fluorescein labeling of cytoskeletal structures

The distribution of actin microfilaments (F-actin) was assessed using fluorescent labeling techniques as previously described (Artursson, Lindmark, Davis & Illum, 1994). Caco-2 cells were grown and differentiated on chamber slides using the growth conditions outlined above and incubated with either gliadin samples or PBS. Monolayers were fixed with 3.75% formaldehyde solution in PBS for 5 min at room temperature, permeabilized in 0.1% TRITON® X-100 in PBS for 5 min, and then washed again with PBS solution. Subsequently, cells were stained with 50 μg/ml fluorescein-labeled phalloidin (Sigma-Aldrich) solution in PBS for 40 min to highlight F-actin. The monolayers were then rinsed with PBS three times, and finally, coverslips were mounted in a 1:1 solution of PBS and glycerol. The fluorescein-labeled structures were blindly analyzed by fluorescence microscopy using a Zeiss AxioVert 200 (*Zeiss, Germany*).

2.9. Statistical analysis

Student's *t* test was used to analyze the statistical significance of the results. Values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were completed using GraphPad Prism 6 software.

3. Results

3.1. General

The biological effects of ID331-WDG on a well-established Caco-2 cell line were assessed in comparison to those of *T. aestivum*-WDG. The Caco-2 cell system was used as a predictive model of the intestinal barrier due to its intrinsic capability to differentiate spontaneously into polarized cells with morphological and functional features of small intestinal enterocytes (Sambuy et al., 2005). TEER values of Caco-2 monolayers, zonulin release, viability assay, and cytoskeleton reorganization were determined after challenging cells with *T. monococcum*- or *T. aestivum*-WDG (Fasano et al., 2000; Iacomino et al., 2013; Picariello et al., 2013; Sander, Cummins, Henshall, & Powell, 2005).

3.2. Effect of WDG exposure to cell permeability

After 21 days, at confluence, the TEER value of the differentiated Caco-2 cells stabilized at >800 Ω/cm^2, confirming the integrity of the membrane and indicating that the TJs were well formed and functionally active. According to ours and other previous observations, cells incubated with 1.0 mg/ml of *T. aestivum*-WDG for 1 h showed a drastic decrease of cell monolayer TEER (Iacomino et al., 2013, Sander, Cummins, Henshall, & Powell, 2005). In contrast, TEER remained almost unchanged after parallel treatment with 1.0/ml mg of ID331-WDG (Fig. 1 panel A).

It has previously been established that the apical secretion of zonulin, which regulates the status of the intercellular TJs, may be one of the key factors affecting cell permeability (Fasano, 2011; Fasano et al., 2000). Consequently, we evaluated how WDG affected apical zonulin release by differentiated Caco-2 cells. Zonulin in the cell surnatant was detected by ELISA at increasing time intervals following incubation with WDG (Fig. 1 panel B). As expected (Clemente et al., 2003), exposure to *T. aestivum*-WDG induced a release of zonulin that peaked at 1 h post-incubation and returned to baseline after 4 h. Cells treated with ID331-WDG behaved similarly to the untreated control with a slight release of zonulin at 2 h (Drago et al., 2006), which was probably due to manipulation of differentiated monolayers (i.e. cell washing and incubation medium replacement).

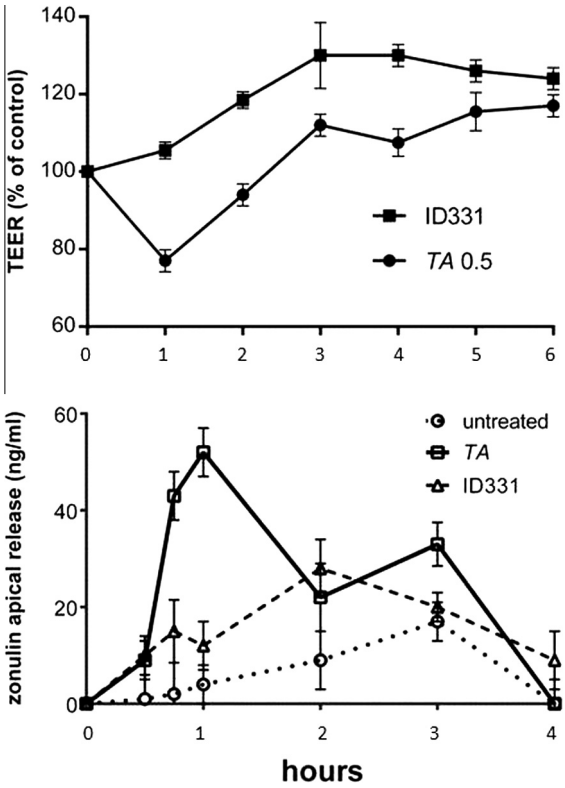

Fig. 1. Effects on the paracellular permeability of differentiated Caco-2 cells of WDG from *T. monococcum* (ID331) and *T. aestivum* (TA) were evaluated by assessing the TEER value in insert chambers (panel A) and apical zonulin release (panel B). All experiments were performed in triplicate. The results represent the mean ± SD.

3.3. Effect of WDG exposure on viability of Caco-2 cells

The cytotoxic effect of WDG on cells was tested by measuring the ATP levels as a metabolic marker (Fig. 2). ATP is the primary form of energy storage in all cells and can be used as a marker for the functional integrity of live cells, as it indicates the energy state of cellular systems even before the membrane integrity is compromised. The exposure to 1.0 mg/ml *T. aestivum*-WDG induced an adverse reaction after 24 h, with a significant reduction in the ATP level of cells if compared to that of cells exposed to media alone. The viability of cells treated with ID331-WDG appeared unchanged, confirming the lack of apparent toxicity on Caco-2 cells.

3.4. ID331. ω-gliadin peptide

In our previous report (Gianfrani et al., 2015), we found that upon *in vitro* gastrointestinal digestion, ID331 ω-gliadin released a stable peptide 105–123 (QSFPQQPQRPQPFPQQPEQ sequence). This sequence has a clear homology with the decapeptide QQPQRPQQPF, except for the lack of a Q residue (Fig. 3), first identified from ω-secalin and supposed to contrast the CD mucosa immune activation induced by toxic gliadins (De Vita et al., 2012).

To evaluate if ω(105–123) has the potential to protect against the toxicity induced by *T. aestivum*-WDG, differentiated Caco-2 cells were pre-incubated with purified digested ω-gliadin or synthetic ω(105–123) peptides before the exposure to *T. aestivum*-WDG.

3.5. Effect of digest ω-gliadin and ω(105–123) peptide exposure on Caco-2 F-actin microfilaments and cell viability

The effect of digested gliadin exposure on Caco-2 cytoskeletal morphology was determined by fluorescein labeling of F-actin filaments (Fig. 4). Cytoskeleton status was evaluated because

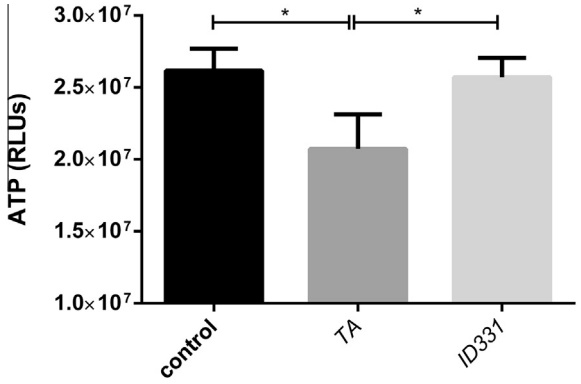

Fig. 2. Relative cytotoxicity for differentiated Caco-2 cells of WDG from *T. monococcum* (ID331) and *T. aestivum* (TA) was assessed by monitoring cellular ATP levels with a bioluminescence assay. Intracellular ATP levels were expressed as relative light units (RLUs). The results represent the mean of five multiple measurements ± SD ($^{*}p < 0.05$).

ID331 ω-gliadin (gi|294998449)

ARQLNPSDQELQSPQQLYPQQPYPQQPYPPQQPFPTPQQYFPHQSQQPFS
PPQQPFPQPQQATPLQPQQPFPQQPQQPQQAFPQPQQQFPLQPQQQFPQP
QQPQ**QSFPQQPQRPQPFPQQPEQ**IISQQSFLLQPQQPFPQQPEQIVSQQP
QQLFSQSQQPFPQQPQQPFPLQPQQPFPQQPAQIIAQQPQQPSPLRPQQP
FLWQPQQPFLQQPQQPFPQPQQVVQIIPQQPQQPFSQSQQPFPQQPQQPF
PLQPQQPFPQQSAQIIPQQPQQSFPLQPQQSFLRQSQQPFLQQPQQPSPQ
PQQVVQIIPQQPQQPFPLHTNQPQQPYPQQQPSGVVE

ω-gliadin (105-123) ^{105}QSFPQQPQRPQ | PFPQQPEQ^{123}
ω-secalin decapeptide QQPQRPQQPF

Fig. 3. ID331 ω-gliadin (gi|294998449). The region in bold indicates the gastrointestinal-resistant peptide ω-gliadin (105–123). This sequence exhibits clear similarities to the decapeptide QQPQRPQQPF previously identified from ω-secalin, with the exception of a missing glutamine residue (De Vita, Ficco & Luciani, 2012).

gliadin-derived peptides are known to induce an early F-actin reorganization affecting zonulin release in enterocytes (Clemente et al., 2003). In differentiated Caco-2 cells, as well as in intestinal epithelial cells (Madara, 1992), F-actin filaments are finely localized at the apical perijunctional area as a continuous band encircling the cells and interacting with the junctional complexes (Fig. 4A). Incubation with 1.0 mg/ml of *T. aestivum*-WDG led to intense reorganization of intracellular actin filaments, which was clearly visible by fluorescence microscopy after only 1 h and was characterized by a deep redistribution of the F-actin from the cellular subcortical compartment (Fig. 4B). Conversely, no significant changes were observed when cells were exposed to similar concentrations of ID331-WDG (Fig. 4C).

Remarkably, a 1 h pretreatment with 200 μg/ml digested ω-gliadin prior to *T. aestivum*-WDG exposure significantly prevented cytoskeleton reorganization (Fig. 4D). Similarly, pretreatment with 200 μg/ml of ω(105–123) peptide followed by a 1 h *T. aestivum*-WDG incubation prevented F-actin reorganization, confirming that this peptide was capable of protecting against the toxic effect of *T. aestivum* (Fig. 4E).

3.6. Effect of digested ω-gliadin and ω(105–123) peptide exposure on Caco-2 cell viability

A 1 h pre-incubation with digested ω-gliadin (200 μg/ml) before a 24 h incubation with 1.0 mg *T. aestivum*-WDG significantly prevented epithelial toxicity because ATP levels were

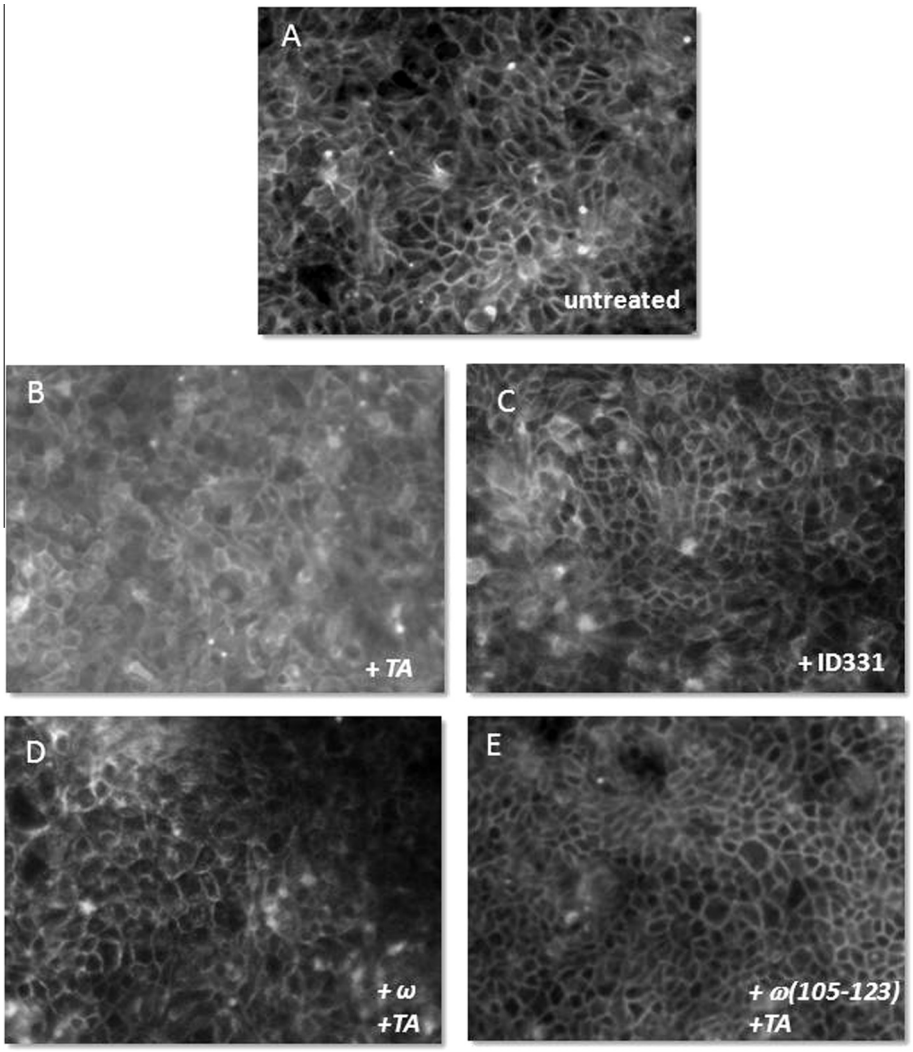

Fig. 4. Distribution of F-actin microfilaments was assessed using fluorescent labeling techniques. Caco-2 cells were grown and differentiated onto chamber slides. At appropriate time-points after treatment, cells were stained with fluorescein-labeled Phalloidin to highlight F-actin (X 400 magnification). (A) Untreated control; (B) cells incubated with WDG from *T. aestivum* (TA); (C) cells incubated with WDG from ID331; (D) cells initially incubated with digested ω-gliadin followed by incubation with WDG from *T. aestivum* (TA); (E) cells initially incubated with ω(105–123) followed by incubation with WDG from *T. aestivum* (TA).

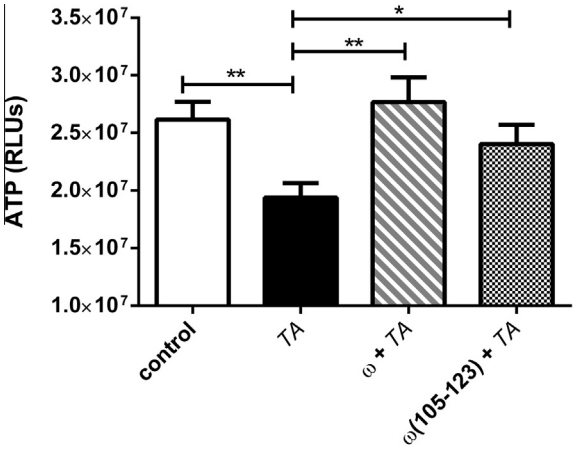

Fig. 5. Protective effects of ω-gliadin fraction and ω(105–123) peptide on *T. aestivum* gliadin-induced cytotoxicity were evaluated on differentiated Caco-2 cells by monitoring the cellular ATP levels. Intracellular ATP levels were expressed as relative light units (RLUs). The results represent the mean of five measurements ± SD ($^{*}p < 0.05$; $^{**}p < 0.005$).

comparable to the untreated control (Fig. 5). To establish whether the protective effect of ω-gliadin was dependent on the peptide ω (105–123), the experiments were also conducted in the presence of the synthetic counterpart. Also in this case, Caco-2 showed no loss of viability when incubated for 24 h with 1.0 mg/ml *T. aestivum*-WDG following a 1 h pre-incubation with the ω(105–123) peptide.

4. Discussion

In the current study, we demonstrated that the gliadin proteins of ID331 are sufficiently different from those of common *T. aestivum* to possess a lower toxicity on differentiated Caco-2 cells. An early event in CD pathogenesis is the alteration of paracellular permeability through which immunogenic gliadin initially gains access to the gut submucosa (Van Elburg, Uil, & Mulder 1993). Unlike *T. aestivum*, ID331 *monococcum* did not enhance permeability and did not trigger zonulin release, as well as not affecting cell viability or inducing cytoskeleton reorganization.

The lower toxicity of ID331 in this *in vitro* system supports previous results finding that, unlike other *T. monococcum* species (cultivar Monlis), ID331 is less effective in inducing CD, because of its inability to induce the synthesis of interleukin IL-15 by the small intestine cells and activate the innate immune pathway (Gianfrani et al., 2012). The α-gliadin peptides 31–49 and 31–55, which have been identified as important activators of the innate immune response (Lahteenoja et al., 2000; Maiuri et al., 1995, 2003; Marsh et al., 1995), were expressed in *T. monococcum* cultivar ID331 as well as in *T. monococcum* cultivar Monlis and *T. aestivum* gliadin (Gianfrani et al., 2015). The presence of peptides 31–49 and 31–55 strongly suggested that the missed activation of innate immunity by ID331 could be due to the counteraction of some other protective factor(s). The current results indicate that ID331 ω-gliadin and its gastrointestinal-resistant peptide ω(105–123) might be considered "*candidate protective factors*" that interfere with the activation of innate immunity. In fact, the pre-incubation of Caco-2 cell-based models of the intestinal epithelium with digested ω-gliadin or ω(105–123), significantly prevented gliadin-dependent F-actin reorganization and the gliadin-induced epithelial damage. Further studies are necessary to improve our knowledge of the molecular mechanisms responsible for the protective effect exerted by the ω(105–123) peptide. Identification of the mechanism might explain some of the large genetic diversity in the amount of toxic peptides present in wheat germplasm. The elucidation of these aspects might also support specific breeding programs with low toxicity genotypes, adequate for the preparation of food products for patients suffering from gluten-related disorders (Sapone et al., 2012) and to develop non-nutritional therapies that improve the health of patients with CD.

5. Conclusion

Using an *in vitro* model of the intestinal epithelium we demonstrated that ID331 gliadin proteins do not induce effects associated with cell toxicity exerted by *T. aestivum* gliadin, due to the protective effect of ID331 ω-gliadin and its gastrointestinal resistant peptide ω(105–123). The ID331 ω-gliadin sequence is absent in monococcum wheat genotypes lacking ω-gliadins such as cultivar Monlis as well as in a number of einkorn lines possessing ω-gliadins in their prolamin patterns (data not shown), suggesting that variation in toxicity may exist in the monococcum wheat germplasm as well. These results open new research perspectives related to a possible protective action of *T. monococcum* on the small intestine of CD subjects. In particular, a diet based of *T. monococcum* could delay or even prevent the onset of CD in at-risk subjects such as first-degree relatives of coeliac patients.

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Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease

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ABSTRACT

Microwave based treatment (MWT) of wet wheat kernels induced a striking reduction of gluten, up to <20 ppm as determined by R5-antibodybased ELISA, so that wheat could be labeled as gluten-free. In contrast, analysis of gluten peptides by G12 antibody-based ELISA, mass spectrometry-based proteomics and in vitro assay with T cells of celiac subjects, indicated no difference of antigenicity before and after MWT. SDS-PAGE analysis and Raman spectroscopy demonstrated that MWT simply induced conformational modifications, reducing alcohol solubility of gliadins and altering the access of R5-antibody to the gluten epitopes. Thus, MWT neither destroys gluten nor modifies chemically the toxic epitopes, contradicting the preliminary claims that MWT of wheat kernels detoxifies gluten. This study provides evidence that R5-antibody ELISA alone is not effective to determine gluten in thermally treated wheat products. Gluten epitopes in processed wheat should be monitored using strategies based on combined immunoassays with T cells from celiacs, G12-antibody ELISA after proteolysis and proper molecular characterization.

1. Introduction

Wheat gluten proteins play an extremely important role in determining the quality of baked-foods, due to their ability to form a cohesive mass in dough once the flour is mixed with water (Bonomi et al., 2013). On the other hand, wheat proteins induce "*gluten-related disorders*", a term referred to all conditions related to food intake of gluten-based products, including celiac disease (CD), dermatitis herpetiformis, gluten ataxia, and non-celiac gluten sensitivity (NCGS) (Sapone et al., 2012). CD is an immune-mediated systemic disorder elicited by the ingestion of gluten-containing cereals (*i.e.* wheat, barley, rye). Prevalence of CD ranges between 0.5 and 1% over the general population. Etiopathogenesis of CD involves a complex series of genetic and environmental factors (Abadie et al., 2011; Sollid, 2000; Lundin and Sollid, 2014). CD is strictly associated with the HLA class II genes encoding for DQ2.5 and/or DQ8 molecules, as the majority of individuals with CD (>97%) carry either the DQ2.5 or DQ8 genes. The HLA DQ2.5/DQ8 molecules have a key role in CD pathogenesis, as they present immunogenic gluten peptide sequences to gluten-sensitive $CD4^+$ T lymphocytes (Abadie et al., 2011; Sollid et al., 2012). Tissue transglutaminase (tTG)-mediated deamidation of specific glutamine residues to glutamic acid increases the affinity of gluten peptides to the HLA molecules (Molberg et al., 1998; van de Wal et al., 1998).

Gluten proteins are classified into gliadins and glutenins, which account for nearly 80% of the total protein content of wheat kernel (Wieser, 2007; Bonomi et al., 2013). Because of the high percentage of proline residues, gluten proteins are resistant to gastrointestinal

Abbreviations used: AMBIC, ammonium bicarbonate; CD, celiac disease; IFN-γ, interferon gamma; MWT, microwave based treatment; NCGS, non-celiac gluten sensitivity; tTG, tissue transglutaminase; TCL, T cell line.

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digestion, so that large gluten fragments can reach the gut lumen at a high concentration, eliciting adverse immune responses in predisposing individuals (Hausch et al., 2002).

Numerous strategies have been developed for preventing or reducing gluten toxicity of wheat flour, as alternatives to a strict gluten-free diet of CD patients. The most interesting approaches are based on proteolytic degradation of gliadin epitopes or chemical modification of target residues of transglutaminase (Rizzello et al., 2007; Wolf et al., 2015; Gianfrani et al., 2007; Wieser and Koehler, 2012; Ribeiro et al., 2015).

Physical treatments of wheat using microwave (Leszczynska et al., 2003; Lamacchia et al., 2016) or pulsed light (Panozzo et al., 2016) irradiation have been recently proposed to reduce the immunoreactivity of gluten proteins. In these cases, the reduction of gluten immunoreactivity has been typically assessed by sandwich ELISA (*e. g.* R5-antibody ELISA).

In the present study, we investigated the chemical and structural modification induced by microwave based treatment (MWT) of wet wheat kernels on gluten proteins. The effect of MWT on either gliadin extracts or peptides derived from enzymatic hydrolysis (pepsin-trypsin or chymotrypsin) of gluten were investigated using R5 and G12 ELISA, *in vitro* assays with T cell from gut mucosa of celiac subjects, Raman spectroscopy and mass spectrometry-based proteomics.

2. Materials and methods

2.1. Chemicals

Enzymes (pepsin, trypsin, chymotrypsin, tTG), Tris-HCL, ammonium bicarbonate (AMBIC), KCl, EDTA and HPLC-MS grade solvents were all provided by Sigma Aldrich (Italy). T-cell culture medium and supplement were from Lonza-BioWhittaker (Verviers, Belgium). The interferon (IFN)-γ antibodies from MabTech (Nacka Strand Sweden).

2.2. Microwave-based treatment

Kernels from *T. durum* (Ofanto cultivar) and *T. aestivum* (Centauro cultivar) were furnished by Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA-QCE) and Borrelli farm (Foggia, Italy), respectively. Different MWT of wheat kernels were performed. Briefly, wheat kernels (100 g) were soaked in tap water (150 or 500 mL), and then irradiated in a commercial MW oven (Whirlpool Corporation, Benton Harbor, Michigan, US). Combinations of MW oven power, wetting water volume, wetting and drying time are detailed in Table 1. The granulometry of milled treated and untreated kernels were measured by Horiba laser granulometer LA-950 (Retsch Technology GmbH, Haan, Germany).

2.3. Raman spectroscopy

Raman spectra of flour samples were obtained with a micro-Raman spectrometer (LabRam, Jobin-Yvon Horiba), operating at room temperature by using a He-Ne laser ($\lambda = 632.8$ nm). The laser beam was focused on samples by an Olympus optical microscope (LMPlanFl, Olympus). A laser power of 3.5 mW was measured at the sample position, in order to obtain an acceptable signal/noise ratio, but avoiding thermal damage of the sample. Each Raman spectrum was measured in the 1550 to 1725 cm^{-1} range from, with an integration time of 10 s averaged over 15 accumulations. Scattered light was collected in backscattering geometry and a notch filter was used to suppress the elastic component of the scattered radiation. The collected light beam was dispersed into a spectrophotometer equipped with a 600 grooves/mm grating. The spectral resolution was ~3 cm^{-1} per pixel. A cooled charge coupled device (CCD) system, cooled at 223 K, detected the Raman spectrum, and a separate CCD camera collected white light microscope images of the sample being probed. For each flour sample, 20 different points were randomly selected and investigated. Spectra were processed by PeakFit 4.12, Systat Software.

2.4. Extraction and enzymatic hydrolysis of gliadin

Alcohol soluble proteins (gliadin) from milled MWT and untreated (control) kernels were extracted according to the classical fractionation procedure (Wieser et al., 1998). Gliadin aliquots were analyzed by SDS-PAGE. Pepsin and trypsin (PT) digests of gliadins were prepared according to Gianfrani et al. (2007).

2.5. Digestion of gluten proteins

Unextracted gluten proteins from milled MWT and untreated (control) kernels were digested by chymotrypsin. To this end, milled kernels (100 mg) were depleted of the saline buffer soluble protein fraction (albumins and globulins) with 100 mM KCl, 50 mM Tris-HCl, pH 7.8, 5 mM EDTA in a rotary shaker for 10 min at room temperature (twice). After centrifugation at 10.000 rpm for 15 min, the pellet was re-suspended in 100 mM AMBIC, at pH 7.8 and incubated overnight at 37 °C in a thermomixer with chymotrypsin (1:50 enzyme:substrate, w/w ratio). Sample was then centrifuged at 10.000 rpm for 20 min. Supernatant was collected and stored at −80 °C until further analysis. Prior to T cell stimulation assays, gliadin/gluten digests were deamidated by tTG as reported previously (Gianfrani et al., 2007).

2.6. Evaluation of gluten content by R5 sandwich and G12 competitive ELISA

Gluten content of MWT wheat kernels was measured by R5 sandwich ELISA, using a Ridascreen® Gliadin Elisa Kit (R-Biopharm AG Darmstadt, Germany) according to the manufacturer's and to

Table 1
Chemical and physical parameter soft microwave treatment of whole wheat kernels.

Treatment	Kernels (g)	Water (mL)	Time in water (min)	External drying	MW (W)	Time in microwave (min)	Granulometry μm	Milling	R5-ELISA (ppm)	G12-ELISA (ppm)
Control	100	/	/	/	/	/	<529	immediately	>600	>600
MWT	100	500	1min	RT	1000	2	<185	After 24 h	70 ± 10	>600
MWT0	100	500	2 h	drained	1000	2	<200	After 24 h	41 ± 8	>600
MWT1	100	150	1 h	damp	600	5	<196	immediately	29 ± 5	>600
MWT2	100	150	1 h	damp	600	3	<584	immediately	42 ± 8	>600
MWT3	100	150	1 h	wet	600	3	<269	immediately	42 ± 8	>600
MWT4	100	150	1 h	damp	600	6	<185	immediately	<5	>600

the AOAC guidelines. The analysis was blindly performed by Istituto di Ricerche Agrindustria, (Modena, Italy).

The determination of gluten on the of enzymatic digest of milled MWT wheat was performed by competitive G12 ELISA kit (GlutenTox ELISA Competitive KT-4758 by Biomedal Diagnostics, Sevilla, Spain), according to procedure recommended by the manufacturer. Samples were assayed at 1:500–1:2000 dilutions, in order to obtain test values within the standard calibration curve.

2.7. LC-MS/MS analysis

Mass spectrometry analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Lyophilized digests (~1μg/analysis) were suspended in 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 μm id pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C18 column (2 μm, 15 cm × 75 μm) 3 μm 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 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 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order 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. Spectra were elaborated using the Proteome Discoverer 2.1 software (Thermo Fisher), restricting the research to the *Triticum* protein database (Uniprot, May 2016).

2.8. SDS-PAGE analysis

The entire protein fraction and fractionated gluten proteins (gliadins and glutenins) from untreated and MWT wheat were compared by SDS-PAGE. To extract whole proteins, 1 g of milled kernels was suspended in 10 mL of 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, pH 6.8, for 2 h at room temperature under magnetic stirring. After centrifugation at 13.000 rpm for 10 min, the supernatant was collected for analysis. Gliadins and glutenins were purified from 100 mg of milled kernels according to Wieser et al. (1998).

Proteins were quantified using the modified Lowry kit protein assay (Sigma), diluted in Laemmli buffer (Bio-rad) and separated on 12.5% SDS-PAGE under reducing conditions, using a vertical electrophoresis system Hoefer SE 600, (GE Healthcare, Milan, Italy), at 25 mA for 3 h at room temperature. The gels were stained with 0.25% w/v R-250 Coomassie Brilliant Blue overnight.

2.9. T cell immunogenicity assay

The T cell immunostimulatory properties of gliadin/gluten from MWT kernels were assessed on intestinal T cell lines (TLC) highly reactive to gliadin from common soft wheat. T cell lines were generated from intestinal mucosa explants obtained from five different patients (mean age, 13 years; range 3–34 years) at the time of diagnosis of CD. All patients were consuming a normal diet at the time of endoscopy. With the exception of one patient, the intestinal mucosa had villous atrophy in all of them. Patients were randomly recruited from local gastroenterology units of Moscati Hospital (Avellino, Italy) and of Section of Pediatrics at University of Naples Federico II, and gave their full informed consent to the study. Enrolled subjects were typed for DQA1 and DQB1 genotypes using commercial HLA typing kits (Dynal Biotech) and resulted HLA DQ2.5 positive. The procedure to generate gliadin-specific TLC from intestinal biopsies of CD patients and relative ethical committee approval have been previously described in details (Camarca et al., 2012; Gianfrani et al., 2007). TCL were cryo-preserved in liquid nitrogen until the use in functional assay. T cells ($3–5x10^4$) were incubated with autologous immortalized B cells ($0.6–1x10^5$) and enzymatic digests of gliadin/gluten from untreated (positive control) or MWT kernels in 200 μL complete medium (X-Vivo plus 5% human serum), in U-bottom 96-well plates. All samples were assayed at different concentration, as indicated, after deamidation by tTG. Cell supernatants were collected after 48 h for interferon (IFN)-γ measurement. All antigen preparations were assayed in duplicate and in at least three independent experiments for each TCL. The IFN-γ production was determined by sandwich ELISA as previously described (Gianfrani et al., 2012).

3. Results

3.1. Effect of MWT on R5-antibody reactivity of durum kernel

The *T. durum* kernels were processed by MWT preliminarily soaked with water, using a range of soaking time/irradiation time/microwave power parameters (Table 1). To estimate the effect of MWT on the immunological potential of wheat, the gluten content was determined by R5 sandwich ELISA before and after treatment, using the extraction buffer (cocktail) recommended by the producer. Compared to untreated kernels, MWT wheat showed a drastic reduction of gluten, up to 70 ppm in line with previous report (Lamacchia et al., 2015, 2016).

3.2. T cell assay of gliadins (alcohol soluble fraction) from MWT – durum kernels

Gliadin from MWT kernels was further assayed for the ability to activate the production of IFN-γ by $CD4^+$ T cells isolated from the intestinal mucosa of DQ2.5 subjects affected by CD. To this purpose, TCL were challenged with an enzymatic (PT) digests of alcohol soluble proteins (gliadins) from MWT kernels. PT-gliadin digests were deamidated by a short treatment with tTG to increase the immunostimulatory capability on T cells (Molberg et al., 1998; van de Wal et al., 1998; Vader et al., 2002; Dørum et al., 2010). MWT strikingly reduced the immunostimulatory activity of gliadins in TCL from all patients tested (Fig. 1).

3.3. SDS-PAGE analysis of proteins from MWT - kernels

Fractionated gliadins and glutenins as well as whole protein extracts (urea-containing buffer) from MWT and untreated wheat were analyzed by SDS-PAGE. Fig. 2 compares the exemplificative protein patterns of MWT1 with untreated wheat.

MWT1 radically modified the profile of whole protein extract from wheat. In control wheat, gliadins and glutenins showed the typical protein patterns, consisting of α-, γ- and ω-gliadin subfamilies and high- (HWM) and low-molecular weight (LWM) glutenins (Ferranti et al., 2007). In contrast, gliadins were drastically affected by MWT, as all the subfamilies were reduced in terms of either number of components or band intensity. Glutenin subunits only underwent minor changes. Overall, these findings demonstrated that MWT dramatically affect protein solubility.

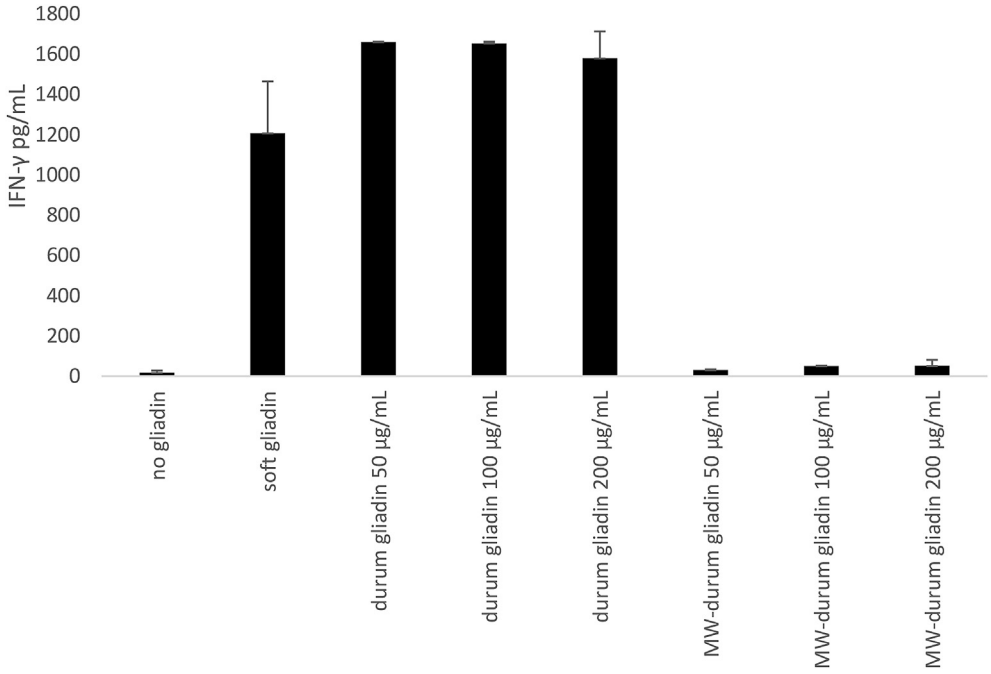

Fig. 1. Effect of MWT on immunostimulatory properties of gliadin alcohol extracts. Polyclonal gliadin reactive TCL were obtained from biopsies of subjects with CD. TCL were pulsed with a deamidated proteolytic digest of gliadin extracts from untreated and MWT *T. durum* kernels. Gliadin from hexaploid wheat (soft gliadin) has been used as internal control antigen (50 µg/mL). As read out of TCL activation, IFN-γ level was measured in cell supernatants after 48 h of stimulation by standard sandwich ELISA. Each experiment has been done in duplicates. Data from one representative patient of five analyzed are illustrated.

3.4. T cell assay of hydrolyzed whole gluten from MWT – T. durum

The relative insolubility of gliadins induced by MWT also justifies at least in part the reduced immunogenicity which was observed when TCL were challenged with gliadin extracts (Fig. 1). To prevent the possible loss of physiologically relevant immunogenic peptides upon MTW, a different approach was applied, consisting in assaying digests of unextracted gluten proteins (instead of fractionated gliadin) for T cell immunogenicity. To this end, after depleting the albumin/globulin fractions from milled kernels, the starchy pellet was directly subjected to chymotrypsin hydrolysis and digests used to test TCL activation. Surprisingly, no differences emerged among MWT and untreated kernels, as demonstrated by the comparable levels of stimulated IFN-γ production at all antigen concentrations tested (Fig. 3). These data confirmed that MWT altered the protein solubility, whereas it did not modify the antigenic properties against TCL. Indeed, immunoreactive R5 gliadin components of MTW kernels were simply not extracted, most likely remaining bound to the starchy pellet due to protein aggregation/coagulation,. Therefore, these proteins released immunogenic epitopes upon chymotrypsin hydrolysis similarly to untreated wheat, suggesting that also *in vivo*, after ingestion of MTW wheat, CD toxic peptides can be liberated during gastrointestinal digestion.

3.5. Effect of changes of MWT parameters on T cell reactivity to gluten - T. aestivum

Above results demonstrated that the time-power regimen of MWT affected the R5-ELISA detection, but not the T cell immunostimulatory properties of whole gluten. In the light of these results, we investigated the margins of improvements of kernel MWT procedure aimed to achieve a gluten epitope detoxification against celiac gut T cells. The MWT parameters were varied within ample ranges also in terms of water volume, hydration time, external drying, and milling in addition to MW power and exposure time (Table 1). Among numerous MWT attempts carried out on *T. aestivum*, only the most representative ones are detailed in Table 1. Method MWT0 was the same as in Lamacchia et al. (2016). After MWT, kernels were milled and gluten content measured by using two different ELISA approaches. The first one, based on the R5 sandwich ELISA performed with the extraction recommended by the producer, revealed a gluten content in the range <5–42 ppm. As expected, the untreated control wheat exhibited a >600 ppm gluten content. The second approach to quantify gluten was based on the G12 antibody ELISA. As G12 test is specifically developed for the detections of hydrolyzed peptides (Morón et al., 2008), gluten was preventively proteolyzed by chymotrypsin. Remarkably, all MWT samples contained high levels of G12 ELISA-detected gluten immunoreactive peptides (>600 ppm), comparable to untreated kernels (Table 1).

To confirm the ineffectiveness of MWT to detoxify gluten, immunostimulatory properties MWT1, MWT2, MWT3 kernels were also investigated. As reported in Fig. 4, MWTs did not induce statistically significant different release of IFN-γ if compared to control when TCL were exposed to hydrolyzed whole gluten.

3.6. Targeted proteomics of digested gluten from MTW kernels - T. aestivum

Further confirmation that CD immunogenic epitopes were not affected by MWT was provided by mass spectrometry (MS) analysis. In particular, we monitored the α-gliadin 33-mer peptide, as a valuable probe of CD toxicity in cereal and wheat based foods in chymotrypsin digests of gluten (Shan et al., 2002). The LC-MS/MS analysis of digested gluten from untreated kernel and MWT1 and MWT2 samples is shown in Fig. 5. Ion extraction of multi-charged ions and chromatographic retention time further proved that chymotryptic digests of MTW kernels (panel B and C) contained the 33-mer peptide at intensity comparable with that of untreated control sample (panel A). Notably, MWT did not induce molecular

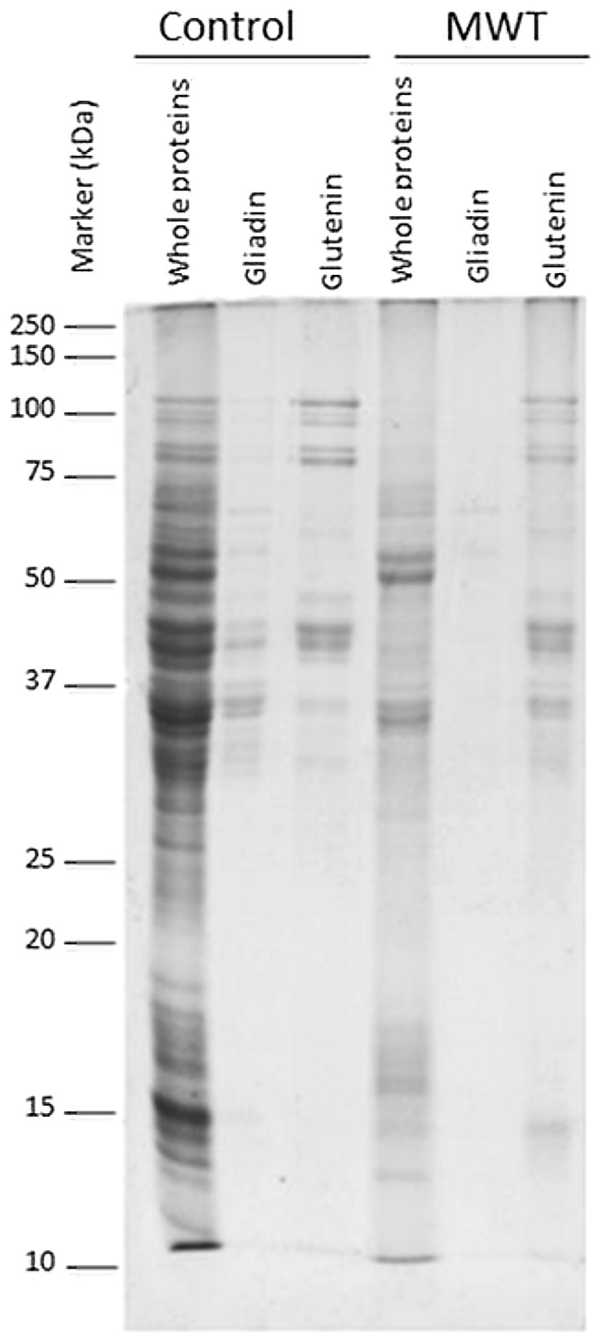

Fig. 2. SDS-PAGE patterns of whole protein extract and fractionated gliadins and glutenins from control (untreated) kernels and treated with MWT0 procedure (Table 1).

weight shift to any of the detected chymotryptic peptides, indicating that primary structure of gliadin proteins, and hence CD-relevant linear epitopes, were not affected by the treatment.

3.7. Raman spectroscopy analysis

Raman spectroscopy was attempted to investigate the changes of gluten protein network induced by MWT. The analysis was performed on MWT1 and MWT2 milled kernels that, according to the R5-based immunoassay, contained 29 and 42 ppm of gluten, respectively (Table 1).

The average Raman spectra, in the range dominated by amide I band, are shown in Fig. 6. Several resolved Raman peaks were assigned to specific vibrational modes according to literature data (Ferrer et al., 2011; Sivam et al., 2013). The secondary structure distributions of gluten proteins were estimated to be 39% α-helix, 56% β-spiral, 3% β-sheet and 1% disordered. In particular, the main bands are centered at about 1620 cm^{-1} (β-sheet), 1645 cm^{-1} (α-helix) and 1672 cm^{-1} (β-turns). Other spectral features related to amide I band were also visible at 1690 cm^{-1} (antiparallel-β-sheet) and at 1590 cm^{-1} (aggregates band). Although the Raman patterns were similar between untreated and MWT flour, the intensity of the bands differed (Fig. 6). The Raman spectra of the control sample and of two modified samples (MWT1 and MWT2) are compared in Fig. 6. In the investigated spectral region, the peaks corresponding to the vibrational modes of β-turns and α-helix increased after MWT. In contrast, the intensity of the band related to features of β-sheet, antiparallel-β-sheet and aggregates in the modified flour samples decreased if compared to the untreated control. Thus, Raman spectroscopy measurements pointed out MWT-induced changes in secondary structure of wheat proteins, which most likely triggers extensive protein aggregation.

4. Discussion

Over the last decades, food technology addressed the development of innovative gluten-free foodstuff, in order to improve the quality life of CD patients. Attempts have been made to convert wheat flour into a gluten free raw material, with gluten content lower than 20 ppm to comply the indication of Codex Alimentarius (directive CE 41/2009; Codex Standard 234–1999, FAO/WHO, 2015). Recently, a MWT of wheat kernel has been proposed to either scientific or to CD patients' community, as a feasible procedure for gluten detoxification (Lamacchia et al., 2015, 2016; Bevilacqua et al., 2016). Early experiments evidenced that MWT of wheat kernels significantly reduced the reactiveness of gliadin to R5 antibody and exerted potentially beneficial effect on the gut microbiota (Bevilacqua et al., 2016). Data herein clearly demonstrated that gluten from the MWT kernels retains unmodified immune toxic potential, as assessed by *in vitro* assay with celiac gut T cells, G12 immunoassay and mass spectrometry analysis.

The R5 sandwich ELISA is a validated method for gluten detection in foodstuff, proposed as a Codex Alimentarius type 1 method (Codex Standard 234–1999, FAO/WHO, 2015) and has been adopted as official method both by the AOAC International (Immer and Haas-Lauterbach, 2012) and the AACC International (Koehler et al., 2013). This test is based on a monoclonal antibody raised against a five-residues motif of rye secalin (QQPFP) and able to recognize major toxic sequences of α-gliadin (Valdes et al., 2003; Mena et al., 2012). However, concerns related to the extraction of gluten from thermally processed wheat-based products had been raised since years (Diaz-Amigo and Popping, 2013). The subsequent introduction of extraction buffers containing reducing agents scarcely improved extraction efficiency of gluten proteins. Actually, the heat-induced aggregation of gluten proteins mainly occurs through the formation of insensitive Lys-Gln isopeptide bonds, involving ε-amino groups of Lys residues, which are relatively abundant in glutenins (Rombouts et al., 2011).

R5 ELISA alone indicated that gluten content was drastically reduced by MWT of wheat kernels. Coherently, immunoassays based on patient cells from gut mucosa of CD patients highlighted the strikingly low immunogenicity of the alcohol soluble gliadin fraction from MWT wheat.

In this work, the MWT-induced structural modifications of gliadin proteins were investigated using a peptidomic approach combined to immunoassays. Overall, the results of these assays demonstrated that the reduced immune response of CD T cells was

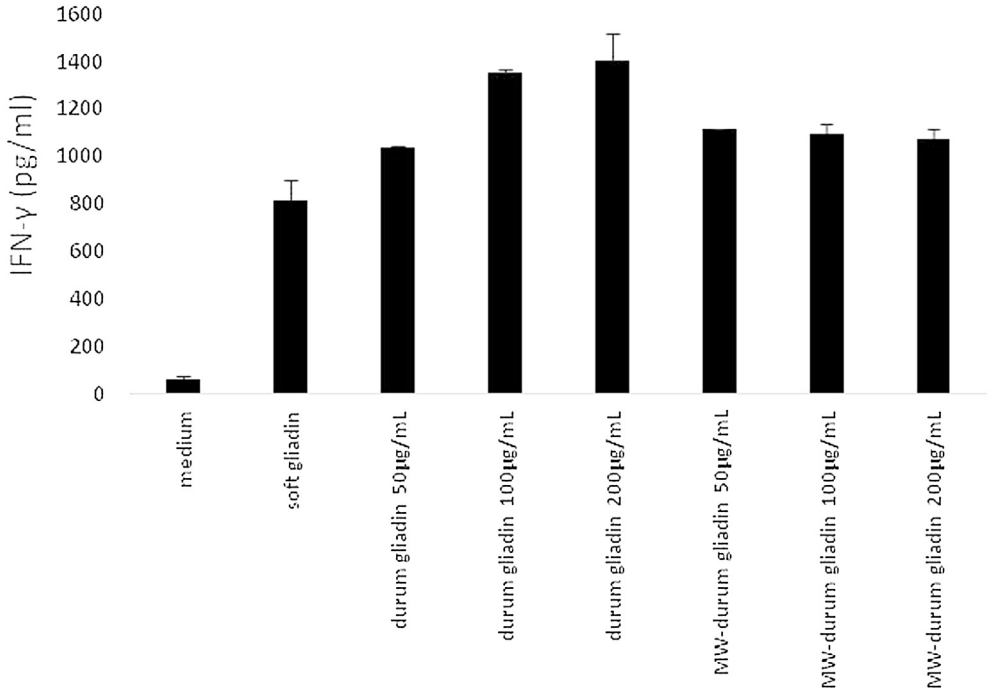

Fig. 3. Effect of MWT on immunostimulatory properties of whole gluten extracts. The gluten proteins obtained from untreated and MWT kernels (Table 1) were assayed for the capability to stimulate TCL from CD gut mucosa. TCL were obtained and *in vitro* stimulated as described in Fig. 1. Each experiment point has been acquired in duplicate. IFN-γ production in TCL from one representative patient of five are illustrated.

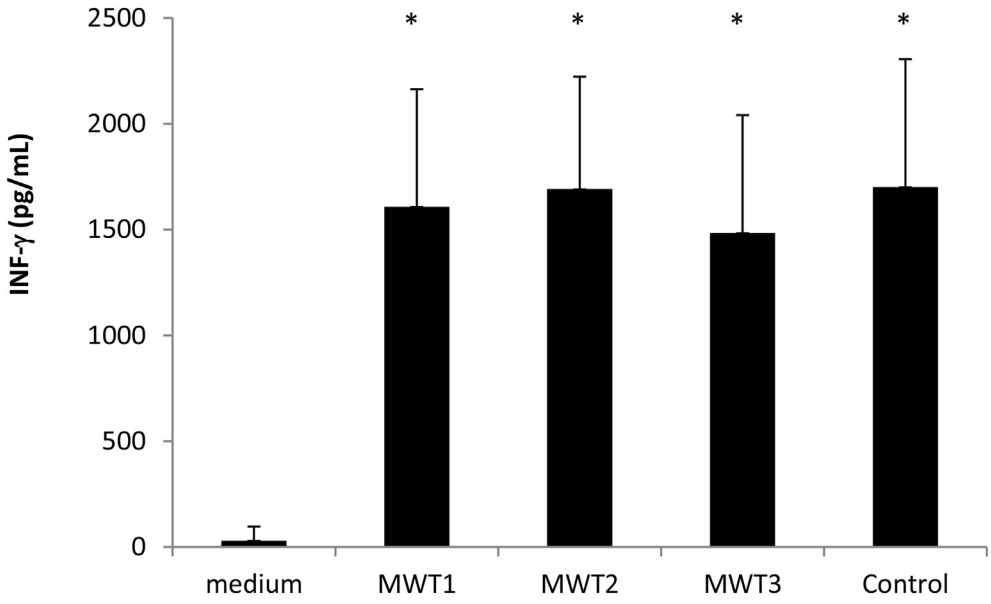

Fig. 4. T cell response to MW treated kernels with R5-ELISA undetectable gluten content. Chymotrypsin digests of gluten (50 μg/mL) from untreated and MWT kernels (MWT1, MWT2 and MWT3) were assayed for TCL stimulation properties. IFN-γ production was measured as indicated in Fig. 1. Data are average plus standard deviation of the IFN-γ response obtained from three different patients. The evaluation of statistical significant differences was performed using Student 2-tailed *t*-test, comparing IFN-γ response of TCL stimulated with gluten and medium alone. *$p < 0.005$.

a consequence of the poor alcohol extractability of gliadins from MWT flour. In any case, when we analyzed the peptides obtained from proteolysis of whole gluten proteins, we observed that MWT kernels retained the T cell immune properties similar to untreated sample, despite a further improvement of the MWT lead to flour with a gluten content lower than 5 ppm (MWT4 sample), when assayed by R5 ELISA. Consistently with our hypothesis, G12 ELISA of hydrolyzed gluten confirmed that MWT did not alter the content of immunoreactive gluten peptides. Finally, LC-MS/MS clearly demonstrated that MWT did not induce any chemical modification to gluten epitopes. In particular, the most immunogenic peptide (Shan et al., 2002), the α-gliadin 33-mer, was detected in all MWT wheat samples at levels comparable to untreated kernels.

Because of the relative insolubility of gliadins, the reduced response of R5 monoclonal antibody reflects drastic MWT-induced protein conformational changes. In support to this hypothesis, a

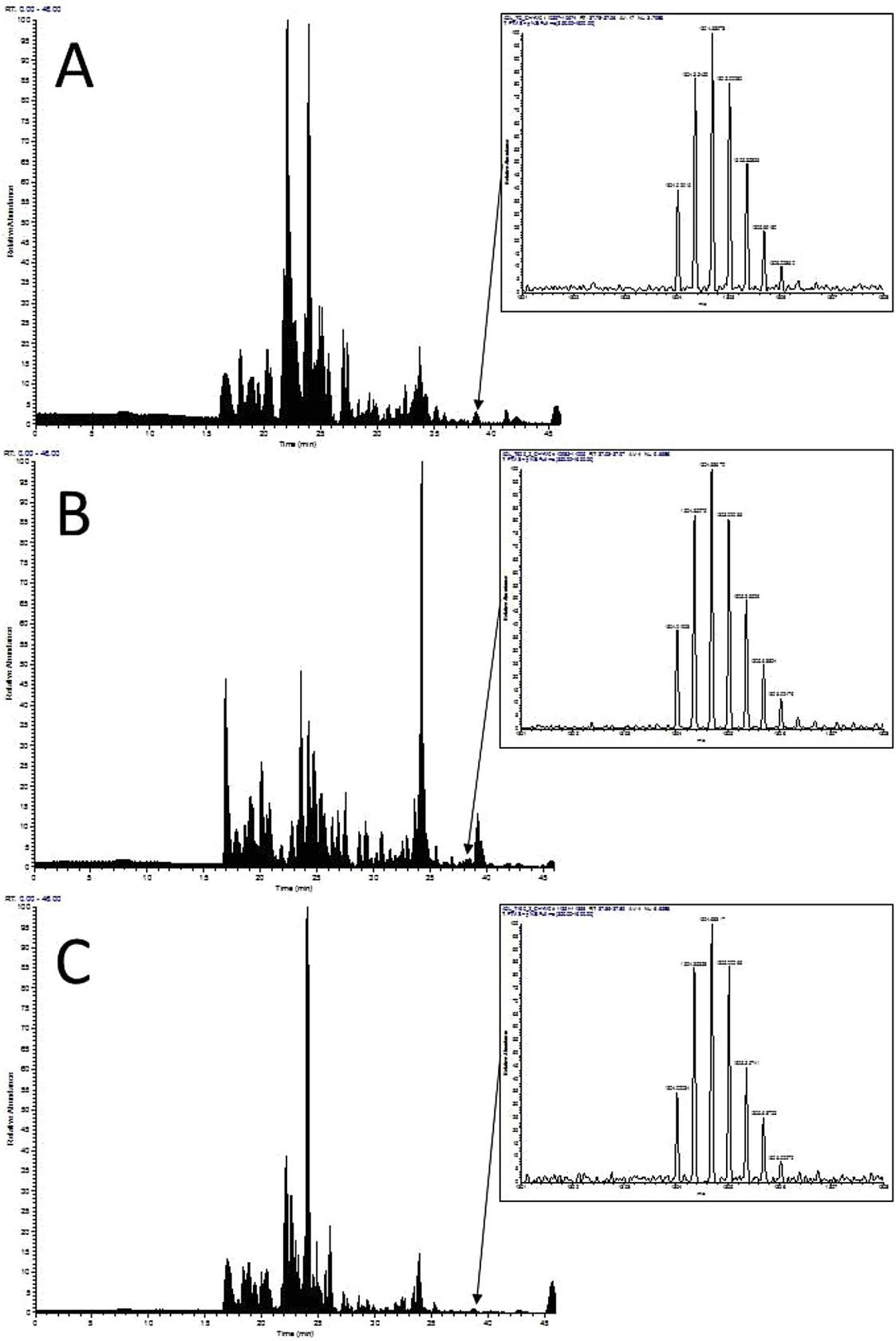

Fig. 5. LC-MS/MS analysis of chymotrypsin digest of gluten from untreated (panel A), MWT1 (panel B) and MWT2 (panel C) kernels. Insets detail the ion extraction of muticharged ion of 33mer α-gliadin peptide. No chemical modification involving 33mer or other main gluten toxic peptides was observed between treated and untreated wheat.

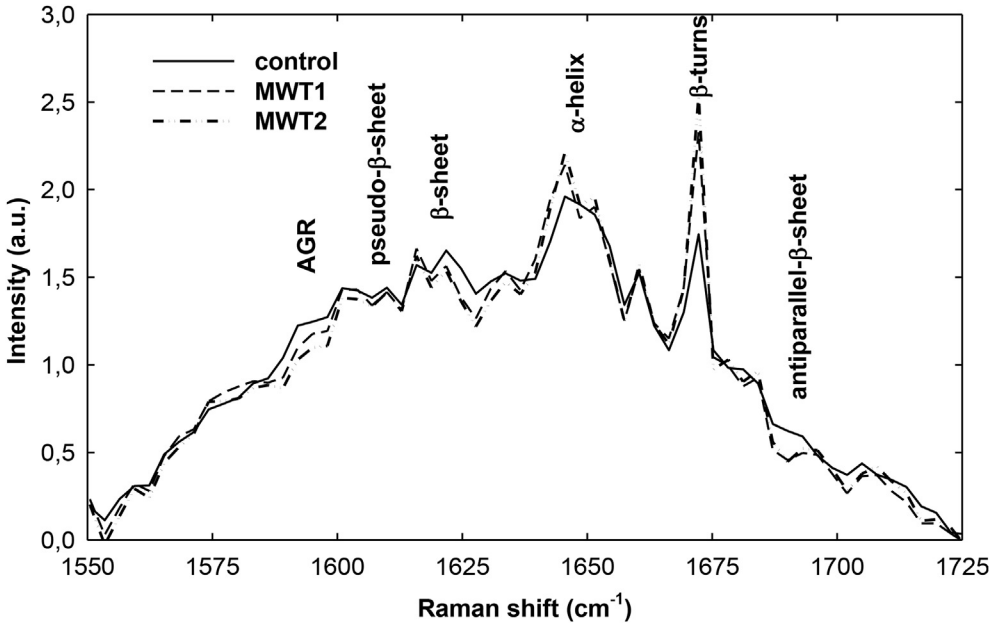

Fig. 6. Average Raman spectra of untreated kernels and MWT (MWT1 and MWT2) kernels. Flour samples in the spectral range were dominated by amide I band. The attribution of main spectral features is indicated in the text.

large body of literature demonstrated that heat treatments, also including MWT, drastically alter the extractability of proteins from foodstuff, due to events such as protein unfolding, denaturation, covalent or non-covalent aggregation, coagulation (van den Broeck et al., 2009; Yalcin et al., 2008). Indeed, Raman spectroscopy clearly evidenced that MWT induced conformational changes to the secondary structure of proteins. In particular, the Raman spectrum exhibited significant protein rearrangement involving the β-turn secondary elements, most likely also including the 33-mer peptide (57–89 residues of α-gliadin) (Tatham and Shewry, 1985). This heat-induced conformational change might hinder in part the access to gliadin epitopes, affecting the R5-immunoreactivity.

Conformational changes in food proteins induced by severe thermal processing are known to affect the immune-recognition properties (Leszczynska et al., 2003; Rumbo et al., 1996). The modification of immunoreactive sequences could have important implication in case of IgE or non-IgE mediated adverse reactions to wheat (Jiménez-Saiz et al., 2015), especially when linear epitopes are chemically modified (e.g. Maillard coupling reactions) or conformational epitopes are unfolded from their native conformation. However, according to a wide consensus, the pathogenesis of CD is triggered by inflammatory T cells reactive to linear gluten peptides, binding the CD-associated HLA DQ presenting molecules. *In vivo*, the immunoreactive peptides are released during gastrointestinal proteolytic digestion and are responsible of the intestinal mucosa inflammation. The findings of this study exclude relevant modifications interesting the primary structure of gluten proteins. Thus, gluten proteins are not destroyed by MWT but remains bound to the starchy milled kernel under ordinary conditions of protein extraction. Nevertheless, CD toxic peptides are released by proteolytic digestion (Gianfrani et al., 2015; Mamone et al., 2015) and retain full toxicity on CD TLC, at each of the MWT conditions investigated.

5. Conclusion

Despite the early encouraging results about the drastic reduction of R5-immunoreactivity of wheat, more in-depth investigations demonstrated that MWT does not affect immune toxicity of gluten. In general, these outcomes demonstrate that R5-assay is not decisive to assess removal of gluten epitopes. Proper antibody-independent molecular-based and immune procedures, including *in vitro* assays with CD patient-derived T cells, are needed to assess the effectiveness of any strategy of gluten "detoxification" and to exclude any residual occurrence of toxic peptides. Furthermore, our results indicate that immunochemical determinations of gluten in processed cereal-based products should be performed by assaying the peptides released through proteolysis from crude foodstuff, rather than with alcohol soluble protein extracts. A rigorous analytical approach, such as the one herein proposed, will have a positive and trustful impact on the celiac community, inviting patients, researchers, producers and clinicians to the right prudence and caution, before generating misleading expectations.

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Transparency document

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Chapter 3

Submitted papers

Eliciting capacity of gastrointestinal digests from raw and roasted peanuts by Rat Basophil Leukemia cell-based assay

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Abstract

The mechanism through which dietary allergens sensitize and elicit adverse reactions remains substantially poor understood. In this work, we determined the stability of peanut allergens as whole raw or roasted food, with the aim of refining the knowledge about peanut allergenic determinants investigated in a physiologically relevant context. To this purpose, peanuts were subjected to gastrointestinal digestion combining the harmonized *in vitro* static digestion models with brush border membrane (BBM) enzymes to simulate the jejunal degradation of peptides. The course of digestion was monitored by SDS-PAGE and HPLC analysis. The effect of processing and digestion on the protein allergenicity was assessed by determining the degranulation capacity of digests with a rat basophil leukemia (RBL) assay in the presence of sera from peanut-allergic patients. As general features, roasting increased digestibility of whole peanuts and reduced their allergenic potential compared to the raw counterpart. These findings provide new and more realistic insights on digestion stability of peanut allergens and can contribute

to identify targets of technological treatments for reducing the allergenic potential of peanuts.

1. Introduction

Allergic reaction to peanuts is an important public health concern that affects an estimated 5% of young children and 4 % of adults within the Western population with an increasing prevalence.[1] Seed storage proteins are the triggering factor of the allergic immune response to peanut whose symptoms may range from mild reaction to life-threatening anaphylactic shock in susceptible subjects.[2] Among fourteen 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. Peanuts are often consumed after roasting in westernized countries, while boiling and frying are the most widespread processing methods in Asia, Africa and China.[3] Protein allergens may be differently affected by heat treatments, alternatively leading to epitope destruction or to formation of IgE-binding neo-epitopes. In general, the heat-induced conformational changes might expose formerly hidden antigenic sites or change the susceptibility to gastrointestinal digestion.[4,5]

Previous studies demonstrated that the dry-roasting process, unlike boiling or frying, increases the IgE-binding to peanut.[6-9] 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.[10] Taking into account the possible correlation between digestion stability and allergenic potential, the behavior of peanut allergens during various *in vitro* digestion steps has been the subject of extensive studies, with

controversial outcomes.[11] Most of these studies have been carried out on purified or recombinant 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.[12-14] Mainly affecting the dynamics of gastrointestinal digestion, food structure and matrix can have a primary impact on the allergenic properties of peanuts.[11,15,] In a previous study, we exploited the harmonized *in vitro* oral-gastro-duodenal and intestinal digestion sequential model to track the metabolic fate of allergens in whole peanuts.[16] 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 before - survived the *in vitro* human digestion. Interestingly, resistant Ara h 3 fragments still harboured IgE-binding sequences, maintaining unaltered immunogenic properties.[16] Generally, the immunogenic potential of peanut allergens has been primarily evaluated with ELISA and immunoblotting assays, using sera from patients suffering from food allergy to peanuts as the source of specific IgE antibodies.[17] The elicitation properties of allergens have also been investigated with cellular assays based on the type I allergenic reaction, measuring the IgE crosslinking and binding to human high-affinity receptor for IgE (FcεRI) that lead to activation of mast cells and basophils.[18] 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.[19] 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.[20] The simultaneous impact of processing and digestion of a peanut-based real food matrix on the elicitation capacity of peanut allergens 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 intestinal 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

Raw and roasted peanuts (*cv Virginia*) were provided by Besana (Milano, Italy). 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). Brush Border membrane (BBM) enzymes were purified from porcine jejunum as previously described.[28] Peptidase activity of BBM enzymes was determined as detailed.[21] 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.[22] Ground raw and roasted peanuts (100 mg) were suspended in 207 µL SSF including human salivary amylase (1500 U mL^{-1}) to reproduce the oral phase. Subsequently, SGF including porcine pepsin (3300 U mg^{-1}, 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 (1.7 mg/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.

2.3. Purification of soluble and insoluble digesta samples

After simulated gastrointestinal digestion, peanut digests were immediately centrifuged at 10000 g, for 30 min. Supernatant (soluble polypeptides) and pellets (insoluble polypeptides) were separated and individually processed as follow:

i)an aliquot of peptides in the water phase was collected and stored at -20°C for reversed phase (RP)-HPLC analysis and RBL assay, whilst the remain sample was precipitated for 30 min in an ice cold bath with 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 analysed by SDS-PAGE.

ii) insoluble polypeptides were extracted with 1 mL Urea buffer (7M 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 10000 *g* for 40 min. The supernatant was 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 [12], 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).

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). Digests (50 µl) were suspended in 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 and 280 nm with an UV-Vis detector.

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

Twenty sera from children with clinically documented food allergy to peanut were used to constitute a pool. They were selected for their concentrations in IgE specific for the peanut allergens Ara h 1, 2, 3 and 6. The serum pool displayed an equivalent reactivity towards raw and roasted peanut; concentrations in specific IgE were calculated at 580 and 528 ng/ml for raw and roasted peanut extracts respectively.

Sera were obtained from the Biological Resource Center (BB-0033-00038) of Clinical Immunology and Allergy Service of Angers University Hospital (France) with the informed consent of the patients and their parents.
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 Rat Basophil Leukemia cell line expressing human FcεRI (clone RBL-SX38), kindly provided by Pr Kinet (Harvard Medical School, New York, USA).[23] Cells were sensitized with the pool of human sera for 48h 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.[24] Samples and controls were analysed in triplicate 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.

3. Results

3.1. Gastrointestinal digestion of whole raw and roasted peanuts

Simulated digestion was carried out on peanuts as whole either raw or roasted food, without any pre-fractionation of the kernel, in order to evaluate the stability of allergens embedded within their natural food matrix. Oral, gastric and duodenal phases were physiologically reproduced according to Infogest protocol.[22] The oral gastric-duodenal digestion (OGD) workflow was also integrated with porcine jejunal BBM hydrolases as already described in previous studies from our group.[16,25-28] 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 a consensus about their use, we adopted conditions (peptidase activity, pH and incubation time) similar to those that have demonstrated physiological correspondence.[29] 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. 1). Protein extract from both raw and roasted peanuts were run as reference control (lanes 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) and roasted (lane 9) peanuts exhibited significant differences, especially in term of intensity of bands. These differences became more evident following digestion with BBM enzymes (lanes 3 and 9), 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,[16] liquid chromatography coupled to tandem mass spectrometry-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 of roasted peanuts were fragments of Ara 3 and Ara h 2/Ara h 6, respectively (data not shown).

In order to assay the presence of possible insoluble protein aggregates trapped in the starchy matrix,[30] the pellet of both OGD and OGD-BBM digests of raw and roasted peanuts were sequentially extracted with Urea-containing (lanes 4-5 and 11-12) and SDS/reducing buffer (lanes 67 and 13-14). Nevertheless, no protein band was detected in these latter extracts by SDS-PAGE analysis, indicating a substantially complete solubilization of hydrolysed polypeptides, either in raw or roasted peanut digests.

In line with SDS-PAGE, the RP-HPLC analyses of soluble peptides 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. 2).

3.3 Rat basophil assay

The RBL assay closely reflects the *in vivo* elicitation of an allergic reaction.[31] Cells were sensitized with a pool of IgE from peanut-allergic patients and 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. 3. The cell response was determined monitoring the release of β-hexosaminidase as a marker of degranulation.[24,32] Table 1 displays the protein concentrations inducing 50% of the maximum release of mediator (EC50). No effect of proteolytic enzymes or digestion buffers was observed for the digest concentrations used in the assay (not shown).

A preliminary assay confirmed that undigested raw peanuts induced a release of β-hexosaminidase, which increased in parallel with the allergen concentrations. An EC50 value of 2.5 ng/mL was determined for this reference sample; roasting only slightly affected degranulation by undigested peanut proteins, with EC50 of 3 ng/mL, indicating that the thermal treatment had minor impact on the potential allergenicity of unhydrolyzed allergens (Fig. 3 panel A; Table 1).

Whereas OGD digestion slightly decreased the degranulation capability of raw peanuts, with a twofold increase of EC50 (EC50 5 ng/mL) (Table 1), the downstream BBM degradation had no detectable impact on the allergenicity (Fig. 3, panel B and C). The degranulation induced by digests of roasted peanuts showed an interesting trend, since EC50 value remarkably increased from 3 to 18 ng/mL after OGD digestion (Fig. 3, panel B; Table 1) and to 25 ng/mL after OGD-BBM digestion (Fig. 3, panel C; Table 1). This result indicated a reduction of allergenicity caused by digestion, which was even more evident for roasted peanuts. In other terms, much higher concentrations

of OGD-BBM roasted peanuts were required to induce a comparable RBL degranulation, if compared to raw or unhydrolyzed peanut allergens.

4. Discussion

Many proteins among the allergens share the common feature of stability to gastric and duodenal enzymes.[33] 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.[34] 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 attempted to simulate human digestion for assessing the metabolic fate of allergens.[35] In this context, use of individual proteins (purified o recombinant forms) make it easier to track the pathway of degradation of allergens, but preclude the investigation of 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 of foods undergo.[35,36] Heat treatments induces chemical/physical modifications, which may affect the stability to enzymatic digestion and consequently the allergenicity of food proteins at 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 capability of sensitization and elicitation. Even in this case, almost all investigations exploitation single purified proteins. 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. 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 developed by Minekus et al, (known as Infogest method).[22] 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.[37] 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.[38] Enzymes involved in this last stage are those of the BBM which include a suite of oligopeptidases (endo- and exo-peptidse), lipases and oligosaccharidases. Together these enzymes tend effectively to hydrolyse those nutrient oligomers that survive the upstream digestion steps. BBM are mainly located on the surface of epithelium gut, but evidence also points at their release in the periapical space of enterocytes mediated by the biliopancreatic secretions.[39-41] 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 [16,26,42] with demonstrated physiological consistence.[29] 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,[16] 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, survived to OGD-BBM digestion of roasted peanuts. Since heat treatment might cause a loss of protein solubility, a 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.[43] 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 more potent allergen than Ara 1.[20] 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.[10] 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 assays highlighted unequivocally that, in turn the increased degradation results in a general reduction of eliciting capacity. The final step of degradation with BBM enzymes contributed to reduce the allergenicity of roasted peanuts, suggesting that BBM 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 survive OGD digestion. For this reason, BBM enzymes had a less evident effect because they mainly consist of peptidases hydrolyzing preferentially small-/medium-sized peptides.[44] The weak bands detected after OGD-BBM of the roasted peanuts are due to persistent fragments of Ara h 3 and Ara h 2, which, although lower, continue to exert an immunogenic effect. Our results are in partial agreement with those by Kroghsbo *et al.* (2014)[45] who demonstrated that peanut roasting does not enhance allergenicity, through investigations carried out with rats.[45] However, our data are not strictly comparable with those obtained in animals due to the different experimental approach.

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.

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, digestion including the BBM stage destroyed most of the epitopes of roasted

peanuts, resulting in significantly lower RBL degranulation. Conversely, allergens of raw peanuts retained part of 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.

In perspective, it would be interesting to compare the specific effects induced by different heat treatments, for instance those induced by roasting frying, boiling or autoclaving at high-temperature.

Abbreviations

BBM, Brush Border Membrane; OGD, oral gastro-duodenal digestion; FcεRI, human high-affinity receptor for IgE; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; RBL, Rat Basophil Leukemia cells;TFA, trifluoroacetic acid

Conflicts of interest

There are no conflicts to declare

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Table 1:
Comparison of RBL activation capacities of raw and roasted peanut digests (OGD and OGD-BBM). The EC50 (ng/ml) values were determined in a semilogarithmic graph depicting the relationship between 5 different concentrations of proteins and the each percentage of degranulation. Data represent the mean ± S.D. of four independent experiments.

	EC50 of degranulation (ng/ml)	
	Raw	**Roasted**
Protein extract	2.5	3
OGD	5	18
OGD-BBM	5	25

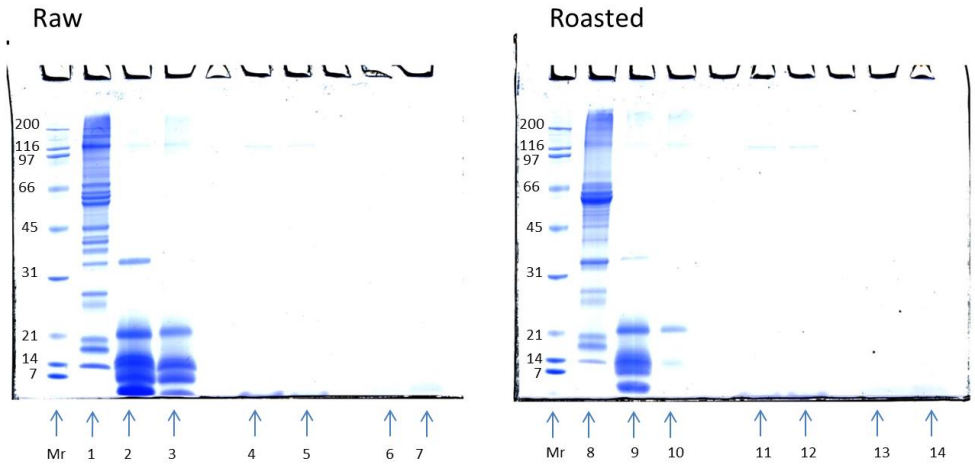

Figure 1. Unreduced SDS-PAGE analysis of raw and roasted digesta peanuts. After centrifugation OGD and OGD-BBM supernatants were individually analysed. The residue pellets were subsequently extracted by 7M Urea buffer followed by 2% SDS, 20mM DTT. Lanes 2 and 9: supernatants of raw and roasted OGD peanuts; 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 7: SDS extract of pellet from raw and roasted OGD digestion; lanes 7 and 8: SDS extract of pellet from raw and roasted OGD-BBM digestion. Peanuts protein extract from raw (lane 1) and roasted peanuts (lane 8), were run as reference control.

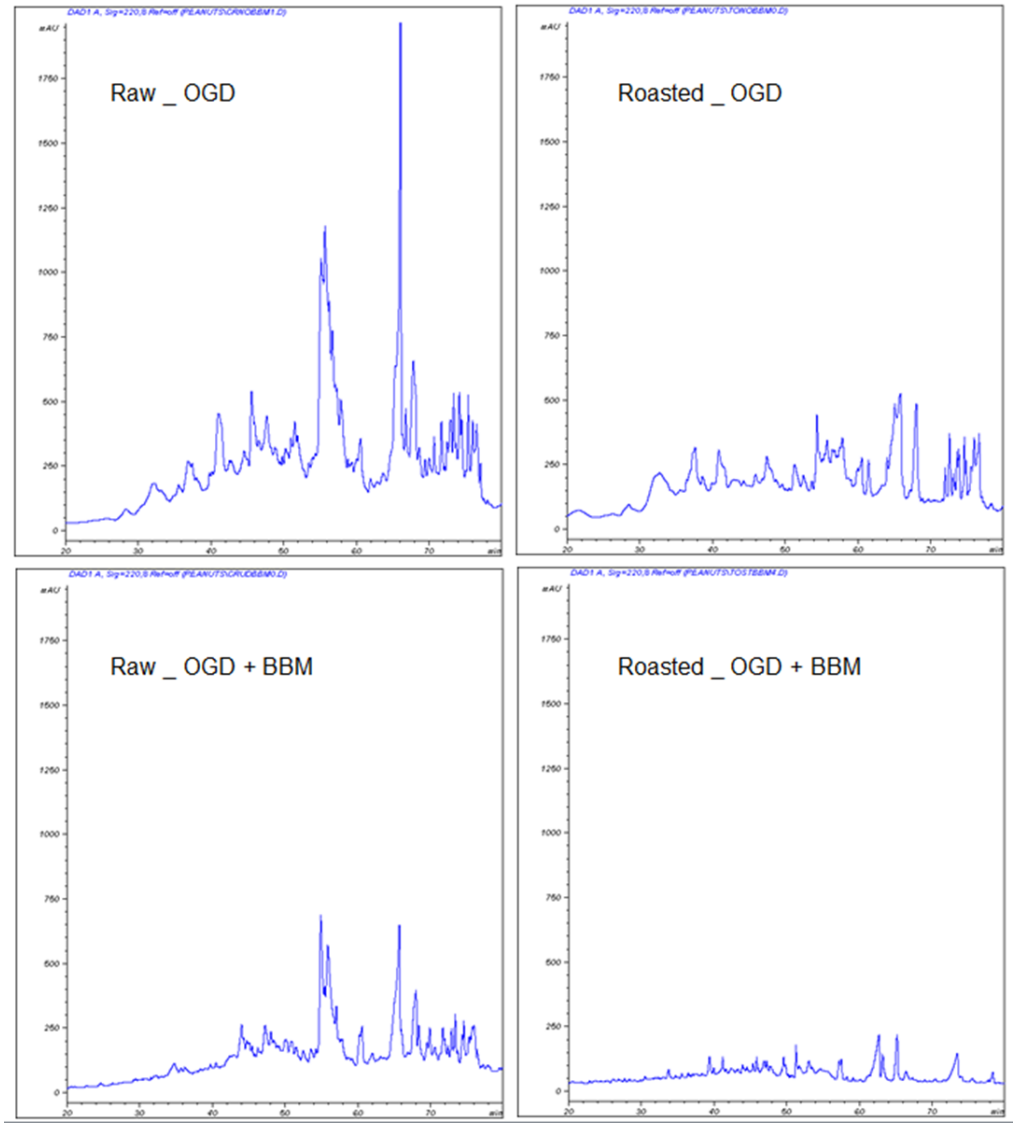

Figure 2: HPLC analysis raw and roasted digesta peanuts before (OGD) and after BBM (OGD-BBM).

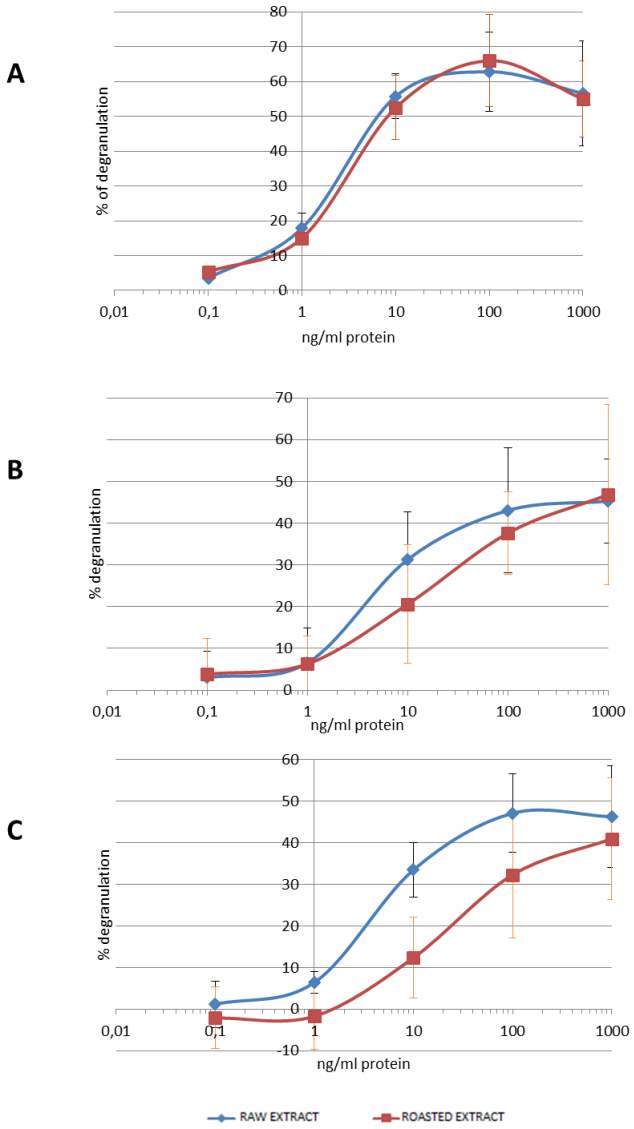

Figure 3. Relative percentages of degranulation in RBL assay induced by undigested protein extracts, raw and roasted peanut. Data points are average of four replicate determinations. The EC50 values (as shown in **Table 1**) were determined in a semilogarithmic graph depicting the relationship between 5 different concentrations of proteins and the each percentage of degranulation. Data represent the mean±S.D. of four independent experiments. Panel A, undigested protein extract; Panel B, OGD digesta digested peanuts; Panel C OGD-BBM digested peanut

Assessment of the metabolic fate of tree nut allergens: the role of intestinal Brush Border Membrane digestive enzymes and food processing

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Abstract

In the present study, the role the intestinal bush border membrane enzymes on the stability of tree nut allergens was elucidated. *In vitro* static oral-gastro-duodenal digestion model, including brush border membrane enzymes phase, was applied to both raw and roasted walnuts, hazelnuts and almonds. Mass spectrometry based proteomic analysis was used to characterized polypeptides sequences that survived to digestion. As general features, roasting increased digestibility of walnut and hazelnut, destroying most IgE binding peptides. The degradation of harmful peptides was significantly enhanced after hydrolysis with brush border membrane enzymes in roasted walnut and hazelnut compared to the raw counterpart. Conversely, almond allergens showed a different behavior, since a large number of peptides harboring harmful sequences survived gastrointestinal digestion of roasted almond; the presence of resistant peptides was more evident after the brush border membrane enzyme phase of roasted almond probably because of the hydrolysis of high molecular weight aggregated forms during roasting.

These outcomes offer the bases for new perspectives on the application of novel food processing in order to improve digestibility of food allergens and to reduce their allergenic potential.

Keywords: almond, brush border membrane enzymes, food allergy, food matrix, hazelnut; *in vitro* digestion; raw and roasted tree nuts; walnut.

1. Introduction

Digestion of protein along the gastrointestinal tract critically affect the risk of food allergy development. Higher resistance of protein to digestion seems to increase the sensitization capacity of a food component making it more immunogenic and/or allergenic (Pekar *et al.*, 2018; Untersmayr & Jensen-Jarolim, 2008). Over the last years, large efforts have been done to assess the stability of food allergen through the digestive tract. For this purpose, several *in vitro* methods have been addressed to reproduce the main physiologically steps occurring during oral, gastric and duodenal phase (Astwood *et al.*, 1996; Fu *et al.*, 2002; Huby *et al.*, 2000; Minekus *et al.*, 2014; Wickham *et al.*, 2009). However, *in vivo* human digestion also includes a relevant stage of degradation by the enzymes of the Brush Border Membrane (BBM) located on the surface of intestinal lumen prior to absorption. BBMs consist of a large set of oligopeptidases (endo- and exo-peptidases), lipases and oligosaccharidases, which, together tend effectively to hydrolyze those nutrient oligomers that survive the upstream gastric and duodenal digestion steps. Although mainly located on the surface of epithelium gut, evidence also prove that BBMs are released in the periapical space of enterocytes mediated by the biliopancreatic secretions (Picariello *et al.*, 2016).

Despite playing a crucial role in determining the metabolic fate of protein and peptides, BBMs have been poorly employed within *in vitro* digestion protocols. Early digestion model including BBM phase were applied for determining the stability of immunogenic or toxic peptides arising from digestion of purified gliadins (Gianfrani *et al.*, 2015; Mamone *et al.*, 2007; Picariello *et al.*, 2013; Shan *et al.*, 2002) and purified casein proteins (Picariello *et al.*, 2010). More recently, BBM enzymes were exploited for the assessment of allergen protein stability within their natural food matrix such as pasta (Mamone *et al.*, 2015), bovine milk (Picariello *et al.*, 2015) and peanuts (Di Stasio *et al.*, 2017)(Di Stasio *et al*., *submitted* 2018). The model of BBM digestion was recently applied to peanut, demonstrating that roasting treatment affect the breakdown of peanut allergens, resulting in a significantly lower of eliciting allergenic properties respect to raw digested peanuts (Di Stasio et al., submitted 2018). Indeed, the compounds naturally occurring in food matrix (i.e. lipids and polysaccharides, other proteins) may affect the proteolysis, delaying or impairing the allergen degradation and altering the pattern of the peptide fragments released during hydrolysis process (Di Stasio *et al.*, 2017; Korte *et al.*, 2017; Pekar *et al.*, 2018; Vissers *et al.*, 2012).

Similarly, food processing may alter the allergenicity, leading to a decrease of allergenic potential due to allergen denaturation and disruption of IgE epitopes by enzymatic digestion (Davis & Williams, 1998) or conversely, to an increase of allergenic potential due to major stability of allergen to digestion as consequence of aggregation with other food components (Teuber *et al.*, 2002).

In the present study, the stability of almond, hazelnut and walnut allergens to oral, gastric and duodenal (OGD) digestion before and after BBM phase was assessed. Tree nuts were processed as both raw and roasted foodstuff,

without any pre-fractionation of the kernel, in order to evaluate the stability of allergens embedded within their natural food matrix and monitoring degradation of allergens by integrated proteomic/peptidomic techniques.

2. Materials and Methods

2.1. Chemicals

Raw and roasted walnuts (cv Cilena), hazelnuts (cv Campana) and almonds (cv Italia) were provided by Besana (Milano, Italy). Pepsin, trypsin, chymotrypsin, dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, urea, ammonium bicarbonate (Ambic), HPLC- grade solvents, phospholipid, trifluoroacetic (TFA), were obtained from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was from Lipid Products (Redhill UK). Brush Border membrane (BBM) enzymes were purified according to Picariello *et al.* (2015). Reagents for electrophoresis analysis were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA).

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

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 walnuts, hazelnuts and almonds (100 mg) were suspended in 207 µl SSF 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 (1.7 mg/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), 6 h at 37 °C, after adjusting pH to 7.2. The reaction was stopped by 5 min immersion in a boiling water bath.

2.3 Purification of soluble and insoluble digesta samples

After simulated gastrointestinal digestion, tree nuts digests were immediately centrifuged at 10000 g, for 30 min. Supernatant (soluble polypeptides) and pellets (insoluble polypeptides) were separated and individually processed as follow:

i) an aliquot of peptides in the water phase was collected and stored at -20°C for Reversed Phase (RP)-HPLC analysis , whilst the remaining sample was precipitated for 30 min in an ice cold bath with TCA up to 30% (w/v) final concentration; the resulting pellet was washed with 1 mL of -20°C cold acetone (3 times) for removing TCA and finally analysed by SDS-PAGE.

ii) insoluble polypeptides were extracted with 1 mL Urea buffer (7M Urea, 1M Tris-HCl, pH 8.5) for 2 h at room temperature; after centrifugation the supernatant was collected. The supernatant was analysed by SDS-PAGE.

2.4. SDS-PAGE

SDS-PAGE was performed on the Mini-PROTEAN cell systems (Bio-Rad). Raw and roasted digesta samples 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 was loaded on precast 12% gel (Bio-Rad). Tree nut proteins, extracted from flour according to Di Stasio *et al.* (2017), were run, as reference control. For insoluble polypeptides, 10 µL of sample was dissolved in 10 µL of Laemeli buffer 2x. After migration gel was stained with Silver blue (Coomassie Brilliant Blue G-250) and digitalized by LABScan scanner (Amersham Bioscience/GE Healthcare, Uppsala, Sweden).

2.5. RP-HPLC

Raw and roasted digesta tree nuts were fractionated by Reversed Phase (RP)-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA). Samples were suspended in 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 10-60% gradient of B over 100 min. The flow rate was 200 µl/min. Chromatographic separation was performed at 37 °C, using a thermostatic column holder. The column effluent was monitored at 220 nm.

2.6. LC-high resolution (HR)-MS/MS analysis

Mass spectrometry analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were resuspended 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 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 1/106 ions and a maximum ion injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were elaborated using the Proteome Discoverer 2.1 software (Thermo Scientific), restricting the search to *Jungladacee* database for walnut, *Corylus avellana* database for hazelnut and *Prunus dulcis* database for almond extracted from both NCBI (https://www.ncbi.nlm.nih.gov/pubmed) and Uniprot (ttps://www.uniprot.org/) downloaded on June 2018. Database searching parameters for identification of SDS-PAGE protein bands were the

following: Met oxidation and pyroglutamic for N-terminus Gln as variable protein modifications; carboxymethylcysteine as a constant modification; a 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. Database searching parameters for identification peptides arising from *in vitro* OGD and OGD-BBM were the same described 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.

3. Results

3.1. OGD and OGD-BBM digestion of whole raw and roasted walnuts, hazelnuts and almonds.

The stability of allergen of raw and roasted tree nut was evaluated by using harmonized physiologically methods developed by Minekus *et al.* (2014) (Infogest method). The oral, gastric and duodenal (OGD) digestion was further complemented with porcine jejunal BBM hydrolases in order to simulate the final step of chyme breakdown occurring at the level of the intestinal epithelial barrier (Mamone *et al.*, 2015; Picariello *et al.*, 2016). The BBM digestion conditions (peptidase activity, pH and incubation time) were similar to those that have been demonstrated physiological correspondence (Shan *et al.*, 2002). The OGD and OGD-BBM digesta samples were purified from lipids and salts prior to proteomic analysis. The course of digestion before and after BBM was first monitored by SDS-PAGE (**Figure 1, 3 and 5**). In order to assay the presence of possible insoluble protein aggregates trapped in the starchy matrix (Gianfrani *et al.*, 2015), the pellet of both OGD

and OGD-BBM digests of tree nuts were extracted with Urea buffer and run by SDS-PAGE as well. The identity of protein bands before and after digestion was achieved by MS-based proteomic analysis. The behaviour of protein allergens during digestion was also monitored by RP-HPLC (**Figure 2, 4 and 6**), while LC-MS/MS was employed in order to obtain the amino acid sequence of peptides surviving digestion (**Table 1 and 2**). The characterisation of digesta sample is described in detail for each tree nut type as follow.

3.1.1 OGD and OGD-BBM digestion of walnut

Preliminary proteomic analysis of undigested proteins (Urea buffer extract) from both raw and roasted walnut (**WP , Figure 1A and 1B**) revealed the migration of of Jug r 4 isoforms at 60 kDa, 42.9 and 35.2 kDa; 11 S globulin (Legumin A-like and legumin B-like) at 56.2 kDa, 42.9 kDa and 35.2 kDa and 2S albumin in the region around 18-19 kDa. After digestion, the electrophoretic profile drastically changed, since large sized proteins were no longer detectable, either in raw or roasted walnut. The digestion of raw walnut (**Figure 1A**) yielded to two bands at 13.8 kDa and 5 kDa, whose intensity was comparable before and after BBM phase. The band at 13.8 kDa corresponded to a co-migrated fragments of 2S sulphur-rich albumin (A0A2I4EX90) and sucrose-binding protein Jug r 6 (A0A2I4E5L6), while the band of 5 kDa was assigned as sucrose-binding protein, identified in *Allergome* as Jug r 6 (A0A2I4E5L6) (**Table 1**).

The SDS-PAGE analysis of the insoluble fraction of digested walnut (**Figure 1A and 1B**) showed the presence of polypeptides entrapped into the walnut matrix. OGD insoluble fraction contained a faint band at 13.8 kDa identified as “2S sulphur-rich albumin” (A0A2I4EX90) and “vicilin like antimicrobial

peptides" (A0A2I4DYE9) fragments. The identification of protein fragment migrated at 5 kDa in roasted walnut before (band "F") and after (band "G") BBM stage failed because database interrogation of MS/MS spectra resulted in an unappreciable protein identification.

Roasting induces a change of digestibility, as low MW bands at around 6.5 kDa were still present before and after BBM, either in soluble or insoluble fractions. In these bands, polypeptides arising from digestion of several walnut protein co-migrated (see **Table 1**).

In line with SDS-PAGE and RP-HPLC analyses of soluble peptides confirmed the increased digestibility of walnut 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 walnut or between OGD to OGD-BBM digests (**Figure 2A and 2B**). The current result demonstrated that thermal processing and the brush border membrane enzyme phase drastically affects digestibility of walnut proteins.

3.1.2 OGD and OGD-BBM digestion of raw and roasted hazelnut

As shown in Figure 3, the major hazelnut allergens, Cor a 11 (7S globulin) and Cor a 9 (11S globulin), were identified at approximately 56.8 kDa and 52.6 kDa respectively. Other isoforms of 11S globulin were also found at 45.4 kDa to 34.8 kDa. The 2S albumin was identified at 10 kDa (**WP, Figure 3A and 3B**).

The electrophoretic profile of OGD-BBM sample demonstrated that no soluble polypeptides (> 6.5 kDa) survived to digestion. A faint band at 14 kDa was detected before the hydrolysis by BBM; in this band fragment of 2S

albumin, 48 kDa protein and oleosin co-migrated (**Table 2**). A similar trend was observed after roasting of hazelnut, where a faint band detected in OGD soluble digesta, disappeared after BBM hydrolysis. Differences were found in the analysis of insoluble fraction as fragments of 2S albumin were detected in OGD but non OGD-BBM insoluble fractions. Weak signal arising from hydrolysis of ATP synthase (subunit beta) were found resistant in insoluble fraction of raw hazelnut both before and after BBM; however, complete digestion of these proteins were observed after digestion of roasting hazelnut.

The RP-HPLC analysis of soluble peptides confirmed the major digestibility of roasted hazelnut after BBM hydrolysis. In fact, the intensity of HPLC peaks clearly decreased either between raw and roasted hazelnuts or between OGD to OGD-BBM digests (**Figure 4A and 4B**).

3.1.3 OGD and OGD-BBM digestion of raw and roasted almond

Proteomic analysis of almond before and after roasting (**Figure 5A and 5B**) revealed that the isoforms of major allergen "amandin", identified in Allergome as Pru du 6 [Q43607, Q43608, E3SH8 (3071591112 NCBI Accession)], were spread out along the SDS-PAGE gels (band at 60 kDa, 50.6 kDa and 23.7 kDa) both in raw and roasted almond (**WP, Figure 5A and 5B**).

Digestion of raw and roasted almond induced a disappearance of all band either before or after BBM phase. Notwithstanding, the electrophoretic profiles of insoluble OGD digesta sample showed two high MW bands at 250 kDa and 116 kDa. MS analysis revealed that major allergen (307159112, Q43608, E3SH9) migrated in these two bands as shown in Table 2. After BBM these proteins were completely digested.

Interestingly, the HPLC analysis of digesta samples showed significant different in term of stability since after BBM the intensity of peptides arising from raw almond was drastically reduced, compared to OGD ones (**Figure 6A and 6B**). Surprisingly, the chromatogram of roasted OGD almond showed a low number of peptides whose intensity increased after treatment with BBM. A plausible explanation of this discrepancy behaviour was that aggregates detected before BBM in the insoluble fraction of roasted sample (**Figure 5B**) were sensitive to intestinal digestion yielding soluble peptides and therefore visible by RP-HPLC.

3.2. LC-MS/MS analysis of digesta tree nuts

The peptide fraction of soluble OGD and OGD-BBM digested samples were analysed by LC-HR-MS/MS. This analysis revealed a heterogeneous mixture of peptides in all tree nuts samples, which escaped to SDS-PAGE analysis. Accession number of identified proteins are listed in Table 3 and 4. The identification of peptides survived digestion was challenged by a series of concomitant factor, including the semi-tryptic nature of the peptides and the unpredictable cleavage specificity of protein hydrolysis resulting from the action of a pool of gastric duodenal and BBM proteases.

3.3. Characterization of IgE binding peptides

The intensity of IgE binding peptides (<6.5 kDa). released from OGD and OGD-BBM digestion were compared with the reference linear IgE-binding epitopes from previous studies (**Table 3 and Table 4**) (Barre *et al.*, 2007; Barre *et al.*, 2008; Poltronieri *et al.*, 2002; Robotham *et al.*, 2009; Robotham

et al., 2002; Willison *et al.*, 2013). The list of sequenced peptide are shown in Table 3 and Table 4.

Epitope sequences REGDIIAFPAGVAHW and IESWDPNNQNFQCAG, (partially found encrypted into the peptide *IEAEAGV*IESWDPNNQQFQ) (Robotham *et al.*, 2009) of Jug r 4 (11 S globulin) detected in OGD raw walnut drastically decrease after BBM digestion. The IgE –binding epitopes QQQQQGLRGEEMEEMVQS (Robotham *et al.*, 2002) and LAGQNNIINQLER (Barre et al 2008) of Jug r 1 (2S albumin) were completely hydrolysed after BBM digestion (**Table 3**).

Similarly, hazelnut allergens showed similar behaviour since IgE-binding epitopes YLAGNPDDEHQRQGQQQFG of Cor a 9, identified by, was degraded by BBM (**Table 3**). The PYSNAPELIYIERGGITGVLF epitope, partially found encrypted into the peptide *TIEPNGLLL*PQYSNAPELI, was significantly hydrolysed by intestinal enzymes (**Table 3**) (Robotham *et al.*, 2009).

IgE binding epitopes of almond allergens have been poorly investigated so far and few information on the nature of IgE binding epitopes are currently available. Among the known harmful almond peptides, the sequence (QQEQQGNGNNVFSGF) of Pru du 6 identified by Poltronieri *et al.* (2002), was detected in OGD sample but completely degraded after proteolytic action of BBM (**Table 3**).

The analysis of roasted tree nut gave interestingly results (**Table 4**). As expected, the amount of immunogenic peptides arising from gastrointestinal digestion of roasted walnut and hazelnut decreased with respect to raw ones, as result of better digestibility as showed by SDS-PAGE analysis. In detail, in roasted walnut digesta, the intensity of immunogenic peptides IESWDPNNQNFQCAG was reduced after the action of intestinal enzymes

(**Table 4**), whilst, YLAGNPDDEHQRQGQQQFG peptide of Cor a 9, was completely degraded after BBM phase. In line with SDS-PAGE analysis, digested roasted almond contained a high number of immunogenic peptides released after BBM hydrolysis. Most of peptides detected in OGD-BBM digested almond arose from digestion of high MW aggregated of almond allergens which were detected in insoluble fraction of OGD digesta almond (**Figure 5**), probably as result of heat treatment (**Table 4**).

In general, as expected, peptides harbouring IgE binding epitopes of major allergens from hazelnut and walnut were shortened or completely hydrolysed because of proteolytic action of enzymes naturally occurring into the gastrointestinal tract. On the contrary, roasting seemed to improve the stability of almond peptides to digestion, indicating that food matrices are differently affected by technological treatment.

4. Discussion and conclusions

It is estimated that food allergies affect up to 8% of children less than 3 years of age and 2% of adults in the worldwide (Sampson, 1999). Among these, tree nuts are common causes of allergenic reactions. Tree nuts are defined as any nut grown on trees, as cashew, walnut, pistachio, almond, pecan, Brazil nut, pine nut, hazelnut and macadamia nut (McWilliam *et al.*, 2015) and they are typically eaten as snacks or included into foodstuff (Willison *et al.*, 2014).

Allergenicity is directly linked to allergen stability (Untersmayr & Jensen-Jarolim, 2008). In most of the studies reported in literature , the relationship between digestion stability and allergenicity has been limited to the purified allergens (Bogh & Madsen, 2016). However such an approach neglected the

important role of food processing and food matrix (Di Stasio *et al.*, 2017; Korte *et al.*, 2017; Schulten *et al.*, 2011). In fact, compounds naturally present in food matrix could affect the digestion of allergen and subsequently the activation of intestinal immune system. Food processing play another important role in determining the stability of food allergens. Chemical/physical treatments, induced by baking, roasting, pasteurization, pressure treatment and other methods, may cause structural protein changes (e.g. chemical modification, protein unfolding, aggregation) to the allergen, affecting their susceptibility to the protease (Besler *et al.*, 2001). Tree nuts are commonly consumed after thermal processing (e.g. roasting or baking) to improve their organoleptic properties (Downs *et al.*, 2016).

Su *et al.* (2004) affirm that heating treatments of walnut (blanching, roasting, microwaving and frying) had no effect on the IgG-binding capacity of Jug r 2 and Jug r 4. On the contrary, autoclave treatment decreased the IgG-binding of Jug r 2 and Jug r 4. Immunologically, Jug r 4 is one of the major allergens in walnut with significant sequence homology with other proteins belong to 11S globulins family, such as Cor a 9 (hazelnut) and Ara h 3 (peanut), contributing to their IgE cross-reactivity (Costa *et al.*, 2014). Cabanillas *et al.* (2014) recently showed that the IgE binding capacity of allergens decreased after pressure treatment at 256 kPa, 138 °C (Cabanillas *et al.*, 2014). Wigotzki *et al.* (2001) described that the IgE-binding of hazelnut allergen from hazelnut based commercial products (e.g. hazelnut chocolates, hazelnut cake, hazelnut cookies) significantly decreased compared to unprocessed hazelnut (Wigotzki *et al.*, 2001). The studies of Cucu el al (2012, 2011) demonstrated that glycation of Cor a 11 allergen, induced by heat treatment in the presence of glucose, caused a reduction of immunoreactivity. (Cucu *et al.*, 2012; Cucu *et al.*, 2011)

BARGMAN *et al.* (1992) showed that thermal treatments drastically reduced the immunoreactivity of the major almond allergens. These phenomena could

be explain by the loss in protein structure that leads to reduction or loss in epitope recognition by IgE antibody (Tukur *et al.*, 1996).

Herein, we assessed the digestion stability of almond, hazelnut and walnut directly in the their natural matrix, also taking into account the impact of thermal processing and looking to the IgE binding peptide fragments produced as a consequence of the physiological digestion. For this purpose, we used an *in vitro* multi-compartmental model, including the additional step with porcine jejunal BBM enzymes, (Picariello *et al.*, 2010; Picariello *et al.*, 2015). Proteomic tools were used to evaluate the digestion stability (Mamone *et al.*, 2011). Nowadays, in fact, proteomic/peptidomic sciences enabled monitoring of the food allergen digestome as well to map the resistant peptides harbouring IgE epitopes sequentially released upon digestion of complex matrices (Di Stasio *et al.*, 2017; Picariello *et al.*, 2011; Picariello *et al.*, 2013).

Our results provide new insights about the relationship between thermal processing and the metabolic fate of tree nut allergens, highlighting the importance of investigating the digestion stability of whole allergenic food, instead of purified proteins. We conclude that roasted walnuts and hazelnuts are more digestible than raw ones leading to the destruction of harmful peptides after intestinal phase, highlighting the fundamental role of BBM enzymes in affecting the digestion stability of food allergens. On the other hand, technological treatments together with the food matrix composition may affect the allergen stability. In roasted almond, in fact, the occurrence of HMW aggregates in the insoluble fraction led to release of harmful peptides after BBM hydrolysis. Therefore, there is a need to investigate accurately these issues by developing new specific process strategies for each allergenic food in order to improve their digestibility and consequentially minimizing their allergenic potential.

Abbreviations

BBM, Brush Border Membrane; HMW, high molecular weight; OGD, oral gastro-duodenal digestion; OGD-BBM, oral gastro-duodenal and intestinal digestion; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TFA, trifluoroacetic acid

Conflicts of interest

There are no conflicts to declare

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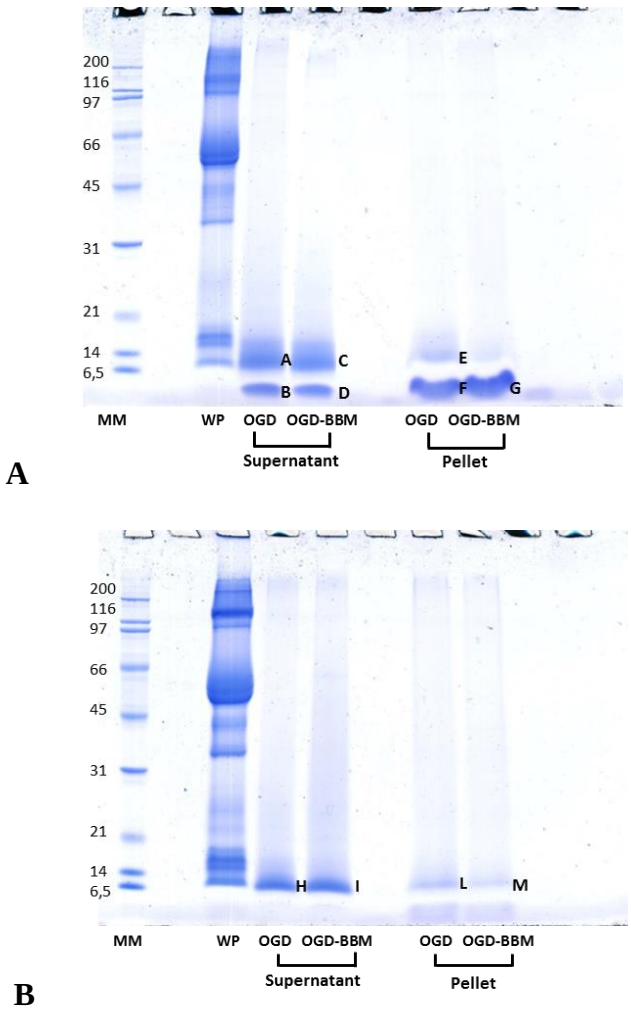


Figure 1. Unreduced SDS-PAGE analysis of raw and roasted digesta walnuts. After centrifugation, OGD and OGD-BBM surpernatants were individually analyzed. The residue pellets were subsequently extracted by 7M Urea buffer. MM: Molecular Marker; WP: Whole protein extracts from raw (A) and roasted (B) walnuts were run as reference control; OGD: oral gastric and duodenal digesta (surpernatant and pellet) from raw (A) and roasted (B) walnuts; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (A) and roasted (B) walnuts

Table 1. Identification of protein bands from SDS-PAGE of walnut digesta through LC-HR-MS/MS of in gel produced tryptic peptides (Fig. 2).

[a] Sequence coverage (%).
[b] Number of peptides identified.
[c] The total number of identified peptide sequences (peptide spectrum matches) for the protein, including those redundantly identified
[d] The number of peptide sequences unique to a protein group
[e] Number of amino acids (AA).
[f] Theoretical and experimental MW and pI values.
[g] Sum of the scores of the individual peptides from the SEQUEST HT search.
[h] Number of distinct peptide sequences in a protein group from the SEQUEST HT search.
N.D.= not detected (below 6.5 kDa)

	Band	Accession	Description	Coverage[a]	Peptides[b]	PSMs[c]	Unique Peptides[d]	AAs[e]	MW Exper [kDa][f]	MW Theor [kDa][f]	Calc pI[f]	Score Sequest HT[g]	Peptide Sequest HT[h]
Raw walnut	A	A0A2I4EX90	2S sulfur-rich seed storage protein	43	8	107	8	146	13.8	17.1	6.52	211.40	8
		A0A2I4E5L6	sucrose-binding protein-like	10	8	91	8	502		57.4	7.30	150.66	8
	B	A0A2I4E5L6	sucrose-binding protein-like	3	3	12	3	502	<6.5 kDa	57.4	7.30	8.41	2
	C	A0A2I4EX90	2S sulfur-rich seed storage protein	43	6	15	6	146	13.8	17.1	6.52	18.06	6
		G8H6H9	Oleosin	8	1	2	1	139		14.7	10.14	4.64	1
	D	A0A2I4E5L6	sucrose-binding protein-like	3	3	12	3	502	<6.5 kDa	57.4	7.30	8.41	2
	E	A0A2I4EX90	2S sulfur-rich seed storage protein	43	8	296	8	146	14.3	17.1	6.52	895.63	8
		A0A2I4DYE9	vicilin-like antimicrobial peptides	6	6	42	6	796		95.2	6.74	116.5	6
Roasted walnut	H	A0A2I4EX90	2S sulfur-rich seed storage protein	38	9	274	9	146	7.2	17.1	6.52	766.36	9
		A0A2I4E5L6	sucrose-binding protein-like	14	13	72	13	502		57.4	7.30	143.39	13
		A0A2I4DYE9	vicilin-like antimicrobial peptides	12	12	64	12	796		95.2	6.74	143.29	12
		G8H6H9	Oleosin	12	2	33	2	139		14.7	10.14	94.20	2
	I	A0A2I4EX90	2S sulfur-rich seed storage protein	30	7	189	7	146	6.3	17.1	6.52	607.66	7
		A0A2I4DYE9	vicilin-like antimicrobial peptides	8	7	27	7	796		95.2	6.74	43.51	7
		A0A2I4E5L6	sucrose-binding protein-like	6	4	9	4	502		57.4	7.30	8.28	4
		G8H6H9	Oleosin	8	1	3	1	139		14.7	10.14	7.41	1
	L	A0A2I4EX90	2S sulfur-rich seed storage protein	30	7	152	7	146	8.9	17.1	6.52	607.66	7
		A0A2I4DYE9	vicilin-like antimicrobial peptides	17	14	85	14	796		95.2	6.74	43.51	7
		A0A2I4E5L6	sucrose-binding protein-like	12	10	57	10	502		57.4	7.30	8.28	4
		G8H6H9	Oleosin	8	1	1	1	139		14.7	10.14	7.41	1
	M	A0A2I4EX90	2S sulfur-rich seed storage protein	22	3	31	3	146	9.9	17.1	6.52	67.72	3
		A0A2I4DYE9	vicilin-like antimicrobial peptides	3	3	8	3	796		95.2	6.74	2.42	3

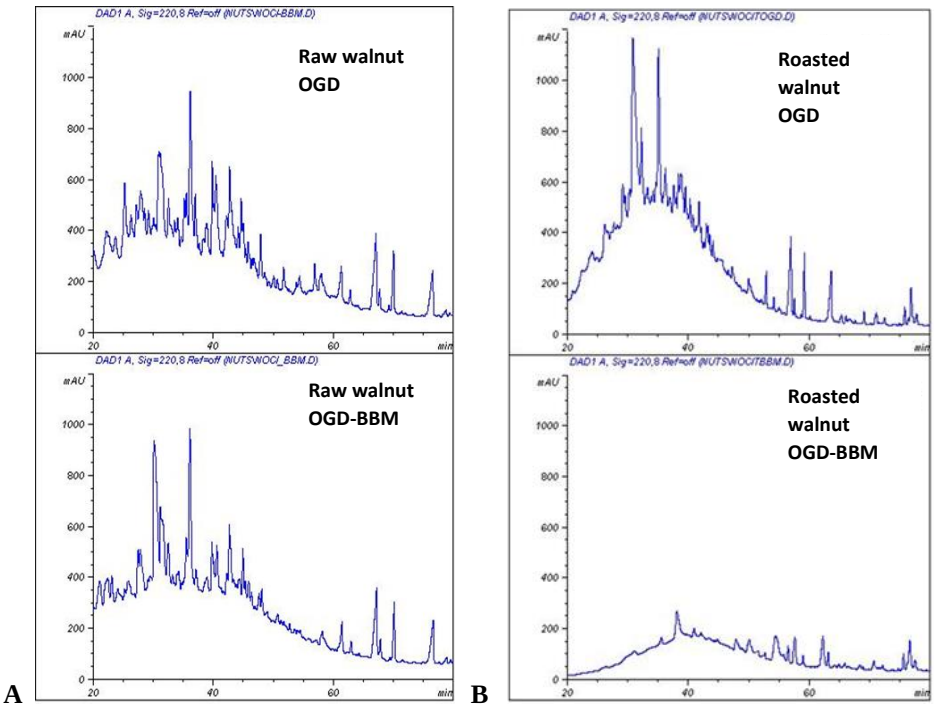


Figure 2. HPLC analysis raw and roasted OGD digesta walnuts before and after BBM digestion.

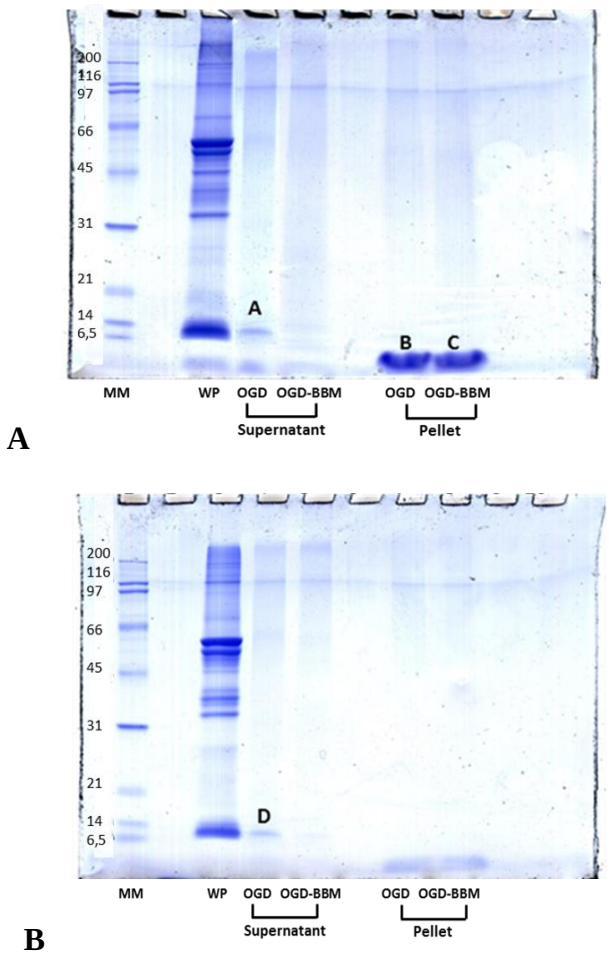


Figure 3. Unreduced SDS-PAGE analysis of raw and roasted digesta hazelnuts. After centrifugation, OGD and OGD-BBM supernatants were individually analyzed. The residue pellets were subsequently extracted by 7M Urea buffer. MM: Molecular Marker; WP: Whole protein extracts from raw (A) and roasted (B) hazelnuts were run as reference control; OGD: oral gastric and duodenal digesta (supernatant and pellet) from raw (A) and roasted (B) hazelnuts; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (A) and roasted (B) hazelnuts.

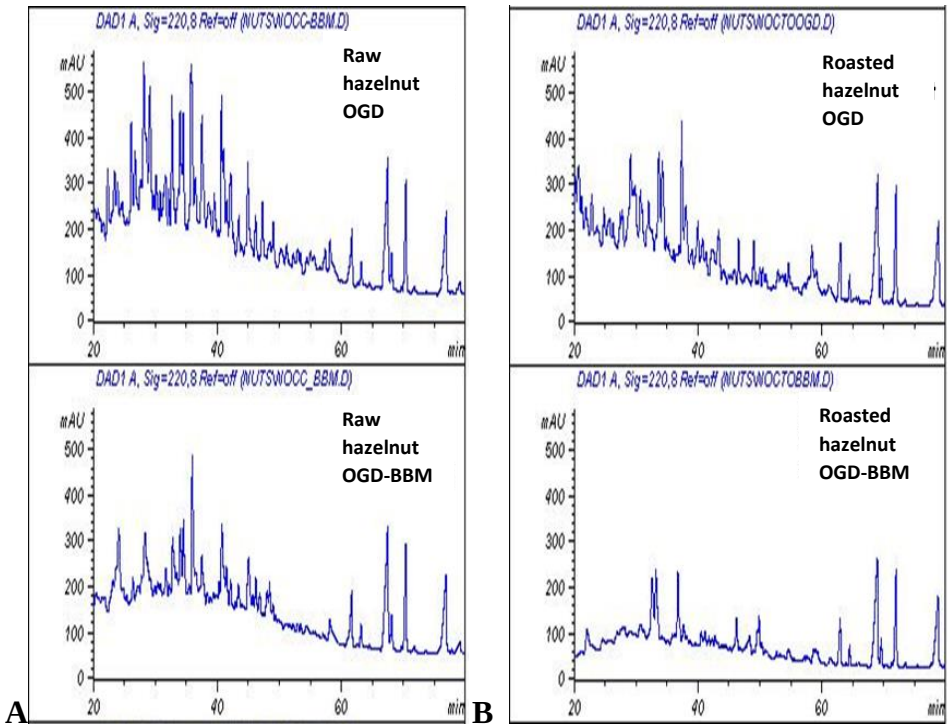
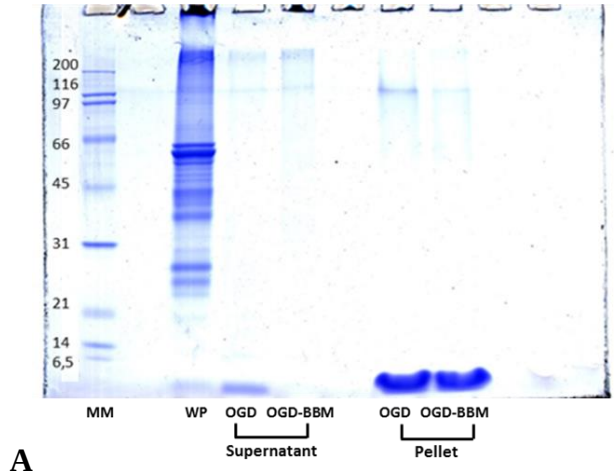
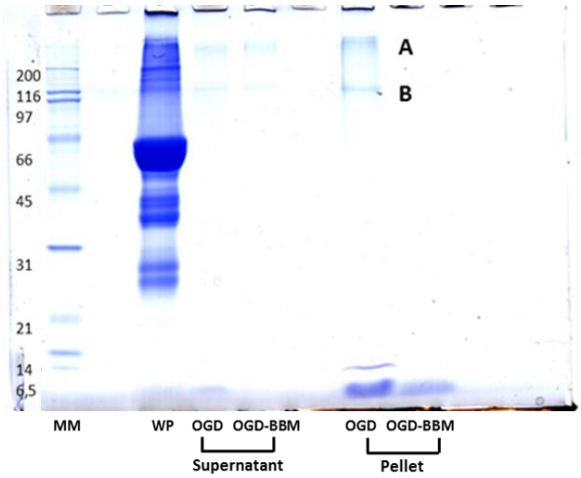


Figure 4: HPLC analysis raw and roasted OGD digesta hazelnuts before and after BBM digestion.



A



B

Figure 5. Unreduced SDS-PAGE analysis of raw and roasted digesta almonds. After centrifugation, OGD and OGD-BBM supernatants were individually analyzed. The residue pellets were subsequently extracted by 7M Urea buffer. MM: Molecular Marker; WP: Whole protein extracts from raw (A) and roasted (B) almonds were run as reference control; OGD: oral gastric and duodenal digesta (supernatant and pellet) from raw (A) and roasted (B) almonds; OGD-BBM: oral, gastric, duodenal and intestinal (BBM step) digesta (supernatant and pellet) from raw (A) and roasted (B) almonds.

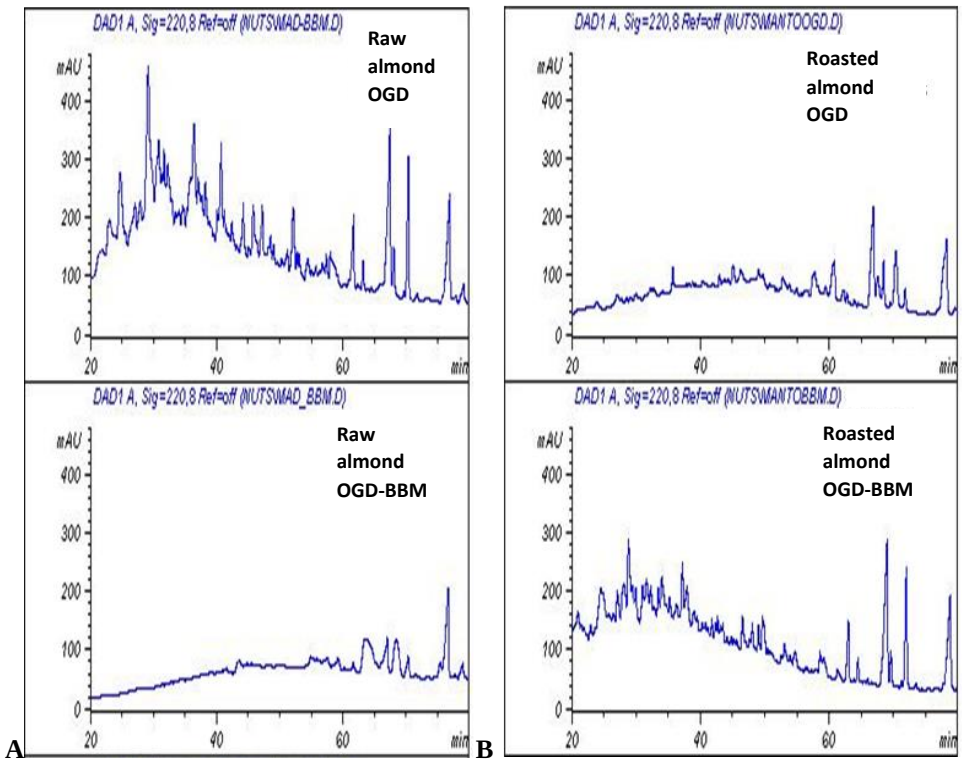


Figure 6. HPLC analysis raw and roasted OGD digesta almonds before and after BBM digestion.

Table 2. Identification of protein bands from SDS-PAGE of hazelnut and almond digesta through LC-HR-MS/MS of in gel produced tryptic peptides (Fig. 3 and 5).

[a] Sequence coverage (%).
[b] Number of peptides identified.
[c] The total number of identified peptide sequences (peptide spectrum matches) for the protein, including those redundantly identified
[d] The number of peptide sequences unique to a protein group
[e] Number of amino acids (AA).
[f] Theoretical and experimental MW and pI values.
[g] Sum of the scores of the individual peptides from the SEQUEST HT search.
[h] Number of distinct peptide sequences in a protein group from the SEQUEST HT search.
N.D.= not detected (below 6.5 kDa)

	Band	Accession	Description	Coverage[a]	Peptides[b]	PSMs[c]	Unique Peptides[d]	AAs[e]	MW Exper [kDa][f]	MW Theor [kDa][f]	calc. pI[f]	Score Sequest HT[g]	Peptides Sequest HT[h]
Raw hazelnut	A	D0PWG2	2S albumin	15	4	47	4	147	9.4	17.1	6.96	154.02	4
		AAL86739.1	48kDa glycoprotein	6	4	20	4	448		50.8	6.55	63.06	4
		Q84T91	Oleosin	8	1	12	1	140		14.7	9.98	40.99	1
	B	D0PWG2	2S albumin	10	2	2	2	147	<6.5 kDa	17.1	6.96	5.62	2
		A0A1I9RGB9	ATP synthase	7	2	5	2	491		52.7	5.57	4.33	2
	C	A0A1I9RGB9	ATP synthase	5	1	3	1	491	<6.5 kDa	52.7	5.57	5.43	1
Roasted hazelnut	D	D0PWG2	2S albumin	15	4	47	4	147	9.8	17.1	6.96	154.02	4
		AAL86739.1	48-kDa glycoprotein	6	4	20	4	448		50.8	6.55	63.06	4
		Q84T91	Oleosin	8	1	12	1	140		14.7	9.98	40.99	1
Roasted almond	A	1220073913	oleosin 1	19	2	31	2	148	304.1	15.5	9.58	86.86	2
		307159112	prunin 1]	8	5	26	5	551		63.0	7.27	82.46	5
		595793949	legumin type B	8	3	7	3	511		57.6	6.18	17.72	3
	B	1220073913	oleosin 1	19	2	21	2	148	125.1	15.5	9.58	58.78	2
		Q43608	Pru2 protein	10	4	18	1	504		57.0	5.76	50.45	4
		E3SH29	Prunin 2	10	4	18	1	504		57.0	5.64	49.81	4

Table 3. Sequences harbouring IgE binding peptides (in bold) identified through LC-HR-MS/MS of raw walnut, raw hazelnut and raw almond digesta before and after intestinal digestion.

	Allergen	Accession	IgE binding (AA sequence)	AASequence	MW (Da)	Peak area -BBM	Peak area +BBM	References
Raw walnut	Jug r 4 (11S gloulin)	Q2PW5	**IESWDPNNQNFQCAG**	IEAEAGV**IESWDPNNQQFQ** IEAEAGV**IESWDPNNQQF**- IEAEAGV**IESWDPNNQ**----- -EAEAGV**IESWDPNNQQFQ** -------AGV**IESWDPNNQQFQ**	2175 2047 1772 2062 1733	5,5E+10 1.4E+10 8,2E+09 1E+10 6,8E+09	5,9E+10 1,2E+10 1,1E+10 9,2E+09 5,9E+08	Robotham *et al.*, 2009
			GDIIAFPAGVAHWSY	RE**GDIIAFPAGVAHW** -E**GDIIAFPAGVAHW**	1639 1483	4,8E+09 2E+09	1,2E+09 1,2E+09	
			VFSGFDADFLADAFN	**SGFDADFLADAFN**VDTETAR	2162	5,8E+08	2,6E+08	
			VVKRAARNEGFEWVSF	**ARNEGFEWVSF**K **ARNEGFEWVSF**--	1470 1342	2,4E+08 9,2E+08	5E+07 3,4E+08	
	Jug r 1 (2S albumin)	P93198	**QGLRGEEMEEM**	RQQQQ**QQGLRGEEMEEM**VQSAR -QQQQ**QQGLRGEEMEEM**VQSAR	2534 2378	8,3E+09 8,8E+09	0 5,8E+08	Robotham *et al.*, 2002
	Jug r 2	Q9SEW4	**QRDFLAGQNININQLEREAKE**	**LAGQNNIINQLER** **-AGQNNIINQLER**	1483 1370	2,1E+09 6,2E+08	0	Barre *et al.*, 2008
Raw hazelnut	Cor a 9	Q8W1C2	**PYSNAPELIYIERGGITGVLF**	TIEPNGLLL**PQYSNAPELIYIER**	2643	1,4E+09	4,8E+08	Robotham *et al.*, 2009
			YLAGNPDDEHQRQGQQQFG	**YLAGNPDDEHQ**	1259	8E+10	4,1E+10	Robotham *et al.*, 2009
			RAESEGFEWVAFKTN	**RAESEGFEWVAFK** **--AESEGFEWVAFK** **RAESEGFEWVAF--** **RAESEGFEWV-----** **--AESEGFEWVAF-**	1556 1400 1428 1209 1272	2,4E+08 9,7E+08 2,5E+09 2,7E+09 2,6E+08	0 1,6E+09 0 0 0	Robotham *et al.*, 2009
			CQIESWDHNDQQFQC	**IESWDHNDQQFQ** **IESWDHNDQQ**----	1546 1272	3,2E+09 1,6E+09	1,4E+09 0	Robotham *et al.*, 2009
	Cor a 1	Q9FPK4	**GPGTIKKITFA**	TGAENLEGNG**GPGTIK** ----AENLEGNG**GPGTIK** ---------NLEGNG**GPGTIK**	1515 1357 1157	6,5E+09 2,5E+08 6,3E+08	3,8E+09 1,7E+08 0	Bohle *et al.*, 2005
Raw almond	Pru du 6	Q43607	**QQEQQGNGNNVFSGF**	**QQEQQGNGNNVFSGF**	1654	2E+09	0	Willison LN *et al.*, 2013
	7S vicilin	595824423	**SQTHVPIRP**	**TKSQTHVPIRPNKLVLKV**	1035	8,4E+08	0	Poltronieri *et al.*, 2002

Table 4. Sequences harbouring IgE binding peptides (in bold) identified through LC-HR-MS/MS of roasted walnut, roasted hazelnut and roasted almond digesta before and after intestinal digestion.

	Allergen	Accession	IgE binding (AA sequence)	AASequence	MW (Da)	Peak area -BBM	Peak area +BBM	References
Roasted walnut	Jug r 4 (11S gloulin)	Q2TPW5	**IESWDPNNQNFQCAG**	IEAEAGV**IESWDPNNQQFQ**	2175	3,6E+08	9E+07	Robotham *et al.*, 2009
			QQEYEQHRRQQQRQQRPQ	AGNPDDEFRPQG**QQEYEQHR**	2401	3,6E+07	0	
Roasted hazelnut	Cor a 9	A0A0A0P7E3	**PQQQSQQGQ-GQGQ**	FED**PQQQSQQGQGQGQ**	1790	1,0E+08	2,8E+07	Robotham *et al.*, 2009
			YLAGNPDDEHQRQGQQQFG	**YLAGNPDDEHQR**	1415	7,6E+07	0	
Roasted almond	Pru du 6	Q43608	**RPSRQEGGQGQQQFQ**	**QEGGQGQQQFQ**GEDQQDR	2063	0	5E+07	Willison LN *et al.*, 2013
			QNDQNQLDQVPRRFYLAG	**NDQNQLDQVPR**	1327	0	4E+08	

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Chapter 4

General discussion and conclusions

General discussion and conclusions

The mechanism by which dietary proteins sensitize and elicit an allergic reaction remains substantially unresolved. In particular, it is still debated whether gastrointestinal digestion stability could be an effective predictor of allergenicity. It is widely accepted that many among the most common food allergens are resistant to proteolytic digestion inducing sensitization at the level of the intestinal tract. Unquestionably, the digestion stability increases the probability that a food protein (or its derived peptide) can sensitize an individual, because in addition to the skin, respiratory way and oral mucosa pathways, the intestinal route is a crucial path for the sensitization.

Noteworthy, the majority of scientific studies have focused exclusively on the use of single (purified or recombinant) allergen proteins, neglecting the relevant effect of food matrix (e.g. protein-protein cross-links, allergens interaction with polysaccharides and/or lipids).

Food are usually exposed to several chemical /physical treatments, which may induce protein modifications leading to either destruction of existing epitopes or creation of new ones. For this reason, understanding the behavior of wholefood in the gastrointestinal tract is a crucial step in assessing the mechanisms triggering the food allergy.

In this context, this PhD thesis evaluated the metabolic fate of proteins of whole peanut and tree nut allergens (hazelnuts, walnuts and almonds), and ancient wheat (*T. monococcum*). A harmonized *in vitro* static digestion model, also including the intestinal Brush Border Membrane (BBM) enzymes was applied for this purpose. The jejunal phase of peptide degradation is a fundamental step for assessing the intestinal stability of large protein fragments produced upstream, during the gastric and duodenal phases. Following,

proteomics and immunological techniques were exploited for the characterization of the digestion products.

In a preliminary study (Di Stasio *et al*., 2017), we explored digestion stability of the major peanut allergens directly in the natural matrix using an *in vitro* static model that simulates the gastrointestinal digestion including the oral, gastric, duodenal and intestinal (brush border membrane enzymes) phases. Compared to the previous studies that involved assessment of allergen stability using standard purified proteins, we herein introduced a further complexity factor represented by the whole peanut matrix to reproduce more realistically what happens after consumption of peanuts. The most striking result of the current study was the additional identification of digestion stable Ara h 3 large sized fragments (7-21 kDa). Such a finding contrasts with most of the previous literature, which claimed the almost complete susceptibility of Ara h 3 to gastrointestinal proteases. A reasonable explanation of this finding may be the "masking effect" by the peanut matrix, delaying or impairing the protein degradation and altering the pattern of the peptide fragments released by proteolysis. Our results point out the importance of investigating the digestion process of whole food, instead of purified allergen proteins, increasing the relevance of model systems with human physiology.

Considering the possible correlation between digestion stability and allergenic potential, in Di Stasio *et al*., (*submitted* 2018), we determined the stability of peanut allergens as whole raw or roasted food, with the aim of refining the knowledge about peanut allergenic determinants investigated in a physiological relevant context. To this purpose, peanuts were subjected to gastrointestinal digestion combining the harmonized *in vitro* static digestion

models with brush border membrane (BBM) enzymes to simulate the jejunal degradation of peptides. The effect of processing and digestion on the protein allergenicity was assessed by determining the degranulation capacity of digests with the RBL assay in the presence of sera from peanut-allergic patients. 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. 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, digestion including the BBM stage destroyed most of the epitopes of roasted peanuts, resulting in a significantly lower RBL degranulation. Conversely, allergens of raw peanuts retained part of 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. In perspective, it would be interesting to compare the specific effects induced by different heat treatments, for instance those induced by roasting frying, boiling or autoclaving at high-temperature.

More recently (Mamone *et al*., 2018), we explored the "deep" seed peanut proteome by using both two dimensional electrophoresis (2-DE)-based analysis run under reducing and non-reducing condition (protein-centric) and LC-MS/MS gel-free proteomic (peptide-centric). The former approach allowed the identification of high molecular weight disulfide-linked Ara h 1 and Ara h 3 heteroligomers and Ara h 1 homoligomers linked through covalent

bonds other than disulfides. The occurrence of these protein complexes revealed natural interactions between Ara(s) subunits with a possible involvement in the allergenic potential of peanut. Differently from the 2-DE, shotgun proteomics allowed the identification of minor allergens and metabolic enzymes in addition to the dominant storage protein components (e.g. isoallergens of the Ara h 9; Ara h 7 isoallergen of conglutin 2S albumin). The complementary exploitation of two proteomic approaches enabled the access to new relevant information about the complexity of the peanut proteome, with special emphasis to the complement of allergens (allergome).

In Bavaro *et al*., (2017), we investigated the combination of high temperature and pressure on the modulation of peanuts immunoreactivity after simulated gastro-duodenal digestion. To reduce the risk of triggering allergic reactions, several technological strategies have been devised to modify or remove allergens from foods, representing potential alternatives to a strict peanuts-free diet. The most interesting ones are based on enzymatic hydrolysis, physical approaches or genetic modification methods. Among the physical methods, there are heat-based treatments which involve chemical modification such as denaturation or formation of covalent bonds between protein allergens with other nutrients including lipids and carbohydrates (Maillard reaction). These modifications can impact the final allergenicity that might vary considerably depending on the temperature, type and duration of the treatment, the intrinsic characteristics of the protein and the physicochemical conditions of the food matrix under investigation. Herein we investigated the effect of autoclaving with or without preliminary hydration, performed at the temperature of 134 °C and the pressure of 2 atm, on peanut seeds in order to evaluate any alteration on the final immunoreactivity assessed on the soluble protein fraction by ELISA and western blot analysis by using allergic patients' sera. Furthermore,

autoclaved peanuts were submitted to a standardized static *in vitro* digestion protocol in order to assess any change in allergen protein stability as a consequence of the technological process applied. The results point out that pre-hydrating peanuts before autoclaving is likely to extensively promote digestion of peanut allergens thus facilitating proteolysis of the major protein allergens. In particular, hydration prior to autoclaving proved to increase the efficacy of the thermal treatment contributing to the disappearance of the main allergenic protein bands and significantly altering the final immunoreactivity.

Food matrix and food process strategies have a strong impact on allergen digestion stability and thereby could lead to negative or positive consequences in susceptible individuals. To date, there is limited knowledge about the relationship between the interaction of food processing and enzymatic degradation in the gastrointestinal tract of tree nut allergens and subsequent interaction with the intestinal immune system. For this purpose, we assessed the stability of raw and roasted tree nuts allergens (walnuts, hazelnuts and almonds) applying the same analytical strategy previously developed (Di Stasio *et al*., 2017). We observed that roasting increased digestibility of hazelnuts and walnuts, destroying most harmful peptides and in particular, this effect was more evident after BBM hydrolysis. Interestingly almond allergens were differently affected by heating treatment, since stable IgE binding peptides were released following BBM stage from roasted almond maintaining intact their allergenic properties. A plausible explanation of this result is the formation of high MW aggregates of almond allergen after roasting. This finding show that technological treatments differently affect food allergens, strictly related to food matrix composition, either reducing or increasing their allergenic potential.

Another relevant topic, which was investigated during this PhD thesis, is the study of ancient wheats and their role in gluten related disorders. A growing interest in developing new strategies for preventing coeliac disease has motivated efforts to identify cereals with null or reduced toxicity. In Iacomino *et al* (2016), we investigate the biological effects of ID331 *Triticum monococcum* gliadin-derived peptides in human Caco-2 intestinal epithelial cells. *Triticum aestivum* gliadin derived peptides were employed as a positive control. Using an *in vitro* model of the intestinal epithelium we demonstrated that ID331 gliadin proteins do not induce effects associated with cell toxicity exerted by *T. aestivum* gliadin, due to the protective effect of ID331 ω-gliadin and its gastrointestinal resistant peptide (105–123). The ID331 ω-gliadin sequence is absent in monococcum wheat genotypes lacking ω-gliadins such as cultivar Monlis as well as in a number of einkorn lines possessing ω gliadins in their prolamin patterns, suggesting that variation in toxicity may exist in the monococcum wheat germplasm as well. These results open new research perspectives related to a possible protective action of *T. monococcum* on the small intestine of CD subjects. In particular, a diet based of *T. monococcum* could delay or even prevent the onset of CD in at-risk subjects such as first-degree relatives of coeliac patients.

The detection of gluten in processed cereal-based products requires a combination of different proteomic and immunochemical strategies, in order to ensure the food safety. In Gianfrani *et al.,* (2017), we observed that microwave based treatment (MWT) of wet wheat kernels induced a striking reduction of gluten, up to <20 ppm as determined by R5-antibody ELISA, so that wheat could be labeled as gluten-free. In contrast, analysis of gluten peptides by G12 antibody-based ELISA, mass spectrometry-based proteomics and *in vitro* assay with T cells of celiac subjects, indicated no difference of

antigenicity before and after MWT. The combination of proteomic and immunological techniques demonstrated that MWT simply induced conformational modifications, reducing alcohol solubility of gliadins and altering the access of R5-antibody to the gluten epitopes. Thus, MWT neither destroys gluten nor modifies chemically the toxic epitopes, contradicting the preliminary claims that MWT of wheat kernels detoxifies gluten. This study provides evidence that R5-antibody ELISA alone is not effective to determine gluten in thermally treated wheat products. Gluten epitopes in processed wheat should be monitored using strategies based on combined immunoassays with T cells from celiac, G12-antibody ELISA after proteolysis and proper molecular characterization.

Summarizing the ongoing results of current PhD project:

Regarding peanuts and tree nuts:

1. The immunoreactivity of proteins/peptides surviving gastro-duodenal digestion of roasted peanuts is significantly reduced compared to raw ones, emphasizing how technological treatments affect the allergenicity of food.
2. Technological treatment differently affect the metabolic fate of food allergens: i) thermal treatment (roasting) of whole almonds induced high molecular weight aggregates of allergens, which are highly resistant to gastrointestinal digestion; ii) conversely, thermal treatment (roasting) of whole hazelnuts and walnuts enhances the digestibility of their allergens.

3. The intestinal phase (BBM enzymes) significantly contributed to reduce the stability of roasted peanuts, hazelnuts and walnuts; such a result suggests that BBM are effective in destroying harmful protein fragments.
4. Understanding the fate of allergenic proteins subjected to novel processing techniques can help to develop useful strategies for food tolerance induction and/or to establish threshold levels of sensitization/elicitation for hypoallergenic foods.

Regarding wheat protein:

1. ID331 (*T. monococcum* cultivar) gliadins do not induce effects associated with cell toxicity linked to *T. aestivum* gliadins, due to the protective effect of ID331 ω-gliadin and its gastrointestinal resistant peptide ω(105–123). These results open new research perspectives related to a possible protective action of *T. monococcum* on the small intestine of CD subjects.
2. Gluten from *Triticum monococcum* cultivars is more easily hydrolyzed by gastrointestinal proteases and, as consequence, immunologically less active. It is important to notify, however, that *Triticum monococcum* wheat is not suitable for the diet of celiac patients, but it can be a valuable candidate for the prevention of *at risk* predisposed individuals.
3. In order to predict the gluten in processed cereal-based products, it is necessary to combine different proteomic and immunochemical strategies – *e.g.* T cells assay, G12-antibody based ELISA, mass spectrometry analysis, in order to ensure the food safety

….RINGRAZIAMENTI….

"*Nella vita ci sono cose che ti cerchi e altre che ti vengono a cercare. Non le hai scelte e nemmeno le vorresti, ma arrivano e dopo non sei più uguale. A quel punto le soluzioni sono due: o scappi cercando di lasciartele alle spalle o ti fermi e le affronti. Qualsiasi soluzione tu scelga, ti cambia, e tu hai solo la possibilità di scegliere se in bene o in male*."
Questa citazione, tratta da uno dei miei libri preferiti di Giorgio Faletti, spiega con chiarezza tutto il mio percorso di dottorato: come il destino lo abbia scelto per me e come poi, con tutta la mia forza, lo abbia affrontato.

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