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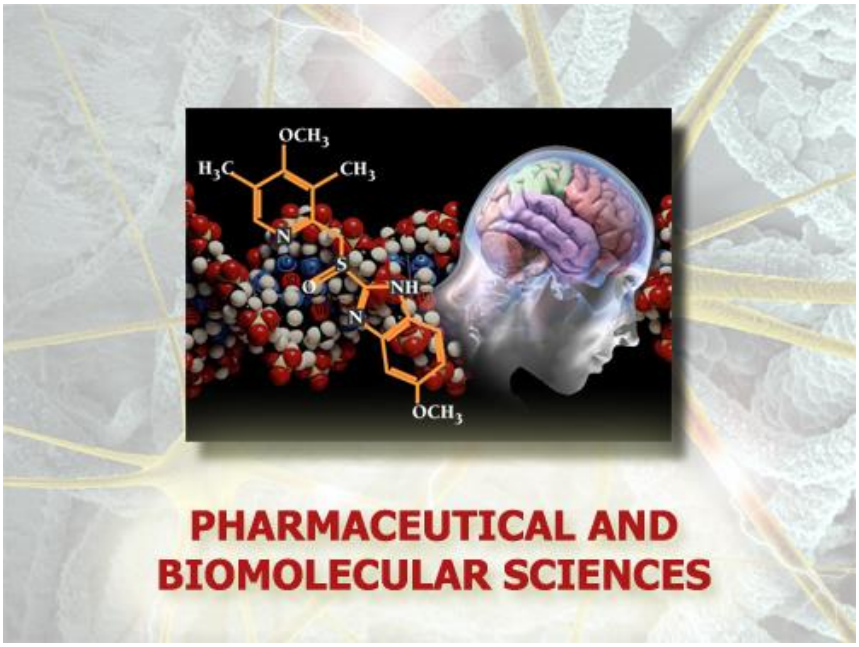

Effect of thermal processing on food protein digestibility and allergenicity

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TITOLO DELLA TESI: Effect of thermal processing on food protein digestibility and allergenicity

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Contents

AIM OF THE WORK AND ABSTRACT

Thermal treatment is the most conventional and most commonly used food processing technique to reduce pathogen loads, to increase the shelf life of a product and to improve quality and palatability. Processing methods alter the structure of food proteins in different ways. Processing may induce the unfolding of a protein molecule, a loss of the secondary and tertiary structures, and/or the formation of intra- and/or inter-molecular covalent- and non-covalent interactions between proteins, carbohydrates and lipids. For this reason, thermal processing may also modify allergenicity, by unmasking hidden epitopes and/or creating new antigenic determinants, or by destroying/masking existing epitopes. Conformational epitopes, which depend on the tertiary protein structure, can be destroyed/created by the denaturation and/or aggregation of native food proteins. Moreover, proteins can become resistant to various degrees to proteolytic digestion in the gastrointestinal tract as a consequence of a heat treatment. Some allergens can resist proteolytic digestion in the gastrointestinal tract, while others may be digested into smaller non-allergenic fragments.

Under this scenario, it seems important to answer the following question:

Does thermal processing effect food protein digestibility and allergenicity?

In order to answer this question, the aim of this PhD project was to investigate the changes in the allergenicity and digestibility of different food matrices, induced as a consequence of thermal processing. To study the effect of thermal processing on allergenicity we selected one traditional food, hazelnut, and one novel food, insects. In order to unveil the effect of thermal processing on food digestibility we selected human milk. Human milk was chosen since it provides complete nutrition in the first months of both pre-term and term infants' life and it is thus of great interest to study how bioavaibility of its nutrients is affected by storing and processing procedures.

Proteins were extracted from hazelnut fruits and insects and the allergenic profile of raw and processed food was revealed by means of immunoblotting, using sera of sensitized patients. The immune-reacting proteins were then identified by means of Mass Spectrometry and the screening of MS data against a protein database.

By studying a cohort of pediatric allergic patients, in collaboration with the Regina Margherita Children's Hospital (Turin), the IgE-binding capacities of hazelnut allergic patients toward proteins in the water-

soluble fraction and toward oil body associated proteins has been investigated. In addition, it has been evaluated whether two different roasting techniques (hot air and infrared) are able to affect the immunoreactivity of hazelnut proteins in different ways. Roasting at a low temperature (140°C) did not affect hazelnut allergenicity for either technique. High temperature (170°C) roasting appeared to reduce hazelnut allergenicity, and more so in an infrared oven than in a hot air oven for both the water-soluble and oil-associated protein fractions. While performing the immuno-allergological characterization of hazelnut proteins, a novel allergen was discovered in the hazelnut oil bodies. The sequence was submitted, accepted and registered as a new allergen, according to the WHO/IUIS Allergen Nomenclature Subcommittee criteria, and was termed Cor a 15. Cor a 15 allergen resulted to be the most frequently recognized oleosin in the considered cohort of patients.

As far as edible insects are concerned, the effect of their boiling and frying on possible cross-allergenic reactions in a cohort of patients allergic to house dust mites (HDM) and shrimps has been investigated, in collaboration with the Mauriziano Hospital (Turin). HDM and shrimps share some ubiquitous proteins that are widely distributed among arthropods and which could be responsible for cross-reaction phenomena in allergic patients. Processing resulted to only affect the cross-allergenicity potential of edible insects slightly and it appeared to be protein-, species- and treatment- specific. The study also led to the discovery of a cockroach allergen-like protein being involved in primary respiratory and food allergies to yellow mealworm (*Tenebrio molitor*).

In vitro human milk gastro-intestinal digestion was performed in order to assess whether a difference in the digestive kinetics of human milk exists, depending on the type of pasteurization technique, that has been adopted (Holder vs High Temperature Short Time, HTST). An *in vitro* dynamic model was used, and the digestion kinetics of proteins and lipids were studied, in collaboration with the Institute of Science and Technology of Milk and Eggs-INRA (Rennes, France). A better retention of native lactoferrin and milk fat globule associated proteins was found in the HTST treated milk, compared to the Holder pasteurized milk. During digestion, the HTST pasteurized milk amino acid release profile was found to be more similar to the raw human milk profile than the Holder pasteurized milk, thus suggesting that HTST pasteurization is more suitable for preserving the original protein bio-availability of raw human milk.

In conclusion, the PhD project activities led to: (i) the discovery of a new major hazelnut allergen in hazelnut, (ii) highlighting that hazelnut immunoreactivity depends on the type of roasting technique, (iii) finding new evidence on the cross-allergenicity of edible insects, and (iv) finding that the bioavailablity and digestibility of pasteurized human milk are improved under mild pasteurizing conditions.

CHAPTER 1

INTRODUCTION

1.1 The Effect of Thermal Processing on Food Quality

Foods can be subjected to different processing treatments in order to improve their organoleptic properties according to culinary traditions, to reduce the pathogen load and to increase the shelf life. Thermal processing methods such as boiling, frying, baking, roasting and microwaving are frequently applied to certain foods prior to consumption.

Native proteins are folded into specific and compact 3D structures, which are determined by primary (a sequence of amino acids), secondary (α-helixes and β-sheets) and tertiary structures. Chemical interactions among the amino acids create a unique protein conformation that could be reorganized at all the different structural levels, during a heat treatment. Heat processing alters the protein structure in different ways. Possible protein structural modifications include: the unfolding of protein molecules, the loss of secondary and tertiary structures, the formation of intra- and/or inter-molecular covalent/non-covalent interactions between lipids, carbohydrates (Maillard reaction) and other proteins (aggregation), and chemical modifications, such as oxidation and hydrolysis of the peptide bonds (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Rahaman, Vasiljevic, & Ramchandran, 2016). At the same time, because of protein denaturation, irreversible intermolecular interactions may result in protein aggregation and cross-linking reactions between a couple of amino acids, e.g., through the formation of lysinoalanine (Teodorowicz, van Neerven, & Savelkoul, 2017).

One of the best-known interactions that occurs between proteins and sugars during the heat processing of food is the Maillard reaction (MR). MR products are important for the development of food colors and flavor (Liu, Ru, & Ding, 2012). MR can be divided into three stages: early, advanced, and final stages. In an early stage, a reducing sugar, such as glucose, mainly reacts with the ε-amino group of lysine and the α-amino group of N-terminal amino acids to form a Schiff base. The Schiff base subsequently cyclizes to the corresponding N-substituted glycosilamine, which then undergoes an irreversible rearrangement to form a Amadori rearrangement product (ARP), 1-amino-1-deoxy-2-ketose. The subsequent degradation of Amadori products depends on the pH of the system. At pH=7, Amadori products undergo 1,2-enolization, mainly with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved). At pH>7, the degradation of the Amadori compound is thought to mainly involve 2,3-enolization, where reductones, acetol, pyruvaldehyde, and diacetyl are formed. All these compounds are highly reactive and take part in further reactions. Carbonyl groups can

condense with free amino groups with the formation of aldehydes and α-aminoketones (Strecker degradation). A range of reactions, involving various pathways, including cyclizations, dehydrations, retroaldolizations, enolizations, oxidations, fragmentations, acid hydrolysis, isomerizations, rearrangements, free radical reactions, and further condensations can take place at an advanced stage (Fig 1) (Liu, Ru, & Ding, 2012). As consequence, a large number of compounds are produced during MR. Some molecules are involved in the flavor and color of food (e.g. melanoidins) while other compounds may have health effects such as carcinogenicity and cytotoxicity (e.g. acrylamide), or beneficial effects, such as antioxidant, antimicrobial and antihypertensive properties (e.g. hydroxymethylfurfural) (ALjahdali & Carbonero, 2019).

Interactions between lipids and proteins occur during food processing and storage. The fatty acid peroxides in the lipids of some foods, such as fish, meat and meat products can interact with muscle proteins to form protein–lipid complexes (e.g. myoglobin with oxidized lipids). This type of interaction mainly occurs through covalent and multiple hydrogen bonds that could modulate protein solubility in water (Alzagtat & Alli, 2002).

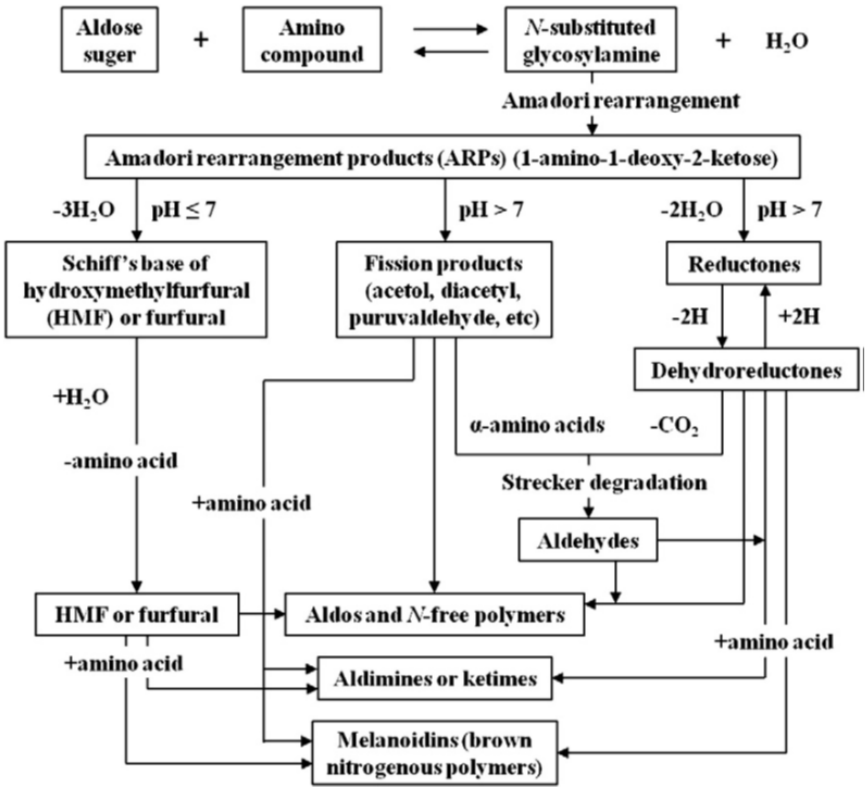

Fig 1. Scheme of the Maillard reaction (Liu, Ru, & Ding, 2012).

Protein structure modifications can affect protein solubility, which in turn contributes to important technological properties, such as emulsification and gelation (Alzagtat & Alli, 2002). The heat-induced modification of food proteins may further affect their enzymatic digestion and peptide absorption by the intestinal epithelium, as well as their presentation to the immune system and thereby influence their allergenicity (Fig 2) (Rahaman *et al*., 2016). A protein aggregate seems to reduce the epithelial uptake, but also promotes protein uptake through Peyer's patches, as has been shown for crossed-linked β-lactoglobulin and α-lactalbumin (Teodorowicz *et al*., 2017). Hydrogen bonds stabilize proteins and can create a compactly folded tertiary structure, thus making it difficult for intestinal enzymes to access cleavable peptide bonds (Gan, Bornhorst, Henrick, & German, 2018). The aggregated proteins that are neither hydrolyzed nor absorbed by the enterocytes, can be further metabolized by intestinal microbes and may result in the formation of new bioactive compounds and in the modulation of the intestinal microbiota composition of humans (Teodorowicz *et al*., 2017).

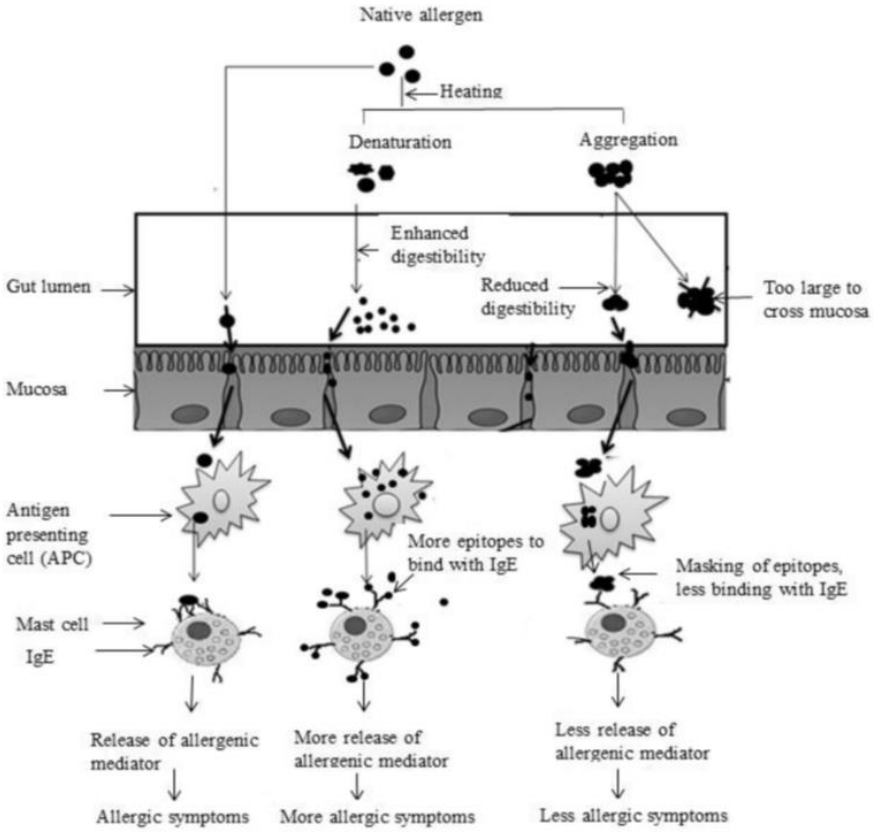

Fig 2. Heat induced conformational changes in the protein structure and modification of protein digestion, absorption and immune reactivity (Rahaman *et al*., 2016).

1.2 The Effect of Thermal Processing on the Protein Structure of Food and Food Allergenicity

1.2.1 Food allergy

Food allergy is an adverse reaction to food caused by a particular hypersensitivity reaction through an immunological mechanism, wich is mainly IgE mediated. The most common allergenic foods in Europe are: cereals containing gluten, crustaceans, mollusks, eggs, fish, peanuts, tree nuts, soybeans, milk, celery, mustard, sesame, lupin and sulphur dioxide (Verhoeckx *et al*., 2015). Food allergies are due to both the IgE-mediated reactions and to non–IgE-mediated disorders (eosinophilic esophagitis, enterocolitis syndrome, proctocolitis). They occur through food sensitization in the gastrointestinal tract, in the skin, and in the respiratory tract (Sicherer & Sampson, 2018). The severity of the clinical symptoms of IgE-mediated food allergies can vary from mild to severe and from local to systemic. The oral allergy syndrome is the most common local food allergic reaction, while anaphylaxis is a severe systemic reaction in which different target organs can be affected. Anaphylaxis symptoms usually appear quickly and can progress in severity in just a few minutes or hours.

IgE binding to the mast cell membrane receptor mediates the allergy reaction. Mast cells are resident cells that are present in the epithelia, the connective tissue and mucous membranes. The binding between this receptor and IgEs causes mast cell degranulation, and liberation of different molecules that mediate the allergic response (such as histamine, serotonin, heparin and chemotactic factors). In order to develop an allergic reaction, a first exposure to tan antigen is needed, a process which is called "sensitization". In this phase, the CD103+ dendritic cells (DCs) in the gastrointestinal tract and the CD11b+ dermal DCs and Langerhans cells in the skin, bind and internalize the allergenic peptides. DCs expose the decomposed peptides on the cell membrane in order to migrate to the lymph nodes and present the antigens to the T-helper (Th) lymphocytes (CD4+). After antigen presentation, the Th differentiate into Th2 lymphocytes that are able to produce pro-inflammatory cytokines and stimulate lymphocytes B to produce allergen-specific IgE. IgEs remain in the blood and tissues for a long time thanks to the bond with basophils, dendritic and mast cells. After the second ingestion of the allergen, degranulation of the mast cell occurs with the subsequent appearance of symptoms (Pelz & Bryce, 2015) (Fig 3).

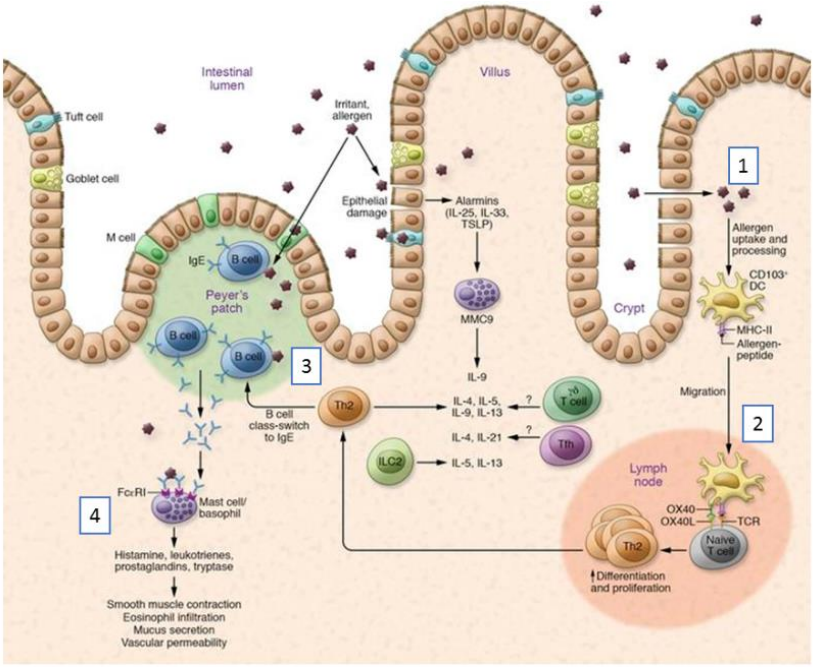

Fig 3. Schematic representation of the food allergy mechanism in the gut. 1) the antigen passes through the intestinal membrane and is internalized by the DCs. 2) the DCs move in the lymph nodes and expose the antigen to the T lymphocytes with their differentiation in T helper 2 (Th2). 3) Th2 stimulates the production of specific IgEs by the B cells. 4) the IgEs bind the mast cells and after a second exposition to the allergen, mast cell degranulation occurs (modified from Sampath & Nadeau, 2019).

Genetic, environmental and epigenetic factors influence the occurrence of food allergies. Numerous risk factors have been identified or proposed to contribute to food allergies or sensitization, including sex (increased frequency in male children), ethnicity (increased frequency in Asian and black children compared to white children), genetics (family associations, HLA and specific genes) and increased hygiene (Sicherer & Sampson, 2018). Moreover, the physiological function of the gastrointestinal tract contributes to antigen degradation and prevents pathogen colonization. The epithelial barrier plays a major role in the immune homeostasis of the intestinal tract by separating the antigens and pathogens from the epithelial layer (Samadi, Klems, & Untersmayr, 2018). The relationship between dietary short chain fatty acids (SCFAs), probiotics, fiber and food allergy onset has recently emerged as a topic of interest. These metabolites can inhibit the inflammatory mechanism, influence CD103+/Treg biology, the IgA/IgE balance and effect allergic leukocytes. SCFAs produced by the microbiome can affect epigenetic and gene transcription programs, which have some immunologic outcomes (Brosseau *et al*., 2019; Licari *et al*., 2019; McKenzie, Tan, Macia, & Mackay, 2017). Furthermore, some studies suggest that

antibiotics are able to determine the risk of developing a food allergy, due to the depletion of particular bacteria that may influence allergy susceptibility (McKenzie *et al*., 2017). An association between the development of food allergies and triacylglycerol (TAG) composition of human milk has recently been found. TAG with longer carbon chains and double bonds can regulate the functions of the fetal immune system through several anti-inflammatory mechanisms and/or result in a reduced Th2 response, thus protecting against allergies (Hong *et al*., 2019).

1.2.2 The diagnosis of IgE mediated food allergies

The double-blind placebo controlled food challenge (DBPCFC) is still considered the gold standard diagnostic test for the diagnosis of food allergies. However, the clinical application of DBPCF for diagnosis purposes has been associated with several practical and ethical limitations. For this reasons, sIgE quantification and/or skin prick tests (SPT) are performed in daily clinical practice, in order to confirm the diagnosis of food allergies. However, the obtained results should be interpreted with care. A positive result does not always indicate the presence of an allergic disease, and could instead reflect a cross-sensitization phenomenon. Alternatively, negative sIgE and/or SPT results do not exclude an IgE-mediated condition. A false negative could be due to the under-representation of minor-allergens and/or the instability or absence of allergenic proteins in the extract used for the diagnosis (Jappe & Schwager, 2017).

In recent years, the *in vitro* basophil activation test (BAT) has proved to be a sensitive and specific instrument that can be used to complement conventional diagnostic tools (both quantification of sIgE and SPT). Briefly, upon activation by a specific food allergen, basophils secrete particular activation (CD203c) and degranulation (CD63) markers that can be cytometrically flow quantified using specific monoclonal antibodies conjugated with a laser excitable fluorochrome (Hemmings, Kwok, McKendry, & Santos, 2018).

1.2.3 Parameters affecting the effect of processing on food allergenicity

Food allergenicity has been reported to either increase or decrease upon thermal processing, depending on on the type of allergen, food, time/temperature settings and methods applied. It has been demonstrated that after thermal processing, protein epitopes may be destroyed, modified, masked or unmasked, after protein denaturation and/or aggregation (Jiménez-Saiz *et al*., 2015). New conformational

and linear epitopes could be created, due to changes in the tertiary and secondary structure, an interaction with other molecules present in the food matrices and exposure to amino acid sequences due to denaturation. Through these mechanisms, MR can induce the production of new molecules with new epitopes and, as a consequence neo-allergens (Teodorowicz *et al*., 2017). The oxidized lipids and plant polyphenols that are found extensively in plants can interact with allergens and modify their allergenicity. For instance, Pru av 1, one of the major allergens of cherries, could interact with epichatechin and caffeic acid and consequently reduce their IgE binding capacity after a heat treatment (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009).

The modification of protein allergenicity induced by processing depends on the **type of allergen.** For instance, Bet v 1 (one of the major allergens from the birch (*Betula verrucosa*) pollen antigen) has the potential to unfold and to lose IgE-binding conformational epitopes upon thermal treatments, while lipid transfer proteins are characterized by a high resistance to denaturation, due the presence of multiple disulfide bonds (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Moreover, the **type of processing** can also affect the food allergenicity potential. For instance, peanuts are eaten boiled, fried or roasted and these different preparation methods seem to have an impact on the prevalence of peanut allergies. In fact, a lower incidence of peanut allergies has been reported in countries where peanuts are consumed after boiling (Cabanillas, 2019). Boiling decreases the IgE binding capacity of the Ara h 1, Ara h 2, and Ara h 3 allergens, compared to roasting, which has been shown to increase peanut allergenicity. The boiling of Ara h 1 induces a partial loss of the secondary structure of the molecule, which is then assembled into branched complexes with a reduced IgE binding capacity, due to a decreased epitope availability. Ara h 1 and Ara h 2 undergo chemical modifications induced by MR during roasting which increase the allergen IgE-binding capacity, produce more stable structures and confer resistance to heat and digestion. The frying of peanuts, but not boiling or roasting, dramatically alters the secondary structure of Ara h 2 by decreasing the content of α-helices in the molecule nd increasing its β-sheets, β-turn and random coil, thus altering the Ara h 2 epitopes and reducing its allergenicity (Palladino & Breiteneder, 2018).

Temperature level also affect the potential allergenicity of a protein. β-lactoglobulin, a major cow milk allergen, when heated to 90°C, unfolds, with the exposure of conformational epitopes, and this results in increased allergenicity. However, further raising the temperature to 100-120°C can lead to an irreversible aggregation of β-lactoglobulin with covalent and hydrophobic interactions, and the consequent masking and/or destroying of conformational epitopes and a reduction of allergenicity (Rahaman *et al*., 2016).

1.2.4 The effect of thermal processing on hazelnut allergenicity

Seeds of *Corylus avellana* species are the most frequently cultivated and consumed hazelnuts and, because of their high potential health benefits, are a common component of different processed foods, such as chocolates, cakes and biscuits. However, hazelnuts have a high allergenic potential, and they are in fact the third food in order of importance to cause anaphylactic shock in children after cow milk and eggs (Costa, Mafra, Carrapatoso, & Oliveira, 2016).

The multicenter and multidisciplinary EuroPrevall (Datema *et al*., 2015) project was conducted, from 2005 to 2010, with the aim of investigating the allergenic prevalence of 24 food allergies across 12 European cities. Hazelnut was reported to be the most common food allergy in a EuroPrevall outpatient clinic survey (32%). The interviewed patients commonly reported oral (84%) and digestive symptoms (20-35%). More severe symptoms, like asthma (13%) and cardiovascular or neurological symptoms (3%), were reported less frequently. Hazelnut sIgE showed a clear variation across Europe, and birch pollen exposure was found to play a dominant role in the occurrence of hazelnut allergies (Datema *et al*., 2015). This is due to the high homology of the pathogenesis related (PR)-10 proteins of seeds (Cor a 1 and Cor a 2) and birch pollens (Bet v 1), although a direct sensitization to Cor a 1 after hazelnut consumption cannot be excluded (Hofmann *et al*., 2013). In general, a sensitization to Cor a 1 was the most prevalent symptom (74%) in the European population, and this was followed by a sensitization to Cor a 2 (19%). The IgEs levels for Cor a 1 were 5 to 10 times higher than those for other hazelnut allergens (Fig 4).

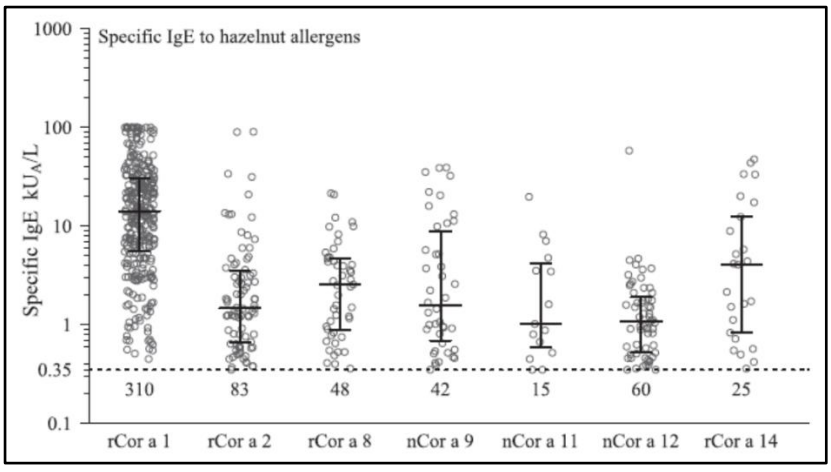

Fig 4. Median sIgE values across the European patients in the EuroPreval study. The dotted lines indicate the cutoff IgE level at 0.35 kUA/L (Datema *et al*., 2015).

Clinical diagnoses should be conducted through the clinical history including the reactivity to raw or processed hazelnuts since hazelnuts are more commonly consumed processed. The ability to react to raw or unprocessed tree nuts, without symptoms related to heated tree nuts, might result in a modification in the current dietary advice. However, the processing conditions of hazelnut in daily life are not standardized, and the clinical reactions are therefore difficult to predict (Masthoff *et al*., 2013). Raw tree nuts are a risk for all tree nut allergic patients, although processed nuts might be tolerated by patients who only recognize PR-10 proteins (a heat-labile allergen) (Table 1). However, reactivity to processed hazelnuts in combination with severe symptoms, makes a reactivity to seed storage proteins more likely (Flinterman, Akkerdaas, Knulst, van Ree, & Pasmans, 2008). Understanding how heat influences allergenicity and whether this effect is reversible, could lead to strategies that could be used to reduce allergenicity in food production with important implications for clinical practices.

Allergen	kDa	Classification	Exposition	Effect of thermal processing on allergenicity
Cor a 1	17	Pathogenesis-related (PR)-10 Bet V1-like protein	Inhalation Ingestion	Decrease
Cor a 2	14	Profilin superfamily	Inhalation Ingestion	Decrease
Cor a 8	9	Prolamin superfamily nsLTP	Ingestion	Decrease
Cor a 9	360	Cupin-11S Globulin Legumin-like protein	Ingestion	Limited effect
Cor a 11	50.8	Cupin-7S Globulins Vicillin-like protein	Ingestion	Limited effect
Cor a 12	16.7	Oleosin	Ingestion	Unknown
Cor a 13	14.7	Oleosin	Ingestion	Unknown
Cor a 14	17	Prolamin superfamily 2S Albumin	Ingestion	Limited effect

Table 1. Hazelnut allergens and the effect of their thermal processing on allergenicity.

1.2.4.1 Heat-labile hazelnut allergens

Cor a 1 is a PR-10 protein that is expressed by the plant in response to external factors, such as environmental stress and infections. This allergen is abundant in pollen and, it has consequently been classified as both food- and aero- allergenic (Costa *et al*., 2016). Cor a 2 is a profilin, that is a protein belonging to cytosolic actin-binding protein family, and it is an important mediator in the communication between membranes and the cytoskeleton. These proteins are widespread in

nature, and for this reason they are considered pan-allergens that are responsible for several cross-reactivity cases between inhaled and ingested food allergens. Cor a 2 has a high amino acid sequence identity with Bet v 2, a common allergen present in birch pollen (Costa *et al*., 2016). Cor a 8 is a non-specific Lipid Transfer Proteins (nsLTP), and one of the biological functions of these proteins is to transport lipids through membranes. They also play important roles in defense, as they help develop antifungal and anti-bacterial activities, as well as a role in the growth and development of the plant (Costa *et al*., 2016).

The clinical symptoms related to Cor a 1 and Cor a 2 are considered mild and are mainly restricted to the oral cavity (Masthoff *et al*., 2013), while the clinical symptoms associated with nsLTP are normally classified as severe (Hansen *et al*., 2009). Anaphylactic reactions, caused by nsLTP, have been documented in 3% of hazelnut allergic patients (Pastorello *et al*., 2002). De Knop and collaborators (2011) in their study observed sensitization to Cor a 8 in 12% of preschool children, 17% of school-age children, and 6.7% of individuals over 18 years old. No sensitization to this allergen was perceived in patients with oral allergic symptoms or in birch pollen allergic individuals without a hazelnut allergy. Cor a 1 is easily denatured after a heat treatment and it could be considered a heat-labile allergen. The loss of the three-dimensional structure of the protein also leads to a variation in the conformational IgE-binding epitopes, and this causes an inability to trigger an adverse reaction in sensitized subjects (Davis & Williams, 1998). Recognition of Cor a 1 by hazelnut allergic patients is completely lost after roasting (140°C, 20–40 min) (Müller *et al*., 2000). A decrease in hazelnut allergenicity after roasting (140°C, 40 min) has also been found in patients with a hazelnut and birth pollen allergy (Pastorello *et al*., 2002; Schocker *et al*., 2000). Cor a 1 detection is lost after roasting at 185°C, while, at 100°C, Cor a 1 maintains its allergenicity resistance (Wigotzki, Steinhart, & Paschke, 2001). The allergenicity of Cor a 2, like Cor 1, tends to decrease after roasting (. The allergenicity of Cor a 1 and Cor a 8 is significantly affected when hazelnuts are submitted to high temperatures and wet processing, such as in an autoclave (121°C and 138°C, for 15 and 30 min, respectively), since autoclaving induces the disorganization of almost all the possible epitopes in these proteins (López *et al*., 2012). nsLTP is slightly less stable when submitted to temperatures above 90°C, probably due to the existence of a lipid-binding tunnel (Costa *et al*., 2016).

1.2.4.2 Heat-stable hazelnut allergens

Cor a 9, 11 and 14 are found exclusively in seeds so they are classified as food allergen and their allergenicity potential seems to remain quite stable after thermal processing (Masthoff *et al*., 2013).

Cor a 9 and Cor a 11 belong to the same group of globulins, that is seed storage proteins that constitute 50 % of the total seed proteins in hazelnuts (Costa *et al*., 2016). Cor a 9 form hexameric structures comprising six subunits that interact non-covalently and which are arranged in an open ring conformation, thereby accounting for 360 kDa. Each subunit consists of an acidic polypeptide (30–40 kDa) linked to a basic polypeptide (20 kDa) by a disulfide bond (Nitride *et al*., 2013). Cor a 14 is a 2S albumin and, like many of these proteins, is cleaved into a larger and a smaller subunit, thereby forming two heterodimers that are bound together by disulfide bridges (Costa *et al*., 2016).

Cor a 9 is associated with severe hazelnut reactions while the sensitization to Cor a 11 has been reported in hazelnut allergic patients presenting mild immunological responses, mainly related to OAS or those experiencing severe systemic reactions (Costa *et al*., 2016). The limited effect of roasting on the ability of hazelnut allergic patient IgE to bind hazelnut proteins has been investigated in several works. No change in immunoreactivity was found after roasting (180°C, 15 min) in two patients with severe hazelnut allergy (de Leon *et al*., 2003). Moreover, Cor a 9, Cor a 11 and Cor a 14 were also recognized after processing in seven hazelnut allergic patients (Wigotzki *et al*., 2000). Likewise, the IgE of five patients sensitized to Cor a 9 continue to recognize this allergen also after roasting (170°C, 10 min) (Dooper *et al*., 2008). The disulfide bonds of Cor a 9 play a key role in stabilization after thermal treatment as they reduce the conformational entropy of the protein in their denaturation state. The number and the distribution of disulfide bridges seem to be the major contributors to its high stability, even in the gastro-intestinal tract (Moreno & Clemente, 2008). (Müller *et al*. (2000) demonstrated the heat stability of Cor a 11 and Cor a 14 after roasting (140°C, 20–40 min), while López *et al*. (2012) found that autoclaving (138°C, 15–30 min) decreased IgE binding, likely as a result of a decreased solubility.

1.2.4.3 Allergens associated to oil bodies: oleosins

Oleosins are the most abundant proteins present on the surface of seed oil bodies (OBs) together with caleosins and steroleosins (Fig 5) (Jappe & Schwager, 2017). OBs constitute the seed plant site for energy storage, that is energy used by plants during seed germination (Huang, 2018).

Among the nut allergens, OB proteins have been receiving increasing interest, since oleosins have been described as major

allergens in peanuts (Schwager *et al*., 2017, 2015) and in sesame (Leduc *et al*., 2006; Teodorowicz, Terlouw, Jansen, Savelkoul, & Ruinemans-Koerts, 2016)

Two type of oleosins are present in the hazelnut seeds, and they were described for the first time by Akkerdaas *et al*. (2006): Cor a 12 and Cor a 13. Cor a 12 has a higher molecular weight (16.7 KDa) and higher basic pI (10.5) than Cor a 13 (MW 14.7 KDa and pI 10). Zuidmeer-Jongejan *et al*. (2014) were the first to report the association of Cor a 12 with severe symptoms. Moreover, it has been demonstrated that oleosins, with their strong hydrophobic structure, are often missed in commercial diagnostical tests because the used extracts are only based on the hydrophilic protein fraction (Costa *et al*., 2016; Jappe & Schwager, 2017).

Hazelnut oleosin sensitization is common in Europe, with 10-25% of the hazelnut allergic patients showing sensitization to Cor a 12 as pointed out in the EuroPrevall study (Datema *et al*., 2015). IgE against Cor a 12 is related to sensitization to many foods, in particular oil-rich tree nuts, seeds and legumes. Oleosins have also been identified in pollen, and it cannot therefore be excluded that pollen play an important role in oleosin sensitization (Datema *et al*., 2015).

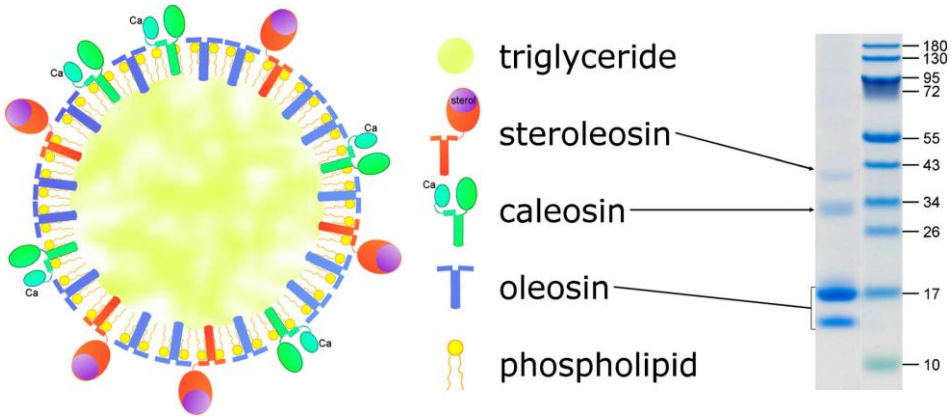

Fig 5. Schematic model of an oil body with its components constituted by a core of neutral lipids, mainly triacylglycerol, and surrounded by a layer of phospholipids and proteins (left). Determination of the molecular mass of oil body proteins from peanuts by means of electrophoresis (right) (Jappe & Schwager, 2017).

1.2.5 The effect of thermal processing on insect protein allergenicity

1.2.5.1 Insects as food

Edible insects are considered a promising solution to meet the growing global demand for high nutritional value proteins, due to the ever increasing world population, which has been estimated will reach up to nine billion by the year 2050. Moreover, climate change is associated with an increase in CO_2 and the livestock activity involves also an air pollution with greenhouse gases. Depending on the farming system, the animal breed, the food supplied to the animals, and the manure management, various concentrations of gases with greenhouse effect may appear in the gaseous emissions. Insects are considered as a new sustainable protein source, since they do not need to have a large quantity of food available and they use less space and water than other animals (Stubbs, Scott, & Duarte, 2018; Patel, Suleria, & Rauf, 2019). Edible insects have a high nutritional value, and the nutrient composition varies according to the species, life stage, sex and diet.

Insects have always been part of the human diet of about 2 billion people, mostly in Asia, Africa and Latin America. However, people in most Western countries dislike the idea of insects as food, as they associate their consumption with primitive behavior. Furthermore, women are more reluctant than men to eat insects, while young adults, especially those with higher levels of education, are more enthusiastic and curious to try insects as food (Melgar-Lalanne, Hernández-Álvarez, & Salinas-Castro, 2019). In some European countries, consumers have shown an interest in new food products that use insects as ingredients in an unrecognizable form. If insects are used in flours or powder, or are added to different products, such as energy drinks and cookies, consumers are more likely to accept them (Ammann, Hartmann, & Siegrist, 2018).

Insects, like many other food products, are subject to microbiological and/or chemical contamination, which may depend on the type of substrate on which they are bred and on the storage conditions. Traditional processing methods, such as boiling, roasting and frying, are often applied to improve their taste and palatability, but they can also have the advantage of removing any microbiological contaminants. EFSA issued a scientific opinion on the topic in October 2015 and it suggested that a specific risk assessment should be performed, taking into account the whole production chain from farming to consumption, pertaining to the species to raise and the substrate to use as well as the farming and processing methods (EFSA, 2015).

The allergenicity potential of insects still has to be evaluated. Different cases of allergic reactions, caused by inhalation and/or contact with larval feces, have been reported in literature and they mainly occurred in people who are regularly exposed to insects, such as entomologists and fish bait breeders (FAO, 2013; Panzani & Ariano, 2001). In this regard, allergic reactions to mealworm (*T. molitor*), after contact through occupational exposure, have been documented (Siracusa *et al*., 1994; Broeckman *et al*., 2017a). There are cases of allergic reactions after insect ingestion (de Gier & Verhoeckx, 2018), and cases of anaphylactic shock in humans have also been reported (Ji, Zhan, Chen, & Liu, 2008).

1.2.5.2 The nutritional composition of edible insects

A specific nutritional composition of insects is hard to evaluate, due to the wide variety of species in the world, of their habitats, diets and cooking methods; moreover, the nutritional characteristics of insects may even be different within the same group, according to their metamorphosis stage. A short summary of the nutritional contents and the wide variety of nutrition quality of nine insect orders is provided in Figure 6 (Rumpold & Schlüter, 2013).

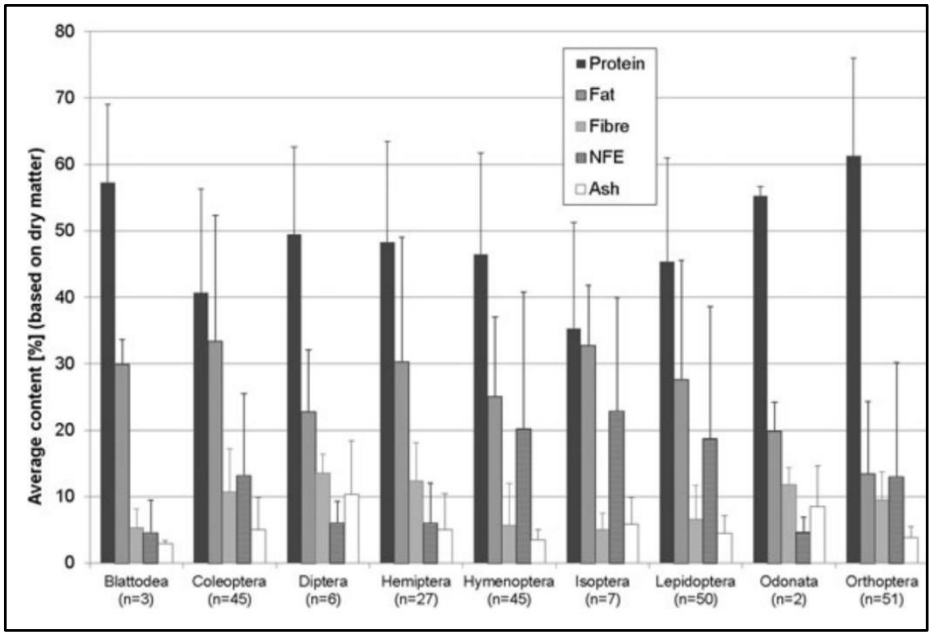

Fig 6. Average nutrient contents [%] (on a dry matter basis) of edible insects from to the same order (Rumpold & Schlüter, 2013). Most of the species analyzed in the Rumpold & Schlüter (2013) study belonged to the Orthoptera order (grasshoppers, locusts, and crickets) and Lepidoptera order (caterpillars) while the least represented orders were Odonata (dragonflies) and Blattodea (cockroaches). n, number of insect samples obtained from literature; NFE, nitrogen-free extract.

The energy value of edible insects is very high and varies from 400 to 770 kcal/100 g (on a dry matter basis), mainly due to two components: proteins and lipids (Rumpold & Schlüter, 2013). Insect proteins contain essential amino acids (in particular lysine, tryptophan and threonine), that are usually present at critical levels in diets, especially in developing countries (Pj & Ko, 2015). Most insect species have a fatty acids content that is comparable with that of fish and, for this reason, insects could play an important role in developing countries, where fish, a source of essential fatty acids, is less availabile. Moreover, insects are not able to synthesize cholesterol. As far as the amount of carbohydrates is concerned, insects are mostly composed of chitin, a substance that is very similar to cellulose, which is the main component of the exoskeleton. Humans are unable to digest and absorb chitin, which becomes fiber in the intestinal tract (Rumpold & Schlüter, 2013).

Overall, insects have the potential to complement and/or replace conventional foods, as they exhibit favorable nutrient profiles. For example, the protein content of grasshoppers (76%) and crickets (70%) are similar to the protein content of whey (87%) and chicken eggs (82%) (Fig 7) (Elhassan, Wendin, Olsson, & Langton, 2019).

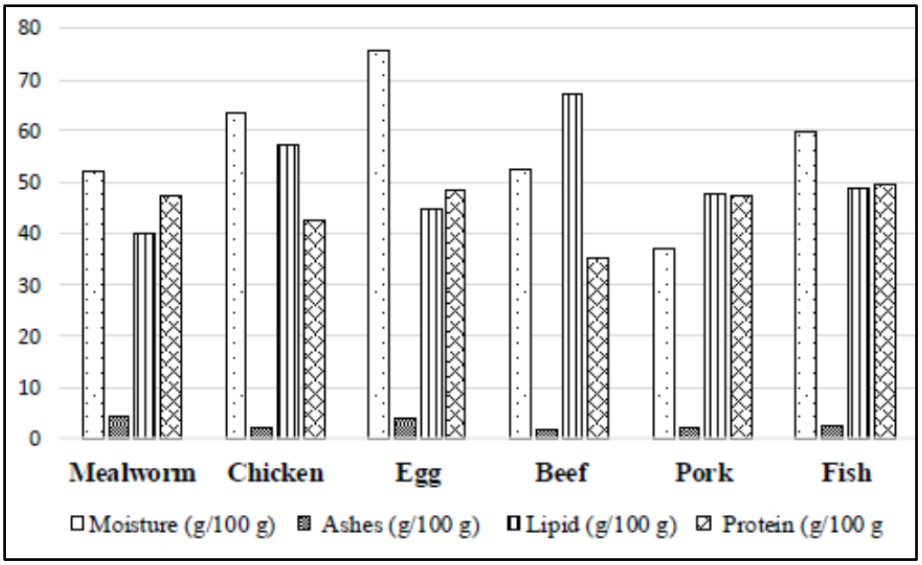

Fig 7. Comparison of the nutritional content of mealworm larvae and conventional foods. The ashes, lipids and protein values are based on the dry weight (Elhassan, Wendin, Olsson, & Langton, 2019).

1.2.5.3 Cross-reactivity of arthropod allergens

The major allergens of house dust mites (HDM) and shrimps correspond to ubiquitous proteins, which are widely distributed over different groups of invertebrate, including insects (grouped in the

same phylum as arthropods) (Fig 8). Because of their high degree of amino acid sequence and structural conservation similarity, IgE-binding cross-reactivity has often been reported to occur among the pan-allergens of invertebrates. This is especially true for tropomyosin and various enzymes, such as the widely distributed α-amylase and arginine kinase (Barre, Simplicien, Cassan, Benoist, & Rougé, 2018).

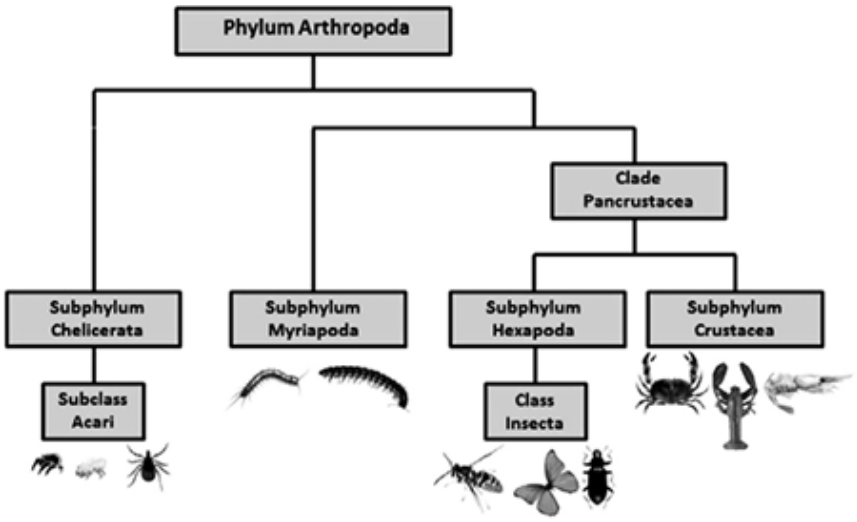

Fig 8. Representation of the Artropoda phylum (Verhoeckx *et al*., 2014).

Tropomyosin itself appears to be the major cause of cross-reactivity among dust mites, crustaceans, some insect species and mollusks (Shafique, Inam, Ismail, & Chaudhary, 2012). Tropomyosin is an α-helix protein with a molecular weight of 37 kDa, which is present in many cell types, especially in muscle cells, since it is fundamental in the regulation of the function of actin filaments. α-amylase and arginine kinase are enzymes that are present in all Arthropods, and they have been identified as being among the most relevant enzymatic pan-allergens in shell-fish, nematodes and insects (Barre, Simplicien, Cassan, Benoist, & Rougé, 2018).

In this respect, the possible cross-allergenic reaction of HDM and shrimps allergic patients to insect proteins needs to be investigated (Ribeiro, Cunha, Sousa-Pinto, & Fonseca, 2018). In the study of Verhoeckx *et al*., 2014) tropomyosin was identified as the major cross-reactive allergen in mealworm in both HDM and crustacean allergic patients. The sera of these patients resulted to be positive to mealworm protein extracts in immunoblotting and basophil activation tests. Broekman and collaborators (2017b) observed that more than 70% of the shrimp allergic subjects in study showed immunoreactivity towards different insect species, and that tropomyosin and arginine kinases are the most frequently recognized proteins. Previously, Broekman *et al*. (2016) evaluated the allergic behavior of 15 shrimp

allergic patients after mealworm ingestion, in DBPCFC trial. After mealworm ingestion, 80% of the patients developed oral symptoms, orticaria, nausea, abdominal cramping, vomiting and dyspnea, while 100% of the patients resulted to be positive to mealworm protein extracts in a basophil activation test. Tropomyosin has also been identified in grasshoppers as the major cross-reactive protein in shrimp allergic patients (Leung *et al*., 1996; Sokol, Wünschmann, & Agah, 2017). Moreover, tropomyosin, in addition to arginine kinase and hexamerin 1B, has also been found to be a cross-reactive allergen in crickets for shrimp allergic patients (Hall, Johnson, & Liceaga, 2018; Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2015).

1.2.5.4 The effect of processing on insect cross-reactivity

The effect of thermal processing (baking, blanching, boiling, frying, and freeze-drying) on insect allergenicity has been assessed *in vitro* by means of immunoblotting experiments. Since immunoblotting depend on the IgE binding capacity, and changes in IgE binding capacity do not always correlate with changes in clinical symptoms, *in vivo* assessments in allergic subjects are still necessary. Different studies have concluded that thermal processing could affect insect allergenicity. For instance, it has been found that tropomyosin and arginine kinase in mealworm retained their IgE binding capacity in mealworms after thermal processing (de Gier & Verhoeckx, 2018).

In the study of H. Broekman *et al*. (2015), BAT conducted on unprocessed and processed mealworm extracts showed no differences in the IgE binding of shrimp allergic patients, because the major allergens, that is, tropomyosin and arginine kinase, were found to be stable after frying. The study conducted on grasshopper by Phiriyangkul and collaborators (2015) concluded that the IgE binding of shrimp allergic patients remains stable after frying for the Hexamerine, decreased for Arginine kinase and Enolase, and increased for Glyceraldehyde-3-phosphate dehydrogenase and Pyruvate kinase. Van Broekhoven and co-workers (2016) studied the effect of lyophilization, boiling and frying treatments and *in vitro* digestion of mealworm proteins on the IgE-reactivity of HDM and shrimp allergic patients. Among the proteins identified as being responsible for cross-reactivity, tropomyosin seemed to remain stable during *in vitro* digestion in both lyophilization and boiling processes while the cross-reactivity to tropomyosin seemed to decrease in fried samples and disappear in the fried samples after digestion. Pali-Schöll *et al*. (2019) found a decrease in IgE biding in HDM and crustacean allergic patients after the enzymatic and heating processes of mealworms, grasshoppers and crickets. The allerginicity of the 27-kDa Glycoprotein allergen increased in silkworm after boiling in silkworm allergic patients (Jeong *et al*., 2016).

1.3 The Effect of Pasteurization on the Nutritional Quality of Human Milk

1.3.1 Human milk composition

Human milk (HM) is an extremely complex and highly variable bio-fluid that has evolved over millennia to nourish infants and protect them from disease during the growth of their immune system. The composition of HM changes in response to many factors, for example, the lactation cycle throughout the day, the maternal diet and the length of gestation, and it matches the infant requirements (Andreas, Kampmann, & Mehring Le-Doare, 2015). HM contains adequate nutritional and immunological components that ensure the optimal development of infants (American Academy of Pediatrics, 2012). HM from healthy women is able to support, by itself, the growth of term infants, and as a consequence, is the reference to determine the nutritional requirements and dietary intakes of infants (American Academy of Pediatrics, 2012). In addition, an exclusive HM diet is associated with lower rates of necrotizing enterocolitis in premature infants than a diet with an infant formula (Sullivan *et al*., 2010).

HM is a natural oil-in-water emulsion composed of milk fat globules (MFG) dispersed in an aqueous phase where proteins are solubilized in the soluble fraction (whey proteins, ~70% of the total proteins), suspended in the colloidal phase (caseins, ~30%), or bound to MFG membrane (1–2 %) (Lönnerdal, 2003; Lönnerdal, Erdmann, Thakkar, Sauser, & Destaillats, 2017). HM is composed of a variety of proteins, which provide a well-balanced source of amino acids. Moreover, proteins improve the digestion and uptake of other HM nutrients, thereby contributing to immune functions, to gut development and exerting antimicrobial activity. Among the various whey proteins, the most abundant is α-lactalbumin (37-47% of the total whey proteins, Chatterton, Nguyen, Bering, & Sangild (2013)). During digestion, this protein releases important active peptides that are implicated in the binding and adsorption of iron, zinc and calcium. Lactoferrin constitutes 22-28% of the whey proteins (Chatterton, Nguyen, Bering, & Sangild 2013), it is involved in the iron uptake from breast milk and it is able to stimulate epithelial cell proliferation and differentiation (Demmelmair, Prell, Timby, & Lönnerdal, 2017). Immunoglobulins are abundant in HM (18-22% of the whey proteins, Chatterton, Nguyen, Bering, & Sangild (2013)). IgAs are the major immunoglobulin (90%) and they play an important role on the protection of the infant against pathogens (Lönnerdal, 2016). Serum albumin is one of the less representative protein (6-7%) of the whey proteins (Jenness , 1979). β-

casein constitutes more than 60% of the total casein present in HM, and it is followed by κ-casein and αs1-casein (Dereck E.W. Chatterton *et al*., 2013). β-casein is highly phosphorylated, and it releasing phosphorylated peptides during digestion, which enhance the solubility and bioavailability of calcium and zinc. K-casein is highly glycosylated and contributes to the stabilization of casein micelles and, with its glycosyl-residues, acts as a decoy for pathogens (Demmelmair *et al*., 2017).

HM contains less protein and a higher level of lactose than bovine milk. Bovine milk has a casein/whey protein ratio of 80:20, while this ratio is 30:70 in HM. Moreover, β-lactoglobulin, a major whey protein of bovine milk and αs2-casein are absent in HM (Dereck E.W. Chatterton *et al*., 2013). The lipid concentration (30–50 g/L) and the organization in the MFG of HM and bovine milk are similar (Jensen, Hagerty, & McMahon, 1978). Nevertheless, the fatty acids (FA) composition is very different. Bovine milk contains less long-chain polyunsaturated fatty acids and more short-chain fatty acids than HM (Conti, 2010).

More than 98% of HM fat is present as TAG, around 1% as phospholipids and 0.5% as sterol (mostly cholesterol). MFG presents a complex physicochemical structure. TAG are exclusively located in the core of MFG while the membrane is a double layer of polar lipids placed on an inner monolayer of polar lipids. The choline-containing phospholipids, phosphatidylcholine and sphingomyelin, and the glycolipids, cerebrosides and gangliosides, are mostly located on the outside of the membrane, while phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine are mainly concentrated on the inner surface of the membrane (Dewettinck *et al*., 2008). Phospholipids, polar lipids, cholesterol, proteins, glycoproteins and enzymes in particular compose the surrounding membrane (Lopez, Cauty, & Guyomarc'h, 2015; Lopez & Ménard, 2011). The main MFG membrane proteins are Mucin 1 (MUC 1), Xanthine oxidoreductase (XDH/XO), Butyrophilin (BTN), Lactadherin (PAS 6/7), Cluster of differentiation (CD 36), Proteose peptone 3 (PP3), Fatty acid-binding protein (FBP), Adipophilin (ADPH) and Periodic acid Schiff III (PAS III). MFG associated proteins seems to have potential beneficial effects on the immune system of newborn infants and on their cognitive functions (Fig 9) (Demmelmair *et al*., 2017).

Despite the important variations related to the maternal diet and nutritional status, the vitamins and minerals contents in HM from healthy mothers are generally considered as the normative standard for infant nutrition. HM contains some enzymes that favor digestion. Bile salt-stimulated lipase (BSSL) is a lipase that is able to liberate fatty acid during the digestive process but also to contribute to the pre-lipolysis phenomena that take place during HM storage (Bertino *et al*., 2013). BSSL is more likely to act in the intestinal phase due to the presence of bile salts and more favorable pH, but it could also act in

the stomach, as a result of being triggered by bile salts carried by active HM over a wide range of pH (4.5–8), corresponding to the gastric pH in newborns infants (Bourlieu *et al*., 2014; Bakala *et al*., 2012). This potential for contributing to fat digestion is believed to be important for newborn infants, and in particular for preterm ones, who present a limited coefficient of fat absorption due to the immaturity of their pancreatic and biliary systems, which results in a low production of pancreatic lipases and bile salts (Bourlieu *et al*., 2014).

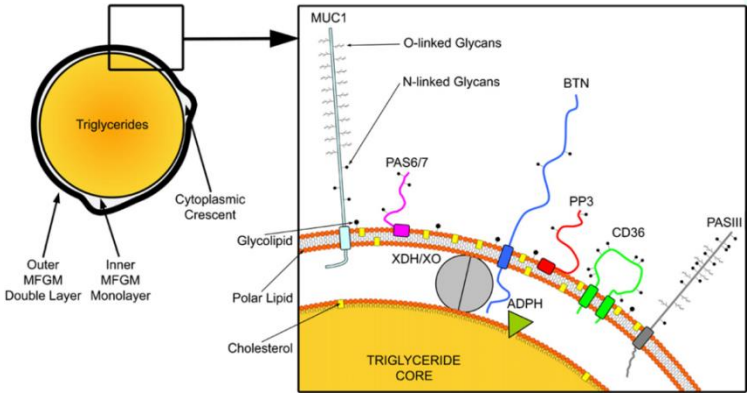

Fig 9. Structure of fat globule with detailed arrangement of the main MFG membrane proteins. ADPH is located in the inner polar lipid layer, while XDH/XO is located between both layers. MUC1, BTN, CD36 and PASIII are located in the outer layer. PAS6/7 and PP3 are only loosely attached on the outside of the MFG membrane (Dewettinck *et al*., 2008).

HM contains numerous growth factors that have wide-ranging effects on a newborn infant. The Epidermal growth factor (EGF) and Insulin-like growth factor (IGF) have an effect on intestinal maturation, while the Vascular endothelial growth factor (VEGF) and Erythropoietin (Epo) regulate the vascular system. Moreover, hormones (Calcitonin, Somatostatin and Adiponectin), Cytokines and Chemokines (IFN-γ, TNF-α, IL-1β and IL-10) have also been found in HM.

1.3.2 The collection and storage of human milk: Human Milk Banks

A human milk bank (HMB) is a facility that has been established to collect, screen and store donor HM after pasteurization in order to support the feeding of vulnerable hospitalized infants with severe allergies, feeding intolerances and/or gastrointestinal problems and for very low weight preterm newborn infants (Picaud, 2015). HM is provided by the infant's mother when the hospitalized infant has not yet developed the suck-swallow-respiration coordination and then

needs enteral nutrition through a nasogastric tube (Lau, 2015). In these cases, mothers collect their breast milk, which is then frozen and sent to the HMB for storage and processing. Moreover, anonymous donors can give their extra-HM to the HMB after careful screening for general health and infections, alcohol, medication or drug consumption (Picaud, 2015).

Two freeze-thaw cycles are applied during HM storage: the first for pasteurization and the second for administration. HM is stored at -20°C in the HMB. Thawing is usually carried for a few hours or overnight at 4 °C. The HM is then pooled and pasteurized. The pasteurized HM is then frozen and cannot be delivered before the results from bacteriological analyses have been confirmed. The thawing procedure is again carried before the milk is administered to the infants.

The most common practice to assure the microbiological quality of banked-HM is Holder pasteurization (HoP). This is a long-time and low-temperature method, which consists in heating pooled bottles of HM in a water bath at 62.5 °C for 30 min, and then rapidly cooling them (Picaud, 2015). However, the time taken to reach this temperature and to cool the milk is not standardized, as it varies according to several factors, such as the material of the bottles and the milk volume.

1.3.3 The impact of pasteurization on the macro- and micro- components contents of human milk

The HoP process has an effect on the immunological and nutritional components. IgAs are the most extensively investigated class of compounds and almost all the published studies have reported its reduction following HoP. The other Ig classes have been investigated in a smaller number of studies, but the results are partially contrasting due to the extremely low Ig concentrations, and the subsequent difficult detectability in HM. However, the majority of the studies have found some degree of reduction (Peila *et al*., 2016). Vitamin C is the highest heat sensitivity water-soluble vitamin while the lipophilic vitamins, together with irons and saccharides, seem to resist to the thermal treatment. Cytokines and growth factors have shown different degrees of thermal resistance, and large variations have been observed over the studies; this may be a result of the different times/temperatures to which HM was exposed, as well as of the different analysis methods used for the compound quantification (O'Connor, Ewaschuk, & Unger, 2015; Meier, Patel, & Esquerra-Zwiers, 2017). A complete degradation (in both concentration and enzymatic activity) has been found for both lipoprotein lipase and BSSL (Peila *et al*., 2016).

α-lactalbumin and serum albumin concentrations are not significantly altered either by storage at -20°C or pasteurization (Akinbi

et al., 2010) although a strong reduction in lysozyme and lactoferrin has been reported (O'Connor *et al*., 2015; Peila *et al*., 2016; Meier *et al*., 2017; Moro *et al*., 2019). The apparent decrease in lactoferrin concentrations is due to heat denaturation and the formation of carbonyls, with a subsequent formation of disulfide-bonded high molecular aggregates, which could lead to a underestimation of the protein content (Baro *et al*., 2011). The total fatty acid profile has always been found to be unaffected by pasteurization, while a large variation of the lipid concentration between raw and HoP milk has been observed across studies (Table 2) (Peila *et al*., 2016).

Heat treatments, together with freeze–thaw cycles, can induce the disruption of the MFG membranes and protein denaturation, and HoP induces an aggregation of proteins in the soluble phase and around the human MFG membrane (de Oliveira, Bourlieu, *et al*., 2016; de Oliveira, Deglaire *et al*., 2016).

Some alternatives to Holder pasteurization have been proposed to ensure the elimination of pathogens while preserving the bioactive components of HM as much as possible. A promising alternative seems to be HTST pasteurization (72°C for 15 s.). HTST has been found to be at least equivalent to HoP in ensuring HM microbiological safety (Goldblum *et al*., 1984; Dhar, Fichtali, Skura, Nakai, & Davidson, 1996; Hamprecht *et al*., 2004; Escuder-Vieco, Espinosa-Martos, Rodríguez, Fernández, & Pallás-Alonso, 2018). However, HTST is better at preserving the HM antioxidant potential, lactoferrin and vitamin (B and C) contents and structures and the cytokine activities (Baro *et al*., 2011; Mayayo *et al*., 2016; Peila *et al*., 2017; Donalisio *et al*., 2018) (Table 3). Discordant results have also been reported for HTST. Almost all of the analysis were performed by simulating HTST pasteurization using a water bath, which is very different from the methods commonly used in HMB practice (Peila *et al*., 2017).

Component	Maintained (>90%)	Maintained (50–90%)	Maintained (10–50%)	Abolished (<10%)
Macronutrients	Carbohydrate (Lactose, Oligosaccharides)	Protein Total fat		
Micronutrients	Calcium Copper Magnesium Phosphorus Potassium Sodium Zinc	Iron		
Vitamins	Vitamin A	Folate Vitamin B6 Vitamin C		
Biologically active (immune)	IL-8, IL-12p70, IL-13 TGF-α	IgA, sIgA IgG IGF-1, IGF-2 IGF-BP2,3 IFN-γ IL-1β, IL-4, IL-5, IL-10 TGF-β Gangliosides	CD14 (soluble) Il-2 Lactoferrin-iron binding capacity Lysozyme	IgM Lymphocytes
Biologically active (metabolism)	Epidermal growth factor Heparin-binding growth factor	Adiponectin Amylase Insulin	Erythropoeitin Hepatocyte growth factor	Bile salt-dependent lipase Lipoprotein lipase

Table 2. Effects of HoP HM components (O'Connor *et al*., 2015).

Components	Preserved (>90%)	Partly retained (50%–90%)	Strongly decreased (10%–50%)	Destroyed (<10%)
Immunological components	IgA	IgA, IgG	IgA	IgM
	Lactoferrin Secretory IgA Lysozyme	IgM Secretory IgA Lysozyme	Lactoferrin	
Macronutrients	Protein			
Micronutrients	Available lysine Folic acid, vitamins B, vitamin C	Available lysine		
Enzymes	Bile salt stimulated lipase Total antioxidant capacity		Glutathione peroxidase	Bile salt stimulated lipase Alkaline phosphatase Lipase
Others	Malondialdehyde Cytokines	Glutathione Bactericidal capacity		

Table 3. Effects of HTST on HM components (Peila *et al*., 2016).

1.3.4 Studying the impact of pasteurization on preterm newborn infants digestion through *in vitro* models

This section presents the key parameters of the digestion in preterm newborn infants, the infant fisiological state that was studied in the experiment reported in Chapter 6. The section is focused mainly on the gastroduodenal digestion of lipids and proteins. In addition, digestion models for studying infant digestion and the effect of milk pasteurization on the preterm newborn health are presented.

1.3.4.1 Gastrointestinal digestion in preterm newborn infants

The digestion process comprises three principal steps: oral, gastric and intestinal phases. The digestive conditions depend on the ingested food and on the physiological stage of the subject (infant, adult, elderly). There are various differences between infants and adults, mainly concerning some digestive enzyme activities and concentrations, gastrointestinal mobility and the value of the gastric pH. The digestion process of infants neglects oral phase as liquid meals transit rapidly through the oral cavity (5-10 s). The gastric pH of infants is less acidic than that of adults (4-5 vs. 2), which may change gastric proteolysis, where the optimal activity of pepsin takes place at a pH of 1.5-2.2. The reduced pepsin secretion in newborn infants (10-20% of adult levels) is another physiological reason that can help explain the limited gastric proteolysis (15%) that has been reported for infants (Shani-Levi *et al*., 2017). The fat absorption coefficient ranges from 74 to 91% in preterm infants after ingestion of HM, 68 to 85% in preterm infants and around 90% in full-term infants after ingestion of infant formula, to >95% in healthy adults (Lindquist & Hernell, 2010). This limited coefficient of fat absorption in infants is likely to be due to the maturation of the digestive system, of the digestive function (i.e. the enzyme and bile salt levels), of the absorption capacity, or a combination of all (Fig 10) (Abrahamse *et al*., 2012). Moreover, preterm infants have a less mature digestive system than older infants (> 6 months), which leads them to have an underdeveloped digestive function (Bourlieu *et al*., 2014).

The stomach is the first main digestive compartment in an infant and it is a key step for the digestion of newborn infants. This compartment stores, mixes, initiates the hydrolysis and regulates the emptying of milk bolus from the stomach into the small intestine. The gastric juice, secreted by the epithelial cells of the gastric mucosa, mainly contains enzymes (pepsinogen and gastric lipase), HCL and mucins. The output and the composition of gastric fluids are influenced by hormones (cholecystokinin, gastrin, secretin) and by other factors (acidity, mixing, gastric emptying rate) (Gan *et al*., 2018).

In the fasting state, gastric secretions and residuals of the previous meal may be present in the stomach (Bourlieu *et al*., 2014).

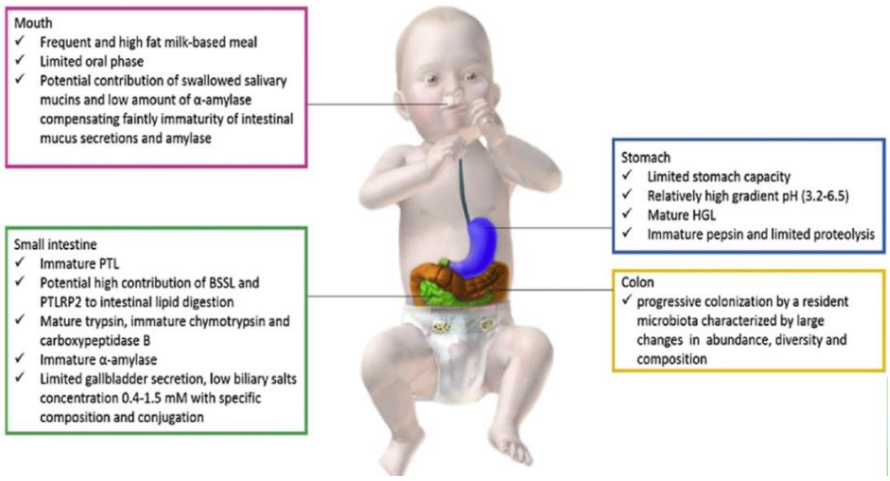

Fig 10. Summary of the developing digestive physiology in human infants. HGL-Human Gastric Lipase, PTL- pancreatic triglyceride lipase, BSSL-bile salt-stimulated lipase, PTLRP2- pancreatic triglyceride lipase-related protein 2 (Shani-Levi *et al*., 2017).

Gastric emptying depends on the composition and structure of the milk, as well as the gestational and postnatal age of the infant. Moreover, hormonal feedback govern the contractions of the antrum and pylorus, and therefore the gastric emptying. The gastric emptying pattern has been reported to be exponential and accurately fitted using the Elashoff model (Elashoff, Reedy, & Meyer, 1982):

$$f = 2^{-(t/t_{1/2})^{\beta}}$$

with: the **f**-fraction remaining in the stomach at a given time; **t, $t_{1/2}$**-the time from the start of the meal until 50% of the meal has been emptied (gastric half-emptying time), **β**-the coefficient that determines the shape of the curve and the intensity or lack of an initial lag time.

There are two main reasons why the gastric pH decreases during the gastric digestion of newborn infants and does not reach adult levels: the immaturity of the acid secretion and the high buffer capacity of milk-based meals. Thus, the acidification capacity of a newborn infant does not seem to depend on the type of meal (Bourlieu *et al*., 2014). A gastric pH decrease seems to follow a polynomial regression, which may be calculated using the average pH values estimated *in vitro* and *in vivo* in published studies (Fig 11) (de Oliverira, 2016b).

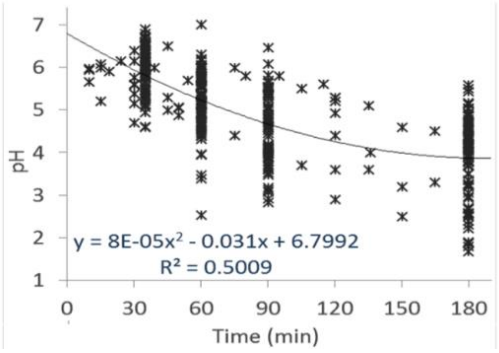

Fig 11. Gastric pH decrease as reported in infants fed every three hours in previous *in vivo* and *in vitro* digestion simulation studies. The proposed fitting for gastric acidification by means of polynomial curves is as reported by de Oliveira (2016b).

Meal and gastric secretions progressively arrive in the small intestine, where nutrients are liberated and absorbed. During the neonatal period, the intestinal phase is marked by the immaturity of the digestive functions of the infant, with lower protein and lipid hydrolysis in relation to the intestinal phase of adults (Shani-Levi 2017). Some accessory organs are crucial for the digestive process, i.e. the pancreas (production of pancreatic secretion containing lipases, proteases and amylase), the liver (production of bile, the metabolism of lipids and proteins, the storage of glycogen and vitamins) and the gallbladder (the storage and release of bile toward the duodenum). Bicarbonate is released from the pancreas into the duodenum to neutralize the acidic chyme from the stomach. The duodenal pH of healthy young and elderly adults is typically between 6 and 7, but it can be temporarily reduced to 5.4 after a meal (Gan *et al*., 2018). The last step takes place in the large intestine where water and electrolytes are absorbed.

Lipolysis in HM starts from the endogenous enzymes. BSSL can hydrolyze any of the TAG position with the liberation of free fatty acids, monoacylglycerol (MAG) and diacylglycerol (DAG) (Lindquist & Hernell, 2010).

In the gastric phase, human gastric lipase (HGL) is mature both full-term and preterm newborns, hydrolyzing between 10 and 30% of the TAG. HGL shows regioselectivity to the external position of the TAG, and a great liberation of oleic, linoleic, stearic and lauric acids have in fact been reported because these fatty acids are mainly positioned in the sn-1,3 position of the glycerol backbone in HM (Fig 12) (Bourlieu & Michalski, 2015).

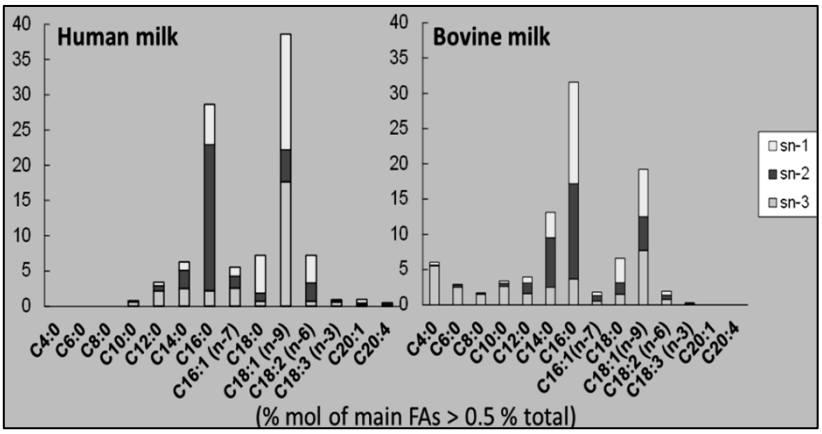

Fig 12. Comparative fatty acid composition and regiodistribution in the TAG of human and bovine milks (Bourlieu & Michalski, 2015).

Lipases secreted by the exocrine pancreas, together with bile salts, complete the digestion of HM fat in the duodenum. Bile salts play a crucial role in the emulsification of chyme, the solubilization of lipolysis products into an absorbable form and in triggering the action of the various intestinal lipases. Pancreatic lipase is the major intestinal lipase; it mainly hydrolyzes TAG in the sn-1,3 positions, leads to the release of sn-2 MAG and free fatty acids, and it needs a colipase to be active in the presence of bile salts. Palmitic acid is more than 70% esterified in the sn-2 position of the TAG (Conti, 2010). Lipase does not hydrolyze the sn-2 TAG position in order to minimize palmitic acid cleavage from the glycerol backbone. This phenomenon protects against the formation of soaps in the intestinal lumen, which may result from free palmitic acid binding with calcium and other minerals (Robinson & Martin, 2017). Pancreatic phospholipase A2 (PLA2) hydrolyzes phospholipids and is stereospecific for the sn-2 position, while the pancreatic lipase related-proteins 1 and 2 (PLPR1 and 2) seem to complement the action of pancreatic lipase, but their functions on infant digestion are not well known (Berton, Sebban-Kreuzer, Rouvellac, Lopez, & Crenon, 2009). Free fatty acids cross the intestinal barrier where they are re-esterificated into TAG and incorporated in chylomicrons in the enterocyte. Chylomicrons are released from the enterocyte, through lymphatic vessels, into circulation.

Proteolysis in the stomach is catalyzed by pepsin. Pepsinogen is secreted by the gastric chief cells and activated into pepsin via autocatalytic cleavage of the N-terminal peptide when it comes in contact with acid. Like aspartic protease, pepsin is more active in acidic environments of around pH 2 and inactivated at a pH above 4.5 (D.E.W Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004). A

lower pepsin secretion coupled with a higher gastric pH indicates less protein hydrolysis by pepsin in an infant's stomach (Gan *et al*., 2018).

In the intestinal phase, pancreatic proteases are secreted as inactive zymogens, which are activated by trypsin. Trypsin is activated from trypsinogen by intestinal enterokinase, and is secreted from the intestinal epithelial cells in response to food stimulation (Bourlieu *et al*., 2014). Trypsin hydrolyzes peptides at the basic amino acid site (lysine and arginine), but other enzymes are also involved in protein digestion. Chymotrypsin hydrolyses peptides with aromatic amino acids (phenylalanine, tyrosine, tryptophan) and elastase splits the protein backbone when uncharged small amino acids (such as alanine, glycine, and serine) are encountered. Carboxypeptidase-A attacks the last amino acid of a target peptide chain, whether it is aromatic, neutral, or acidic amino acid, while carboxypeptidase-B attacks basic amino acids (Whitcomb & Lowe, 2007). Other intestinal peptidases (e.g. γ-glutamyltranspeptidase, oligoaminopeptidase, dipeptidylaminopeptidase IV) help to complete the hydrolysis of peptides into free amino acids. The peptides and free amino acids generated by the digestion are absorbed by carrier-mediated mechanisms across the basolateral membrane in the portal blood system, by the intracellular vesicle mediated transport system (transcytosis) or by paracellular passive diffusion (Wada & Lönnerdal, 2014).

Some proteins, such as lactoferrin and IgA, are detected in intact form in infant stools and result to be resistant to proteolytic degradation. Global impaired HM proteolysis is often considered beneficial, since some intact proteins and peptides exert several bioactive functions directly in the gastrointestinal lumen or at peripheral organs, after being absorbed from the intestinal mucosa (Lönnerdal, 2016). Likewise, the fast protein hydrolysis that occurs after pasteurization, for instance (Deglaire *et al*., 2016, 2019) might not be a negative factor, as different bioactive peptides are released from the lactoferrin of HM and they play important antimicrobial roles in the gastrointestinal tract of babies (Wada & Lönnerdal, 2014).

Emulsion disintegration starts in the infant's stomach, where the emulsion milk is exposed to a decrease in pH and to mechanical agitation, with several structural changes, such as creaming, flocculation and coalescence of the lipid droplets (physical processes), modification of the interfacial layers and hydrolysis (chemical processes), with aggregation and destabilization of the emulsion (Gallier, Acton, Garg, & Singh, 2017). The products of gastric hydrolysis (e.g. peptides and free fatty acids) and other endogenous products (mucins, enzymes) are surface active and influence droplet stability (Golding *et al*., 2011). Once it has arrived in the duodenum, bicarbonate, the product of gastric hydrolysis, together with pancreatic secretions, bile salts and phospholipids will participate in

lipid emulsification. Furthermore, the mixed micelles formed by bile salts and phospholipids are crucial for the solubilization and absorption of the digestion products as well as many dietary hydrophobic molecules, such as vitamins, cholesterol and sterols (Golding & Wooster, 2010).

Milk emulsion droplets may vary from one meal to another. For instance, *in vitro* studies have demonstrated a different particle size distribution in human milk after pasteurization. In the pasteurized milk were found an increase of the MFG size with a different interface, type and organization of proteins compared to raw milk (Bourlieu & Michalski, 2015; de Oliveira, Bourlieu, *et al*., 2016; de Oliveira, Deglaire *et al*., 2016).

1.3.4.2 *In vitro* models

Due to ethical and practical reasons, few *in vivo* studies dealing with infant digestion are available in the literature. Usually, they concern infants frequently fed through a nasogastric tube, which can be used for the collection of gastric contents by aspiration. Studies concerning the intestinal phase of the digestion are rarer due to the access difficulty and necessity of invasive approaches (Bourlieu *et al.*, 2014). To overcome these problems, the use of animals can be an alternative. The most frequently animals as a model of human digestion used in literature for digestion studies are rats and pigs for adult and piglets for infants. Digestion of nutrients can be studied by evaluating their kinetics of hydrolysis, studied by evaluating their bioavailability (proportion of digested and absorbed nutrients reaching the bloodstream) by taking blood samples at specific times before and after food consumption (Bornhorst *et al.,* 2016). This measure is indirect, as the non-digested quantity of nutrients is measured. *In vivo* studies usually provide the most accurate results, but they are time consuming, costly and difficult to implement in terms of ethical and technical limitations. Moreover, due to high inter-individual variability large sample is needed (Bornhorst *et al.,* 2016). In order to face this difficulty, much effort has been devoted to the development of pertinent *in vitro* models.

In comparison to *in vivo* models, *in vitro* digestion models are cheaper, quicker and have better repeatability, reproducibility and a limitation of ethical constraints (Ménard *et al*., 2014). The anatomical and physiological parameters that determine these conditions depend on the age, meal, feed conditions and other specific characteristics of the target that has to be simulated (e.g. preterm versus full-term newborn infants) (Bourlieu *et al*., 2014). The parameters used in *in vitro* digestion models differ among studies, which makes a comparison of the data difficult. To face this challenge, the INFOGEST network was created to bring together the scientific community in the field of *in vitro* digestion. This group has recently developed a

consensus protocol to mimic digestion at the adult stage with a static *in vitro* model (Brodkorb *et al*., 2019; Minekus *et al*., 2014). Although they suffer from various limitations, *in vitro* models are useful for studying the food kinetics of digestive hydrolysis and structural changes under simulated gastrointestinal digestion. *In vitro* models cannot mimic the hormonal feed-back mechanisms or the neural interactions between organs, and the study of absorption by *in vitro* models is still limited (Shani-Levi 2017). Moreover, most of *in vitro* models do not take into account the role of the gut microbiota despite it significantly contributes toward the nutrients digestion. Due to the bacterial diversity within individuals, influenced by the environment, genetics, diet, antibiotic use, and geographical location, the gut microbiome composition is still difficult to standardize in the *in vitro* models (Pearce *et al.*, 2018).

Since static and semi-dynamic models overly simplify their representation of the digestive tract, their direct applicability to digestive processes is limited. Gastrointestinal dynamic models have recently incorporated a more realistic representation of the digestive tract, since they simulate the dynamic changes in pH, the enzymatic output and activity, the emptying rate and the flux between the gastric and intestinal phases. These dynamic models allow samples to be collected from different compartments during the digestion process, and provide data in terms of the kinetics of disintegration and hydrolysis. The Dynamic Gastric Model (DGM) and the Human Gastric Simulator (HGS) are systems developed for simulating only the gastric compartment while the artificial colon (ARCOL) simulates only the intestinal tract. ARCOL can mimic the intestinal passive absorption of microbial products using dialysis fibers. In a similar way, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) mimics the host-microbe interactions, the microbial metabolites and the gut barrier permeability by using human intestinal cells (Dupont *et al.,* 2019). The most popular gastrointestinal dynamic model is the TM-1 (TNO gastrointestinal model 1), which includes stomach and the three parts of the small intestine (duodenum, jejunum, ileum). This model takes into account parameters such as emptying rates, peristalsis movements and nutrient absorption in the intestine by a dialysis system (Dupont *et al.,* 2019). A simplified gastrointestinal dynamic system has been developed at INRA, France. DIDGI® contains two successive compartments that simulate the stomach and the small intestine, and it is controlled by STORM® software (Fig 13). It simulates the flows of ingested food through compartments, the digestive secretions, the gastric pH decrease, the emptying rates and the temperature (Ménard *et al*., 2014). This model was validated by simulating infant digestion in piglets. However, this model does not mimic absorption and peristaltic movements.

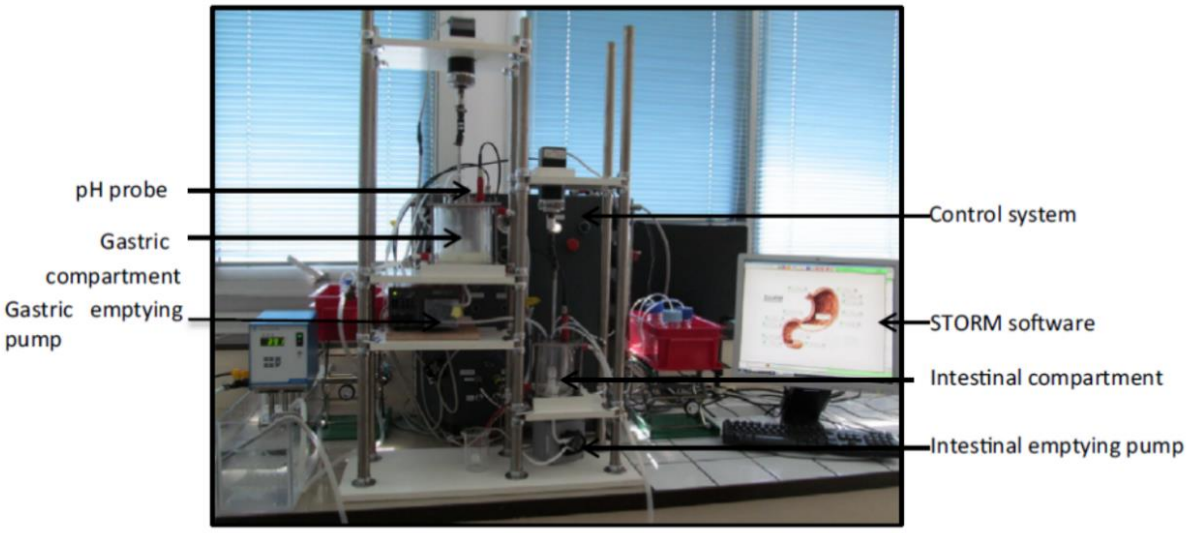

Fig 13. The DIDGI® gastrointestinal dynamic *in vitro* model (Ménard *et al*., 2014).

1.3.4.3 Impact of pasteurization on human milk digestion

Only a few *in vivo* and *in vitro* studies have investigated the effect of pasteurization on HM digestion. All the available evidence has been obtained by comparing raw and Holder pasteurized HM. Williamson and co-workers (1978) studied seven preterm infants fed raw, pasteurized and boiled HM for 3 consecutive weeks. No differences in the absorption of N, Ca, P, or Na were observed for the three types of milk. All the infants gained weight rapidly during the weeks in which they were fed raw HM, and fat absorption was reduced when the infants were fed treated HM. The authors suggested that the improved fat absorption from the raw HM could have been related to the preservation of HM endogenous lipases (i.e. BSSL). Similarly, Andersson *et al*. (2007) studied five preterm infants fed raw or pasteurized HM in a cross-over trial. The authors observed a higher growth rate and higher fat absorption coefficient (17% higher) when the infants received raw HM.

Dicky and collaborators (2017) have recently studied the effects of pasteurization in a wider cohort of preterm infants (n=926) who were randomized into two groups: the first group (n=636) was fed raw HM and the second (n=290) pasteurized HM. The authors did not find any differences in growth of body weight between the two groups, but they found a significant and decreased risk of bronchopulmonary dysplasia in infants who received their own mothers' raw HM. Moreover, Cossey *et al*. (2017) found no differences in the growth of the body weight or in the episodes of feeding intolerance between

preterm infants fed raw (n=151) or pasteurized (n=152) HM. The authors found an increased rate of late-onset sepsis associated with the use of pasteurized HM, that could be explained by the effect of the heat treatment on the immunological quality of HM, in terms of partial destruction of the immune-components, non-pathogenic bacteria present in the mothers' milk and, thus, partial loss of HM bactericidal activity. No effect of pasteurization on necrotizing enterocolitis or retinopathy was found in the meta-analysis of Miller and co-orkers (2019) who examined the effect of HM on preterm infant morbidity.

The results from dynamic *in vitro* digestion showed no impact of HM pasteurization on gastric lipolysis in preterm newborn infants, and BSSL inactivation by pasteurization did not seem to affect gastric lipolysis in preterm infants. As far as emulsion disintegration during gastric digestion is concerned, statistical differences were found *in vitro*, with a marked emulsion destabilization, albeit only for raw HM (de Oliveira, Bourlieu, *et al*., 2016). The *in vitro* results were confirmed, by the same authors, in a randomized controlled trial on 12 preterm newborn infants fed raw and pasteurized HM (de Oliveira *et al*., 2017). However, pasteurization reduced the gastric lipolysis of full-term HM *in vitro* (de Oliveira, Deglaire *et al*., 2016). Intestinal lipolysis was found to be enhanced in raw HM, compared to pasteurized HM, for both full-term and preterm HM (de Oliveira, Bourlieu *et al*., 2016; de Oliveira, Deglaire *et al*., 2016).

As far as proteolysis is concerned, selective protein digestion has been observed for both raw and pasteurized HM, in particular with a great resistance of α-lactalbumin during raw HM digestion and fast Lactoferrin proteolysis in pasteurized HM. *In vitro* intestinal digestion evidenced that the bioaccessibility of AA was selectively modulated by pasteurization (de Oliveira, Bourlieu, *et al*., 2016; de Oliveira, Deglaire, *et al*., 2016). Moreover, the impact of pasteurization has been investigated in terms of peptide release. Following *in vitro* dynamic digestion, pasteurization impacted both the gastric and intestinal kinetics of peptide release in full-term newborn infants (Deglaire *et al*., 2016). No effect on the formation of bioactive peptides was found between the two milks when an *in vitro* system was used (Wada & Lönnerdal, 2015). However, in the latter study, the HM samples were obtained from just one volunteer and were exclusively digested with proteolytic enzymes. No impact of pasteurization was found on peptide release during the *in vitro* dynamic digestion of preterm newborn infants. Only some bioactive peptides from β-casein presented significant and different abundances between raw and pasteurized HM during digestion (Deglaire *et al*., 2019).

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CHAPTER 2

EFFECT OF ROASTING ON HAZELNUT OLEOSINS ALLERGENICITY

Aim of the Work

Since few data are available about the contribution of oleosins to hazelnut allergy in children, we recruited 26 well-characterized Italian pediatric patients with signs of convincing hazelnut allergy or sensitized, but tolerant, to hazelnuts and screened for raw and roasted hazelnut oleosins immune-recognition. A comprehensive proteomic analysis was conducted to investigate the contribution of oleosins to hazelnut allergy and to assess whether it is affected by roasting.

Abstract

Raw and roasted hazelnut oil body associated proteins were analyzed by means of 1-2D electrophoresis and mass spectrometry. Oleosin Ig-E reactivity was assessed by immunoblotting with the sera from 17 children with confirmed hazelnut allergy and from 9 tolerant subjects. A molecular characterization of hazelnut oleosins was performed by interrogating *C. avellana cv.* Jefferson and *cv.* TGL genomes.

An in-depth proteomic and genomic investigation allowed identifying two new oleosins, in addition to the already reported Cor a 12 and Cor a 13, in hazelnut oil bodies. One of the new oleosins was registered as a new allergen according to the WHO/IUIS Allergen Nomenclature Subcommittee criteria and termed Cor a 15. Cor a 15 was the most frequently recognized oleosin in our cohort of patients. Hazelnut roasting increased the oleosins immunoreactivity and led to 100% recognition of Cor a 15 by allergic patients.

The novel oleosin Cor a 15 is clinically relevant for children. The high prevalence of hazelnut oleosin sensitization here reported further confirms the need to include oleosins in routine diagnostic tests.

2.1 Methods

2.1.1 Study population

Pediatric subjects with signs of convincing hazelnut allergy (HA patients, no. =17) and pediatric subjects sensitized but tolerant to hazelnuts (HS patients, no. =9) were retrospectively recruited from the Paediatric Allergy Unit of the Regina Margherita Children's Hospital (Città della Salute e della Scienza, Turin, Italy). Hazelnut allergy was defined as a convincing history of allergic symptoms (oral allergy syndrome (OAS), urticaria, angioedema, respiratory, cardiovascular, and/or gastrointestinal symptoms) appearing within 1 h after hazelnut ingestion, confirmed by an oral food challenge (OFC) (except for severe anaphylaxis), as well as a positive Prick-by-Prick (PbP) result to raw and roasted hazelnuts and/or a hazelnut sIgE level of ≥0.10 kU/L in serum. The HS patients were characterized by atopic dermatitis (AD) and/or other food allergy (FA) and/or other nut allergies (NA), with a positive PbP to hazelnut and/or positive sIgE to hazelnut, but negative to OFC. Five sera of non-nut sensitized non-allergic hazelnut pediatric consumers were pooled and used as a control.

All the HA patients reported a reaction to foods containing roasted hazelnuts (cocoa and hazelnut spreads and chocolates or hazelnut ice cream, chocolate pralines with roasted whole hazelnuts or hazelnut nougat). For this reason, the OFC was performed with roasted hazelnuts. PbP was instead performed using both raw and roasted hazelnuts. Histamine (10 mg/ml) was used as a positive control, and a saline solution was considered as a negative control. PbP was considered positive if the wheal was higher than 3 mm without any reaction to the negative control. The serum IgEs were assayed toward hazelnut extract, rCor a 1, rCor a 8, rCor a 9 and rCor a 14 and rBet v 1 using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). The cut-off for positivity was set at 0.10 KU/l. A subgroup of the HA patients (patients nos. 15, 16 and 17), who were characterized by low rCor a 1, rCor a 8, rCor a 9 and rCor a 14 RAST IgE values, ranging from 0.10 kU/L to 1.78 kU/L, were defined as Low Rast Hazelnut Allergic (LRHA) patients. The study was approved by the local ethics committee of the Città della Salute e della Scienza (Turin, Italy) (approval no. 312 prot. n. 22050). The parents of all the patients gave written informed consent.

2.1.2 Statistical analysis

rCor a 1, rCor a 8, rCor a 9 and rCor a 14 sIgE concentrations were standardized by subtracting the mean values and dividing by the standard deviations. The sIgE values below the limit of detection were

assigned 0.01; the sIgE values above the limit of detection were assigned 100. The standardized data were analyzed by means of nonparametric Kruskal-Wallis one-way ANOVA, because of their non-normal distribution. The correlation between the standardized sIgE concentrations of the different allergens were calculated by means of Kendall's tau. All the analyses were performed using PAST software, version 2.17 (Hammer, Harper, & Ryan, 2001).

2.1.3 Hazelnut water-soluble and OB-associated proteins extraction

Raw hazelnuts (*Corylus avellana cv.* Tonda Gentile delle Langhe, TGL) were obtained from experimental fields and roasted at a lab scale (120°C for 30 min). The same raw and roasted hazelnuts were used for both the PbP and immunoblotting experiments. The water-soluble proteins were extracted according to Platteau *et al*. (2010).

The isolation of the oil body (OB) fraction was performed according to Cao *et al.* (2015), with some modifications. Two g of chopped hazelnuts were sonicated (40 MHz for 2 min) in 12 mL of a grinding medium 1 (GM 1: 0.6 M sucrose, 10 mM sodium phosphate, pH 9.5) and were filtered using two layers of gauze. The filtrated samples were centrifuged at 21460xg for 20 min at 4°C and the OB pads were collected and re-suspended in 10 mL of GM 2 (GM 1 with the addition of 0.1% Tween 20). After sonication (40 MHz for 1 min), the samples were centrifuged (21460xg for 20 min at 4°C) and the OB pads were gathered and re-suspended in 8 mL of GM 3 (GM 2 with the addition of 2M NaCl), sonicated again (40 MHz for 1 min) and centrifuged (21460xg for 20 min at 4°C). The OB pads were collected and sonicated (40 MHz for 1 min) in 6 mL of Urea 9 M, pH 11. The samples were shacken for 10 min at RT and were then centrifuged (21460xg for 20 min at 4°C). The OBs were collected and solubilized in GM 1 at a final concentration of 100 mg/ml. OB-associated proteins were extracted adding 500 µL of diethyl ether to 200 µL of OB fraction, centrifuging at 21460 x g for 5 min at 4°C. After supernatant removal, the washing step was repeated for three times, then the proteins were precipitated according to Wessel and Flügge procedure (Wessel & Flügge, 1984) and quantified by Bradford protein assay (Bio-Rad).

2.1.4 LDS-PAGE and 2-Dimensional Electrophoresis (2DE)

Both the hazelnut water-soluble and the OB-associated proteins were separated by means of LDS-PAGE, only the OB-associated proteins were further separated by means of 2DE.

LDS-PAGE: 5 µg of protein sample were diluted in LDS Sample Buffer (Invtrogen, Life Technologies Ltd., Paisley, UK) under reducing (with 2%

of a NuPAGE Sample Reducing Agent) and non-reducing conditions and separated with 12% NuPAGE mini gels with an MES Running Buffer (Invitrogen), according to the manufacturer's protocol. The gels were then fixed for 2 hours in 30% ethanol and 10% ortophosphoric acid, were stained in Colloidal Coomassie Blue (Candiano *et al.*, 2004), and scanned using a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

2DE: OB-associated protein extract (40 µg) was diluted in 125µL of rehydration buffer (7M urea, 2M tiourea, 1% ASB-14, 10% glycerol, 20 mM Tris, 2.5 % DTT, 0.5% IPG buffer pH 6-11 and pH 3-11NL (1:2)) (GE Healthcare, Chicago, Illinois)) and was then loaded on immobilised pH gradient strips (7 cm, 3-11NL) (GE Healthcare). The strips were passively rehydrated for 16 h at 20°C and isoelectrofocusing was performed on EttanIPGphore (GE Healthcare), using the following focalisation protocol: 200 V for 1 hour, 400 V for 1 hour, 2000 V for 1 hour and finally up to 5000 V to reach 30000 Vhrs. The focused strips were incubated for 15 min at RT in the reduction buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris–HCl, pH 8.6, 2% w/v DTT) and then for another 15 min in the alkylation buffer (6M urea, 30% v/v glycerol, 2% w/v SDS, 50mM Tris–HCl, pH 8.6, 4.5% w/v iodoacetamide (IAA)). The thus equilibrated strips were then embedded at the top of precast homogeneous gels (NuPAGE 12% Bis–Tris, Invitrogen) and the electrophoretic separation was performed in XCell SureLock Mini-Cell System (Invitrogen) at RT, 200 V constant, 125 mA, 100 W for 45 min. The gels were stained with Colloidal Coomassie Blue and scanned using a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

2.1.5 Immunoblotting

After LDS-PAGE protein bands were electro-transferred into Nitrocellulose Membrane (0.2 µm) with XCell II Blot Module (Transfer buffer (Invitrogen) with 10% methanol (v/v)). The membranes were blocked with TBS containing 0.3% Tween 20 for 30 min and incubated o.n. at 4°C with patient sera diluted 1:5 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin).

The hazelnut water-soluble 1DE membranes were incubated with the pooled HA sera (patients nos. 1 to 14) and LRHA (nos. 15 to 17) separately. The OB-associated protein 1DE membranes were incubated (o.n. at 4°C) with the serum of individual patients, while the OB-associated protein 2DE membranes were incubated with the pooled LRHA patient sera. After incubation, the membranes were washed three times with washing solution (TBS, 0.05% Tween 20) for 10 min, and incubated for 1 hour at RT with anti-Human IgE antibody (Sera Care Life Sciences Inc., Milford, Massachusetts) dilute 1:5000 in the

incubation buffer. Membranes were washed three times and developed with Alkaline Phosphatase Substrate Kit (Bio-Rad).

2.1.6 Mass spectrometry analysis: MALDI-TOF/TOF and ESI-Q-TOF

In gel digestion: Spots were cut from the 2DE gel, destained overnight (O.N.) in 40% ethanol/50mM NH_4HCO_3. Bands from 1DE gel were reduced in 10mM DTT/50 mM NH_4HCO_3, for 45 minutes at 56°C, and alkylated in 55mM IAA/50 mM NH_4HCO_3, for 30 minutes in the dark, at RT. The spots and bands were washed with water, and destained with 50%ACN/50 mM NH_4HCO_3 (twice), pure ACN and again with 50%ACN/50 mM NH_4HCO_3. Samples were dried in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) and digested o.n. at 37°C under shaking with modified porcin trypsin (Promega, Madison, Wisconsin) at 75 ng/µl of 25 mM NH_4HCO_3/10% formic acid.
MALDI-TOF/TOF: 0.5 µl of peptide mixture was applied to the target with 0.5 µl of the matrix solution (alpha-cyano-4-hydroxycinnamic acid in 30% acetonitrile, 0.1% TFA) and dried under vacuum. Spectra were obtained by means of Ultraflex II MALDI-TOF/TOF (Bruker, Bremen, Germany), as already described by Zava *et al*. (2009). The tandem mass spectra were acquired running LID experiments using LIFT TOF/TOF acquisition. Manual/visual evaluations were made of the fullscans and the MS/MS spectra were analyzed using Flex Analysis software (Bruker). The MALDI-TOF spectra were searched with MS-Fit software package (http://prospector.ucsf.edu), while the MALDI TOF/TOF LID spectra were searched with MS-Tag software (http://prospector.ucsf.edu/) in homology mode.
ESI-Q-TOF: The peptide mixtures were desalted on a Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO) prior to mass spectrometry (MS) analysis. The LC-MS/MS analyses were performed by means of a micro-LC system by Eksigent Technologies (Dublin, California, USA), which included a micro LC200 Eksigent pump with a 5-50 µL flow module and a programmable autosampler CTC PAL with a Peltier unit (1.0-45.0°C). The stationary phase was a Halo Fused C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in ACN (B), and it was eluted at a flow-rate of 15.0 µL/min and at an increasing concentration of solvent B, that is, from 2% to 40%, over a period of 30 minutes. The injection volume was 4.0 µL. The oven temperature was set at 40 °C. The LC system was interfaced with a 5600+ Triple TOF TM system (AB Sciex, Concord, Canada) equipped with a Duo Spray TM Ion Source and a CDS (Calibrant Delivery System). The mass spectrometer worked in data dependent acquisition mode (DDA) (Cvijetic *et al*., 2004; Martinotti *et al*., 2016).

Peptide profiling was performed using a 100–1300 Da mass range (TOF scan with an accumulation time of 100.0 ms), followed by an MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored per cycle). The ion source parameters were set in electrospray positive mode as follows: curtain gas (N2) at 25 psig, nebulizer gas GAS1 at 25 psig and GAS2 at 20 psig, ion spray floating voltage (ISFV) at 5000 V, source temperature at 450 °C and declustering potential at 25 V (Cvijetic *et al*., 2004; Martinotti *et al*., 2016).

2.1.7 Hazelnut protein identification

Protein database implementation: The NCBI *C. avellana* database included two proteins that had already been annotated as oleosins, Cor a 12 (AAO67349) and Cor a 13 (AAO65960). In order to find further putative oleosins, the *C. avellana cv.* Jefferson genome (Rowley *et al*., 2018), genomic re-sequencing data of the TGL *cv.* (kindly provided by Prof. Roberto Botta, DISAFA, University of Turin, Italy) and *C. avellana* TSA (Transcriptome Shotgun Assembly, NCBI, project GGSA01) were *in silico* interrogated with an oleosin-conserved domain (PLFIIFSPVLVPA) by means of a local tBLASTn search (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/). The TGL genomic reads were *de novo* assembled through the Velvet tool (v1.2.09; https://github.com/dzerbino/velvet/) using a k-mer value of 19 to reconstruct the loci. Assembled contigs were structurally annotated with Augustus (v2.5.5; http://augustus.gobics.de).
Protein database search: The DDA files were searched for using Mascot v. 2.4 (Matrix Science Inc., Boston), and the implemented *Corylus avellana* NCBI database was interrogated. The search parameters were set as follows: an S-carbamidomethyl derivate on Cys as the fixed modification, oxidation on Met, acetylation on N-term, Met-loss on N-term, phosphorylation on Tyr, Thr and Ser as variable modifications and 3 missed cleavages to allow for trypsin digestion. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance at 0.1 Da. The peptide charges (on a monoisotopic mass) were set at 2+, 3+ and 4+. Only proteins with at least two peptides and with a peptide score> the peptide. Oleosin sequences alignment was performed using T-COFFEE (Version_11.00.d625267).

2.1.8 Expression and purification of the recombinant new allergen and inhibition tests

The total RNA was extracted from hazelnut seeds with a Spectrum Plant Total RNA Kit (Sigma). DNA was removed with a TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) and RNA retrotranscription was performed using a High-Capacity cDNA Reverse

Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) with hexamers. Specific primers, designed on the new allergen gene (Forward: 5'- ATGGCTGATTACCAACACCAG-3'; Reverse: 5'- TCATGTCTTCTTTTCCTCGCC-3'), were used to amplify cDNA using a BioRad (Hercules, CA, USA) thermal cycler. The thermocycling conditions were as follows: initial denaturation for 3 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 64°C and 40 s at 72°C, followed by 10 min of final incubation at 72 °C. The complete coding sequence (CDS) of the new allergen was then purified using a DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA), cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α cells. Finally, the plasmid with the new allergen CDS, whose sequence was confirmed by BMR Genomics (Padua, Italy), was sent to PRIMM (Italy) for the expression and purification of the corresponding protein.

Aliquots of 0.2 and 2 µg of the recombinant new full-length allergen and C-terminal fragment (from aa 102 to aa 169) were added to 800 µl of a pool of LRHA patients' sera and to a pool of selected HA patients' sera (nos. 2, 9, 10 and 12), respectively, for the blot inhibition tests.

2.2 Results

2.2.1 Study population

The main characteristics of the pediatric subjects included in the study are summarized in Table 1. All the patients showed positive PbP test to raw hazelnuts: 16/17 HA patients and 2/9 SH patients showed a wheal diameter ≥ 5 mm. One HA patient out of 17 and 4/9 HS patients showed negative PbP results, when tested with roasted hazelnuts.

The median scores of the sIgE of rCor a 1, rCor a 8, rCor a 9 and rCor a 14 for the HA patients were 0.22 (IQR 0.01-15.90), 0.01 (IQR 0.01-0.66), 9.80 (IQR 0.69-27.40) and 4.67 (IQR 1.49-26.10), respectively. The median scores of the sIgE of rCor a 1, rCor a 8, rCor a 9 and rCor a 14 for the HS patients were 0.01 (IQR 0.01-20.32), 0.10 (IQR 0.01-2.65), 0.32 (IQR 0.01-0.71) and 0.14 (IQR 0.01-0.69), respectively. The HA and HS patients differed significantly as far as the rCor a 9 and rCor a 14 values are concerned ($p<0.05$). Furthermore, the rCor a 9 and rCor a 14 values were directly correlated ($r2$ 0.66; $p<0.001$).

Nine patients (5/17 HA patients and 4/9 HS patients) also suffered from a pollen allergy (PA). Finally, 10/17 HA patients and 5/9 HS patients were allergic to other tree-nuts (NA).

2.2.2 Discovery of two new hazelnut oleosins

In order to characterize the OB-associated proteins, 1-2 DE protein separation was performed (Fig 1 and 2) and the proteins were identified by coupling MALDI-TOF/TOF and ESI-Q-TOF MS analyses (Table 2, Table 3). MALDI TOF/TOF analysis pointed out that some high m/z signals were not assigned to any identified protein. By coupling information from MALDI TOF/TOF LID spectra and from both *C. avellana cv.* Jefferson genome and *C. avellana cv.* TGL re-sequencing data (kindly provided by Prof. Roberto Botta, DISAFA, University of Turin, Italy), two new oleosin-like proteins were found, that differed from the already known Cor a 12 (AAO67349) and Cor a 13 (AAO65960) sequences (Akkerdaas *et al*., 2006).

ID	Patient Classification	SEX	AGE (years)	FHA	AD	FA	PA	NA	Hazelnut PbP (ø mm)		IgE CAP-RAST (KU/l)						Roasted OFC: SYMPTOMS	Reduced Immunblotting to RAW hazelnut OB		Reduced Immunoblotting to ROASTED hazelnut OB	
									Raw	Roasted	Hazelnut	rCor a 1	rCor a 8	rCor a 9	rCor a 14	rBet v 1		13 KDa band	17 KDa band	13 KDa band	17 KDa band
1	HA	M	15	yes	yes	yes	no	no	6	6	75,8	58,8	1,02	19,2	67,8	55,8	OS, NS, V, ER, AP, LE, H	no	no	yes	Yes
2	HA	M	5	yes	yes	no	yes	no	13	5	13,7	0.10	0.25	9,8	4,5	< 0,10	OAS, LAE	yes	yes	yes	Yes
3	HA	M	11	yes	no	yes	no	no	12	6	51,7	72,3	<0,10	4,95	5,43	< 0,10	OAS, PI, AP	no	yes	no	Yes
4	HA	F	7	yes	yes	yes	yes	yes	10	10	28,90	<0,10	<0,10	27,4	39,3	< 0,10	V, OAS, PI, ER	no	no	yes	Yes
5	HA	M	8	yes	yes	yes	no	yes	9	8	73	64,3	19,2	11,2	26,1	< 0,10	V, AS, LE	no	no	yes	Yes
6	HA	F	12	yes	yes	yes	yes	yes	9	8	84,20	<0,10	<0,10	73,4	41,2		AN	no	no	yes	Yes
7	HA	M	7	yes	no	yes	yes	yes	6	4	55,9	<0,10	0,54	39,3	4,16	< 0,10	V, AP, ER	yes	no	yes	Yes
8	HA	F	7	yes	yes	yes	no	yes	5	5	9,55	<0,10	<0,10	19,9	3,94	< 0,10	OAS, AP, NS, C, H	yes	no	yes	Yes
9	HA	M	8	yes	yes	yes	yes	yes	8	5	16,6	11,5	<0,10	6,85	4,76	9,8	OAS, C, ER	yes	yes	yes	Yes
10	HA	M	12	yes	no	no	yes	yes	10	14	> 100	29,1	0,11	> 100	> 100	36,5	V, AP, U, LE, AS	no	yes	yes	Yes
11	HA	M	11	no	no	yes	yes	no	3	0	30,7	0,42	36,8	0,69	0,27	4,44	ER	no	yes	no	Yes
12	HA	F	16	yes	yes	yes	no	yes	10	8	41,9	<0,10	<0,10	34,7	19,9	< 0,10	OAS	yes	yes	yes	Yes
13	HA	M	10	yes	yes	yes	yes	yes	10	8	7,45	4,33	<0,10	1,92	1,49	4,4	OAS, PI, ER	no	yes	yes	Yes
14	HA	M	6	yes	yes	no	no	no	6	7,5	3,75	<0,10	<0,10	<0,10	4,67	< 0,10	ER, U, AP, LAE	no	yes	yes	Yes
15	LRHA	M	11	yes	no	yes	no	yes	5	5	0,76	<0,10	1,78	0,13	0,47	< 0,10	OAS, PI, H	no	yes	no	yes
16	LRHA	M	12	yes	yes	yes	yes	yes	12	7	1,29	0,55	0,13	<0,10	<0,10	< 0,10	LAE, A, U	no	yes	yes	yes
17	LRHA	M	16	yes	yes	yes	yes	no	7	5	0,71	<0,10	<0,10	0,36	0,76	< 0,10	LAE	no	yes	yes	yes
18	HS	F	7	yes	yes	yes	no	yes	3	5	7,76	<0,10	6,5	0,32	<0,10	< 0,10	none	no	yes	yes	yes
19	HS	M	6	yes	yes	yes	no	no	5	5	24,7	38.1	0.85	<0,10	<0,10	< 0,10	none	yes	yes	yes	yes
20	HS	F	6	no	yes	no	yes	yes	4,5	0	78	> 100	1,37	1,74	0,69	> 100	none	no	yes	yes	yes
21	HS	M	17	yes	yes	yes	yes	no	10	10	7,42	7,53	<0,10	0,71	0,14	15,8	none	no	yes	yes	yes
22	HS	F	12	yes	no	yes	yes	no	4,5	0	47,40	58,70	<0,10	0,18	<0,10	67,3	none	no	yes	yes	yes

23	HS	M	11	yes	yes	yes	yes	yes	3	0	0.65	<0,10	<0,10	<0,10	0,3	< 0,10	none	no	yes	yes	yes
24	HS	M	11	yes	no	no	yes	yes	5	0	15,20	<0,10	14,60	<0,10	<0,10	< 0,10	none	no	yes	yes	yes
25	HS	M	9	yes	yes	yes	no	yes	5	4	6,44	<0,10	0,18	1,00	0,91	< 0,10	none	no	yes	yes	yes
26	HS	M	7	yes	no	yes	no	no	12	9	6,14	<0,10	<0,10	0,65	1,37	< 0,10	none	no	no	no	yes

Table 1. Patient information (n = 26), allergy symptoms, ImmunoCAP specific IgE values (kUA/L) and immunoblot test reactivity. HA, Hazelnut Allergic patient; LRHA, Low RAST Hazelnut allergic patient; HS, Hazelnut Sensitized patient; M, male; F, female; FHA, family history of atopy; AD, atopic dermatitis; FA, other food allergy; PA, pollen allergy; NA, other nut allergy; U, urticaria; A, angioedema; ER, erythema; LAE, labial edema; OAS, oral allergy syndrome; PI, pharyngeal itching; V, vomiting; AP, abdominal pains; OS, ocular symptoms; NS, nasal symptoms; LE, laryngeal edema; AS, asthma; C, cough; H, hoarseness; R, respiratory; HY, hypotension; AN, anaphylaxis.

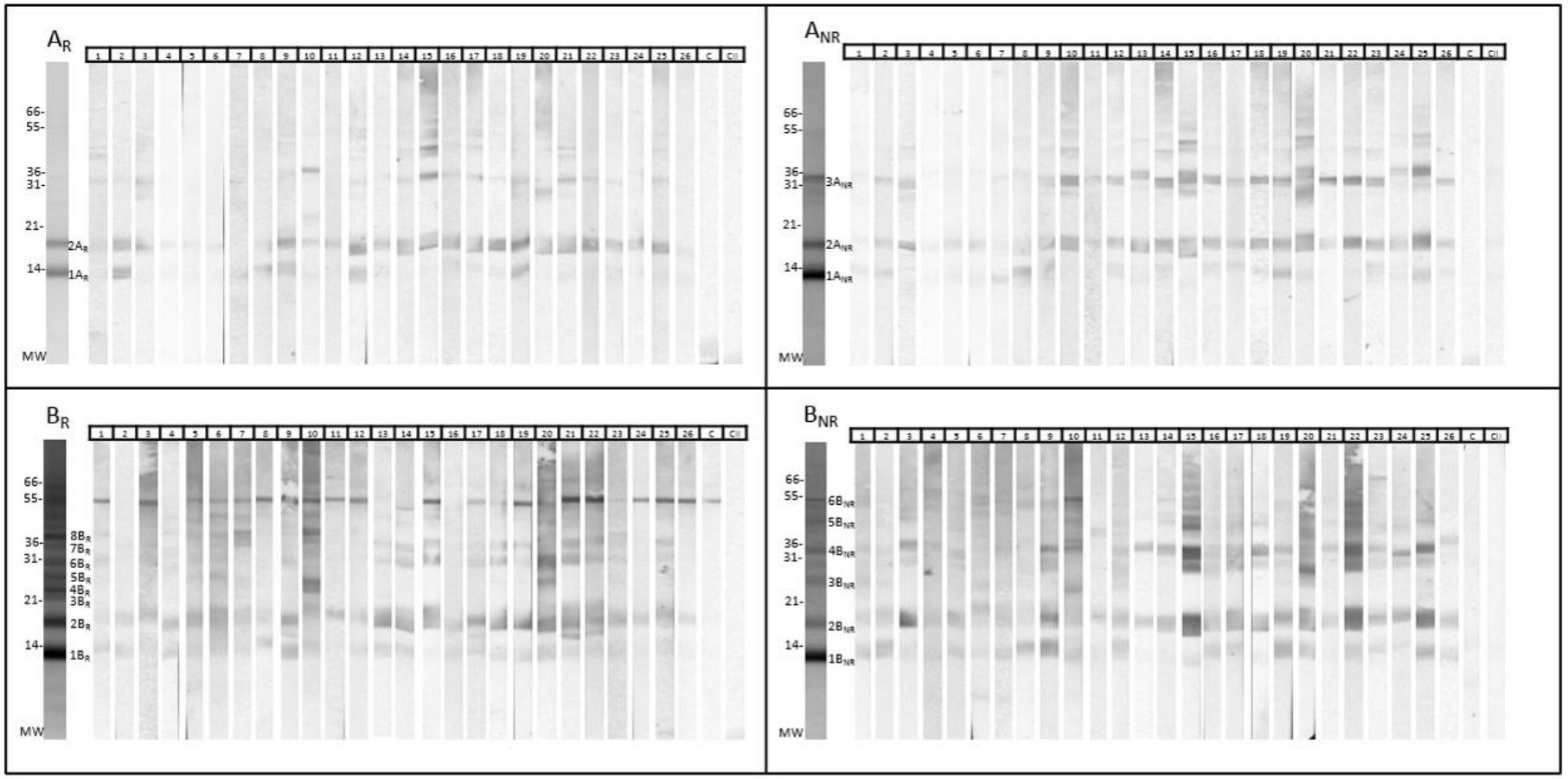

Fig 1. For each panel: first lane, stained LDS-PAGE of raw (A) and roasted (B) OB-associated protein extracts under reducing (R) and non-reducing (NR) conditions; lanes 1 to 26: immunoblotting with individual patient serum. C: a sera pool of five healthy individuals was used as control. CII: secondary antibody control. MW: molecular weight.

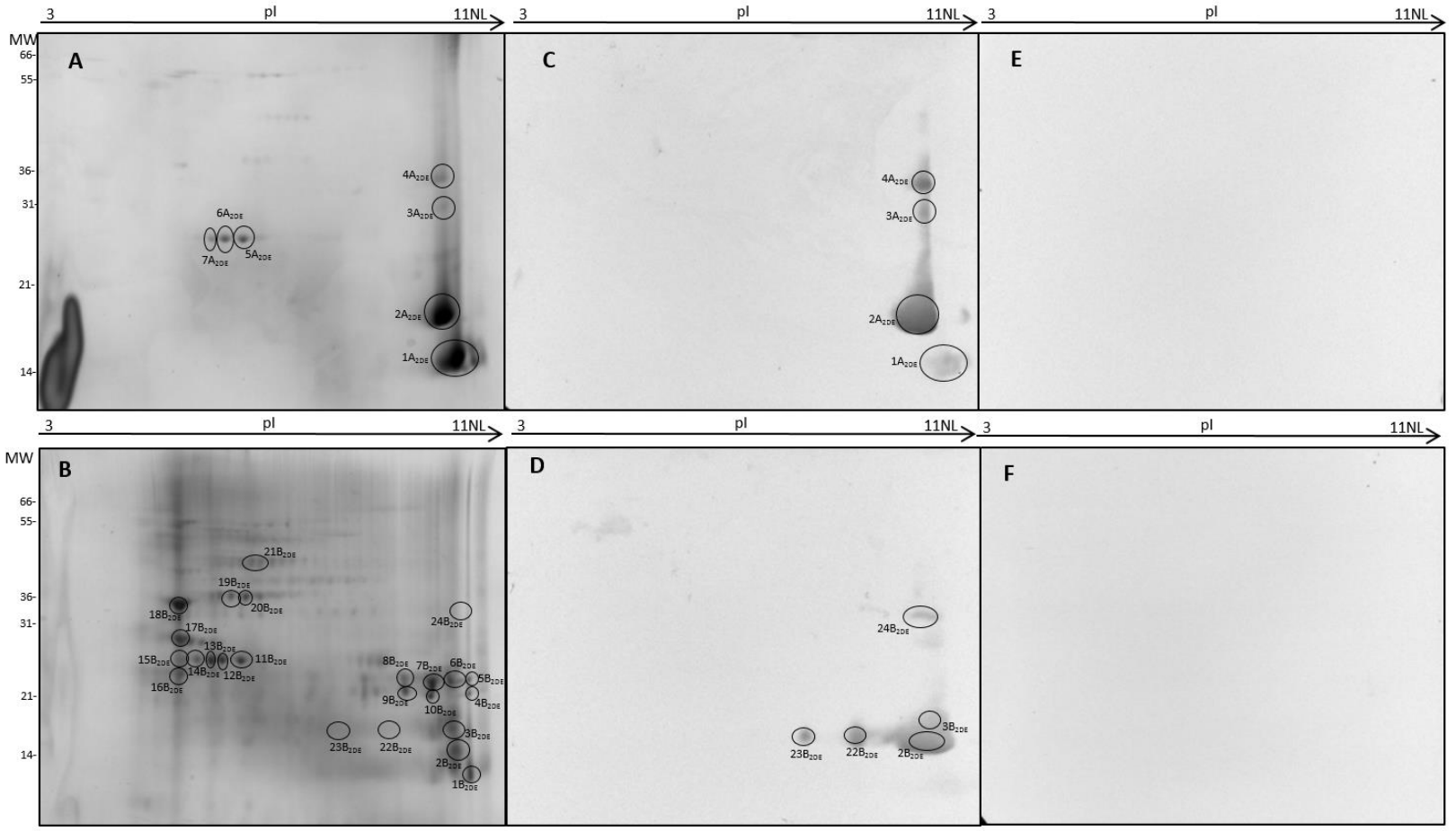

Fig 2. Stained 2DE and immunoblotting of the OB-associated protein extracts. Stained 2DE of raw (A) and roasted (B) hazelnut OB protein extracts. Immunoblotting of raw (C) and roasted (D) hazelnut OB protein extracts with the sera pool of LRHA patients. Immunoblotting of raw (E) and roasted (F) hazelnut OB protein extracts with a sera pool of five healthy individuals. MW: molecular weight; pI: isoelectrical point.

N° band	Entry (NCBI)	Protein name	MW experimental/ MW theoretical (Da)	Protein Score	N° of matching peptides	Protein Coverage (%)	Molar Fraction (%)
Raw hazelnut under reducing conditions (R)							
$1A_R$	AAO65960	Cor a 13	14000/14723	776	7	36.6	78.4
	AAO67349	Cor a 12	14000/16745	455	4	26.9	21.6
$2A_R$	MK737923	Cor a 15	17000/17695	1045	7	45.9	100
Raw hazelnut under non-reducing conditions (NR)							
$1A_{NR}$	AAO65960	Cor a 13	14000/14723	798	4	32.9	55
	AAO67349	Cor a 12	14000/16745	302	4	26.9	45
$2A_{NR}$	MK737923	Cor a 15	17000/17695	377	4	44.6	100
$3A_{NR}$	MK737923	Cor a 15	34000/17695	208	5	45.3	56.9
	AAO67349	Cor a 12	34000/16745	318	4	26.9	43.1
Roasted hazelnut under reducing conditions (R)							
$1B_R$	AAO65960	Cor a 13	14000/14723	1231	6	38.9	64.4
	AAO67349	Cor a 12	14000/16745	624	6	34.8	35.6
$2B_R$	MK737923	Cor a 15	17000/17695	1080	8	45.9	89.5
	AAO67349	Cor a 12	17000/16745	140	3	21.7	10.5
$3B_R$	AHA36627	Cor a 9	22000/59200	1853	14	31.2	69.5
	AAO65960	Cor a 13	22000/14723	203	4	26.9	30.6
$4B_R$	AHA36627	Cor a 9	23000/59200	2268	17	28.4	100
$5B_R$	AAO65960	Cor a 13	27000/14723	456	4	29.1	47
	AAO67349	Cor a 12	27000/16745	378	4	26.9	38.3
	AHA36627	Cor a 9	27000/59200	433	7	16.2	14.7
$6B_R$	MK737923	Cor a 15	29000/17695	293	5	36.6	40.6
	AAO65960	Cor a 13	29000/14723	401	4	29.1	27.2
	AAO67349	Cor a 12	29000/16745	410	4	26.9	22.2
	AHA36627	Cor a 9	29000/59200	539	8	18.6	10.1
$7B_R$	MK737923	Cor a 15	34000/17695	78	4	36.6	53.6
	AHA36627	Cor a 9	34000/59200	181	3	21.7	46.4
$8B_R$	AHA36627	Cor a 9	38000/59200	524	8	19.9	68.8
	AAO67349	Cor a 12	38000/16745	178	4	26.9	31.2
Roasted hazelnut under non-reducing conditions (NR)							
$1B_{NR}$	AAO65960	Cor a 13	14000/14723	692	4	29.1	64.5
	AAO67349	Cor a 12	14000/16745	404	4	26.9	35.5
$2B_{NR}$	MK737923	Cor a 15	17000/17695	879	7	45.3	100
$3B_{NR}$	AAO67349	Cor a 12	27000/16745	562	5	34.2	54.2
	AAO65960	Cor a 13	27000/14723	462	4	31.4	45.8
$4B_{NR}$	AAO67349	Cor a 12	33000/16745	274	4	26.9	83.6
	AHA36627	Cor a 9	33000/59200	149	4	12.8	16.4
$5B_{NR}$	AAO67349	Cor a 12	47000/16745	200	4	29.6	49.8
	AHA36627	Cor a 9	47000/59200	609	11	28.2	35.2
	AAL86739	Cor a 11	47000/51110	189	5	11.2	15
$6B_{NR}$	AHA36627	Cor a 9	53000/59000	1259	15	30.1	38.3
	AAO67349	Cor a 12	53000/16745	177	4	26.9	33.7
	MK737923	Cor a 15	53000/17695	72	3	22.9	20.3
	AAL86739	Cor a 11	53000/51110	217	4	10.8	7.7

Table 2. LDS-PAGE protein bands identification.

N° Spot	Entry (NCBI)	Protein name	MW experimental/ MW theoretical (Da)	PI experimental/ PI theoretical	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
Raw hazelnut								
$1A_{2DE}$	AAO67349	Cor a 12	14000/16745	10/10.54	4430	8	38.8	64.1
	AAO65960	Cor a 13	14000/14723	10/9.98	3846	8	41.1	35.9
$2A_{2DE}$	MK737923	Cor a 15	17000/17695	10/9.56	3709	13	55.8	96.5
	AAO67349	Cor a 12	17000/16745	10/10.54	666	5	28.9	3.5
$3A_{2DE}$	MK737923	Cor a 15	29000/17695	10/9.56	889	8	49.6	62.2
	AAO67349	Cor a 12	29000/16745	10/9.98	404	4	26.9	16.2
	AAO65960	Cor a 13	29000/14723	10/10.54	563	4	29.1	13.7
	MK737924	Oleosin	29000/15790	10/9.43	93	3	18.7	7.9
$4A_{2DE}$	MK737923	Cor a 15	34000/17695	10/9.56	1298	8	45.9	65.8
	AAO65960	Cor a 13	34000/14723	10/9.98	524	4	29.1	18.8
	AAO67349	Cor a 12	34000/16745	10/10.54	312	3	21.7	15.4
$5A_{2DE}$	Not identified							
$6A_{2DE}$	Not identified							
$7A_{2DE}$	Not identified							

Table 3A. Raw hazelnut 2DE protein spots identification.

N° Spot	Entry (NCBI)	Protein name	MW experimental/ MW theoretical (Da)	PI experimental/ PI theoretical	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
Roasted hazelnut								
$1B_{2DE}$	AAO67349	Cor a 12	14000/16745	10/10.54	1679	7	35.5	81
	AAO65960	Cor a 13	14000/14723	10/9.98	1051	5	31.4	19.0
$2B_{2DE}$	AAO67349	Cor a 12	15000/16745	10/10.54	2155	7	39.4	72.6
	AAO65960	Cor a 13	15000/14723	10/9.98	1001	5	31.4	22.2
	MK737923	Cor a 15	15000/17695	10/9.56	134	3	24.8	5.1
$3B_{2DE}$	AAO67349	Cor a 12	17000/16745	10/9.56	920	6	34.2	45.3
	MK737923	Cor a 15	17000/17695	10/9.98	1054	7	45.3	39.8
	AAO65960	Cor a 13	17000/14723	10/10.54	962	4	29.1	14.9
$4B_{2DE}$	AHA36627	Cor a 9	22000/59200	10.5/8.93	1406	13	27.8	100
$5B_{2DE}$	Not identified							
$6B_{2DE}$	AHA36627	Cor a 9	23000/59200	10/8.93	4799	14	30.2	57.1
	MK737923	Cor a 15	23000/17695	10/9.1	132	5	36.6	27.0
	AAO65960	Cor a 13	23000/14723	10/9.56	273	3	34.3	15.9
$7B_{2DE}$	AHA36627	Cor a 9	23000/59200	9/8.93	6112	14	32.3	81.1
	MK737923	Cor a 15	23000/17695	9/9.56	124	4	36.6	18.9
$8B_{2DE}$	AHA36627	Cor a 9	23000/59200	8.5/8.93	3519	14	29	100
$9B_{2DE}$	AHA36627	Cor a 9	22000/59200	10/9.56	3333	18	29.4	84.5
	MK737923	Cor a 15	22000/17695	10/10.54	56	3	30.4	15.5
$10B_{2DE}$	AHA36627	Cor a 9	22000/59200	9/8.93	2197	13	27.4	75.1
	MK737923	Cor a 15	22000/17695	9/9.56	96	3	31	24.9
$11B_{2DE}$	Not identified							
$12B_{2DE}$	Not identified							
$13B_{2DE}$	Not identified							
$14B_{2DE}$	Not identified							
$15B_{2DE}$	AAL86739	Cor a 11	27000/51110	5/6.12	473	8	23.2	100
$16B_{2DE}$	AAL86739	Cor a 11	24000/51110	5/6.15	536	8	22.4	75.2
	AHA36627	Cor a 9	24000/59200	5/6.12	616	5	15.2	24.8
$17B_{2DE}$	AHA36627	Cor a 9	30000/59200	5/6.15	296	5	14.4	60,6
	AAL86739	Cor a 11	30000/51110	5/6.12	145	3	9.2	39,4
$18B_{2DE}$	AHA36627	Cor a 9	38000/59200	5/6.15	189	5	15.7	51.4
	AAL86739	Cora 11	38000/51110	5/6.12	112	5	9.4	48.6
$19B_{2DE}$	AHA36627	Cor a 9	38000/59200	5.7/6.15	1562	16	45.1	100
$20B_{2DE}$	AHA36627	Cor a 9	38000/59200	6/6.46	1702	17	40.1	90.8
	AAL86739	Cor a 11	38000/51110	6/6.12	122	5	13.2	9.2
$21B_{2DE}$	AAL86739	Cor a 11	45000/51110	6.2/6.12	4659	24	55.3	91.4
	AHA36627	Cor a 9	45000/59200	6.2/5.69	985	15	39	8.6
$22B_{2DE}$	MK737923	Cor a 15	17000/17695	8/9.56	803	6	45.3	80.6
	AAO65960	Cor a 13	17000/14723	8/9.98	174	3	28.4	19.4
$23B_{2DE}$	MK737923	Cor a 15	17000/17695	6.7/9.56	17741	7	44.6	83.3
	AAO67349	Cor a 12	17000/16745	6.7/10.54	16745	3	21.7	16.7
$24B_{2DE}$	MK737923	Cor a 15	36000/17695	10/9.56	1055	8	45.9	74.8
	AAO65960	Cor a 13	36000/14723	10/9.98	517	4	29.1	16.4
	AAO67349	Cor a 12	36000/16745	10/10.54	350	3	28.2	8.8

Table 3B. Roasted hazelnut 2DE protein spots identification.

The two new sequences were submitted to GenBank under the accession numbers MK737923 and MK737924 (Fig 3). The MK737923 sequence showed 56% similarity with Cor a 12 and 41% with Cor a 13. The MK737924 sequence showed 63% similarity with Cor a 12 and 76% with Cor a 13. Interestingly, we found a homolog of the MK737923 (169 amino acids long in *C. avellana cv.* TGL) in *C. avellana cv.* Jefferson (213 amino acids long, but with a ~30 residual long gap) (Fig 4).

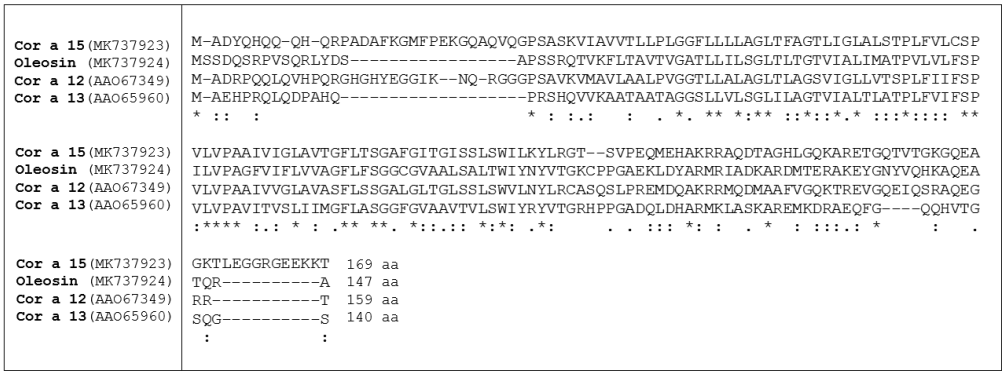

Fig 3. Hazelnut oleosin sequence alignment. Amino acid alignment of the newly discovered MK737923 (Cor a 15) and MK737924 oleosins and the already published AAO67349 (Cor a 12) and AAO65960 (Cor a 13). * identical amino acid, : very good amino acid change, . good amino acid change.

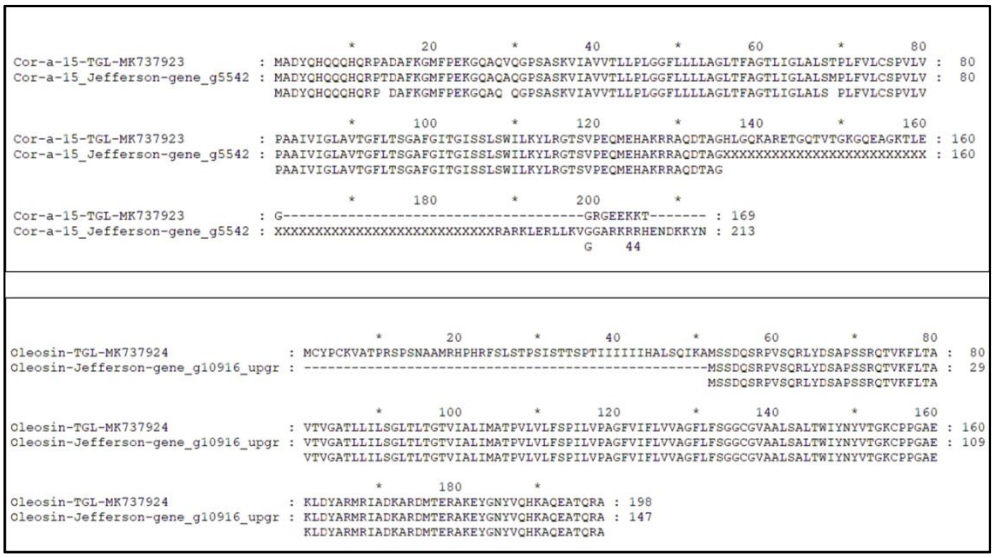

Fig 4. Hazelnut oleosin sequences from *C.avellana*, *cv.* TGL and *cv.* Jefferson: alignment. Amino acid alignment of the newly discovered MK737923 (Cor a 15) and MK737924 oleosins from cvs TGL and Jefferson, respectively

2.2.3 Ig-E reactivity of the HA patients to hazelnut OB-associated proteins and discovery of Cor a 15

The HA patients showed total hazelnut RAST sIgEs values ranging from 0.71 kU/L to 100 kU/L, and three HA patients (nos. 15, 16, 17), in particular, showed both low hazelnut sIgEs levels and low IgEs levels for single recombinant allergens. We termed these patients Low RAST Hazelnut Allergic (LRHA) patients. We hypothesized that other allergens that were not included in RAST could account for their positivity to OFC. We thus preliminarily tested a total hazelnut water-soluble protein extract by immunoblotting against the sera of LRHA patients and against the pooled sera of the remaining HA patient (patients from nos.1 to 14). The IgEs of the LRHA patients did not bind with any protein band, while several protein bands were recognized by the HA pool (Fig 5).

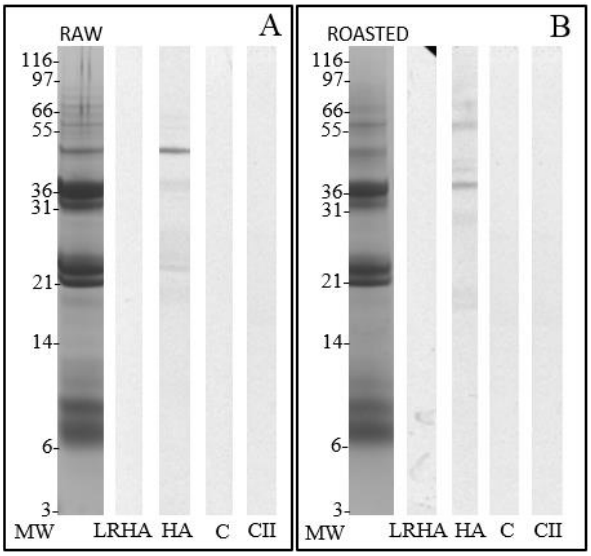

Fig 5. Stained LDS-PAGE and Immunoblotting of the total water-soluble hazelnut protein extract. Stained LDS-PAGE of raw (A) and roasted (B) hazelnut total water-soluble protein extract and immunoblotting with the pooled sera of the LRHA and HA patients; C: pool of 5 healthy individual sera used as control; CII: secondary antibody control; MW: molecular weight.

We then performed immunoblotting toward the OB-associated proteins to evaluate the immunoreactivity of all the patients against other hazelnut protein fractions considering each patient in the study, one at a time. The OB-associated hazelnut proteins were extracted and separated, by means of LDS-PAGE, under both reducing (R) and non-reducing (NR) conditions, in order to evaluate the immunorecognition of both the aggregated and single proteins (Fig 1: A_R and A_{NR} panels). The NR condition, which prevents the cleavage of disulfide bonds between proteins, allowed to observe the naturally occurring protein interaction,

while the R condition allowed to resolve proteins into their constituents. Two oleosin containing bands were found at 14 and 17 kDa, while a third band was also found, under NR conditions, at 34 kDa.

One hundred percent of the HA patients recognized at least one oleosin-containing band. Thirty percent of the HA patients in the R condition recognized the 14 kDa band, containing both Cor a 12 and Cor a 13 ($1A_R$, Table 2). The 17 kDa band, which exclusively contains the MK737923 oleosin ($2A_R$, Table 2), was the most recognized one, and was immunoreactive to 64% of the HA patients in the R condition. Eighty-two percent of the HA patients showed IgE binding to oleosin dimers ($3A_{NR}$, 34 kDa band), containing both MK737923 oleosin and Cor a 12 in the NR condition (Table 2).

The MK737923 oleosin was therefore submitted as allergen candidate to WHO/IUIS Allergen Nomenclature Subcommittee. The biochemical characterization and the proof of allergenicity of oleosin MK737923 were reviewed, and it was approved as a new hazelnut allergen and termed Cor a 15. The three hazelnut oleosins, Cor a 12, Cor a 13 and Cor a 15, were also immune recognized by HS patients, with a similar frequency to the HA patients (Fig 1).

2.2.4 Ig-E reactivity of the patients' sera to roasted hazelnut oleosins

We further assessed whether immune-reactivity was affected by processing, since hazelnuts are usually consumed roasted (Fig 1, panel B). In addition to the 14 kDa and 17 kDa bands, higher MW bands were detected in the LDS-PAGE of roasted hazelnut OB extracts. The 14 kDa band, which contains a mixture of Cor a 12 and Cor a 13, was recognized by 82% of the patients in the R condition ($1B_R$, Table 2). The 17 kDa band, which contains Cor a 15 with traces of Cor a 12 under the R condition ($1B_R$, Table 2), was again the most recognized band (100% of the HA children). The bands around 30-40 kDa, generated by roasting, were recognized by almost all the patients. In addition to the Cor a 12, Cor a 13 and Cor a 15 oleosins, these bands also contained the Cor a 9 or Cor a 11 soluble hazelnut allergens (from $3B_R$ to $8B_R$, Table 2).

2.2.5 Hazelnut oleosins are major allergens and Cor a 15 is the most frequently recognized oleosin by LRHA patients

In order to evaluate the contribution of each oleosin to IgE reactivity towards OB associated proteins, we used the sera of the LRHA patients, since they exclusively immune-recognized oleosins as previously shown (Fig 5). We performed 2DE separation of OB-associated proteins in order to improve protein separation and to obtain unambiguous identification

of the protein responsible for the immunoreaction. This approach was needed in particular for roasted hazelnuts, as the phenomenon of protein aggregation of oleosins with Cor a 9 and Cor a 11 is more marked (Fig 2).

Most of the spots in the 2D map alkaline zone were identified as oleosins, or as the basic subunit of Cor a 9. Most of the spots in the acidic zone were found to contain the acidic subunit of Cor a 9 and Cor a 11 (Table 3), both not showing any immunoreactivity against LRHA patient sera (Fig 2, Panels C and D). In spite of the different 2DE protein profiles of the raw and roasted OB proteins (Fig 2, Panel A and B), the immunoreactivity profiles were very similar (Fig 2, Panel C and D). The most reactive spots in both the raw and roasted extracts contained co-migrating proteins: Cor a 12, Cor a 13 and Cor a 15, with Cor a 15 being the most abundant in most of the spots (Table 3). Interestingly, the $1A_{2DE}$ and $2A_{2DE}$ spots, which showed a similar densitometric intensity in the 2DE gel, resulted in highly different immunoreactivity. Spot $2A_{2DE}$, which contained Cor a 15 at a 96.5% molar fraction, was markedly more immunoreactive for the LRHA patients than spot $1A_{2DE}$, which exclusively contained Cor a 12 and 13 (Fig 2, Panel A and C; Table 3). The other newly discovered MK737924 hazelnut oleosin was only found, albeit in a very low quantity, in spot $3A_{2DE}$ (which also contains Cor a 12, Cor a 13 and Cor a 15), and resulted to be weakly immunoreactive.

In order to verify that Cor a 15 was the most frequently recognized oleosin, Cor a 15 and its C-term fragment (from aa 102 to aa 168) were expressed and purified in *E.coli* (rCor a15; rCor a 15 C-term) (Fig 6, panels A and C). Two µg of full-length rCor a 15 added to both LRHA and HA patient sera completely inhibited the recognition of the 17kDa band in the 1D immunoblotting inhibition experiment (Fig 6, panel B), while the same amount of rCor a 15 C-terminal did not show a complete inhibition of the IgEs patients' sera (Fig 6, panel D).

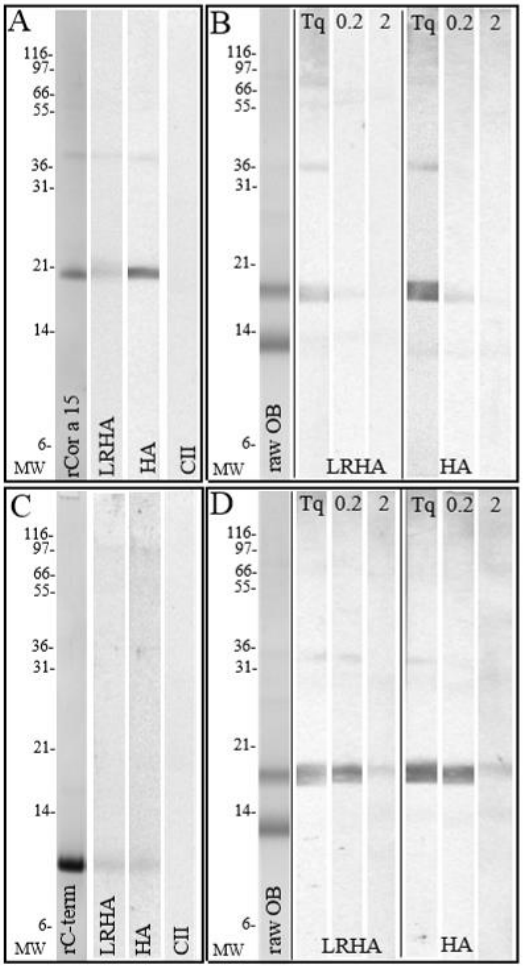

Fig 6. Immunoblotting inhibition assay. **Panel A**: Stained LDS-PAGE of rCor a 15 and immunoblotting with pooled sera of the LRHA and HA patients. **Panel B**: Inhibition assay. Stained LDS-PAGE of raw hazelnut OB protein extracts and immunoblotting with the pooled sera of the LRHA patients and with the pool of HA patient sera, without (Tq) and with the addition of patients' sera with 0.2 and 2 µg of rCor a 15. **Panel C**: Stained LDS-PAGE of rCor a 15 C-terminal (rC-term) and immunoblotting with pooled sera of the LRHA and HA patients. **Panel D**: Inhibition assay. Stained LDS-PAGE of raw hazelnut OB protein extracts and immunoblotting with the pooled sera of the LRHA patients and with the pool of HA patient sera, without (Tq) and with the addition of patients' sera with 0.2 and 2 µg of rCor a 15 C-terminal.
CII: secondary antibody control; MW: molecular weight.

2.3 Discussion

A recent review by Jappe & Schwager (2017) has demonstrated that the absence of oleosins in the commercial extracts of diagnostic tests could be the main reason for the lack of diagnosis of patients with a clear history of tree nut allergy but negative or with low levels of both RAST and SPT.

With the aim of an immuno-allergological characterization of hazelnut oleosins in pediatric patients, we performed isolation, separation by electrophoretic techniques and identification by mass spectrometry of hazelnut OB associated proteins. With this approach, for the first time we were able to separate by 2D electrophoresis the OB associated proteins from water-soluble hazelnut allergens, and to identify two new hazelnut oleosins. In addition, by coupling 2DE protein separation with immunoblotting, we succeeded in unambiguously ascribing the immunoreactivity of LRHA patients, that were characterized by low RAST IgEs values and positive OFC and PbP, to oleosins only, excluding the contribution of other allergens. In this way, we demonstrated the highly immunogenic power of the newly reported Cor a 15 oleosin for both hazelnut allergic and sensitized pediatric patients. We also showed that roasting increased oleosin IgE binding, as already reported for peanuts (Schwager *et al*., 2017).

In our study, the Cor a 9 and Cor a 14 IgE levels were clearly associated with the elicitation of the allergic reaction, according to Datema *et al*. (2018). Although our study referred to a limited number of patients, the Cor a 9 and Cor a 14 values were useful to discriminate between hazelnut allergic and sensitized but tolerant patient groups.

As far as Cor a 12, Cor a 13 and Cor a 15 are concerned, all patients' IgEs recognized at least one oleosin in immunoblotting analyses. The evaluation of LRHA subgroup IgEs against hazelnut oleosins was thus crucial to account for their allergic reaction. Both HA and LRHA patients showed a broad spectrum of symptoms to OFC, ranging from mild to severe (hypotension). The involvement of oleosins did not appear to be indicative of a specific symptomatology, thus not confirming the hypothesis of Zuidmeer-Jongejan *et al*. (2014), who stated that severe symptoms are associated with hazelnut oleosin sensitization.

It was not possible to establish the involvement of IgEs directed against oleosins in the pathogenesis of the allergic reactions for the HA patients, except for the LRHA patients, as the rest of patients were co-sensitized to genuine hazelnut allergens. The HS patients' IgE reactivity to oleosins was probably due to cross-reactivity or cross-sensitivity to pollen or other nuts for six out of nine patients, and an asymptomatic sensitization, like the one already reported for LTPs (Smeekens, Bagley, & Kulis, 2018), could be hypothesized for three out of nine patients.

As for the relevance of oleosins for the diagnosis of hazelnut allergy, our data partly support the findings of Zuidmeer-Jongejan and coworkers

(2014), however, the observation has here been extended to include a pediatric population. We observed the highest frequency of immunorecognition for the 17 kDa band, where we exclusively identified the new allergen Cor a 15, unlike Zuidmeer-Jongejan, who detected both Cor a 12 and Cor a 13 in the same band (Zuidmeer-Jongejan *et al*., 2014). Moreover, as demonstrated by Schwager *et al*. (2017) for peanut oleosins, rCor a 15 and rCor a 15 C-terminal were able to inhibit IgEs against the 17 kDa band in the HA and LRHA patients, although complete inhibition was only obtained by means of full-length rCor a 15. In the 14kDa band, we only identified Cor a 12 and 13, with a recognition frequency of 30%, which is slightly lower than what the Europrevall project reported for Cor a 12 in children (34%) (Mareen R. Datema *et al*., 2015).

This is also the first investigation that has evaluated the effect of roasting on the immunoreactivity of hazelnut oleosins. Our findings show a remarkable resistance of oleosins to high temperatures corresponding to an enhancement of oleosin immunoreactivity after roasting, in our cohort of children, with a more evident effect on the recognition of Cor a 12 and Cor a 13. The immune-recognition of roasted Cor a 12 and Cor a 13 exceeded 80% for the HA patients, while Cor a 15 reached 100% for the HA patients. Similarly, Schwager *et al*. (2017) demonstrated an increase in oleosin immunoreactivity after roasting for in-shell roasted peanuts, thus suggesting that the Maillard reaction could play a key role in explaining the enhancement of the allergenic potential. In fact, as recently reviewed by Toda *et al*. (2019), glycation after roasting may have influenced the affinity and/or the accessibility of oleosins to IgEs. Moreover, glycation may have caused structural modifications of the oleosins, such as aggregation, by means of intra- or inter-molecular crosslinking, between the lysine and arginine residues. Roasting has also been demonstrated to promote protein-lipid complex formation, and this could be particularly evident for oleosins. The formation of in vivo oleosin-lipid complexes could facilitate allergen intestinal uptake, causing alteration of the allergen digestibility and increasing immuneoreactivity (Bublin, Eiwegger, & Breiteneder, 2014).

In conclusion, we demonstrated that Cor a 15, here reported for the first time, was the most frequently recognized hazelnut oleosin in a cohort of hazelnut allergic pediatric patients, and that 100% of recognition was reached when hazelnuts were roasted. Cor a 15 was clinically relevant for a sub-group of HA patients with low sIgE levels to genuine hazelnut allergens. Furthermore, the presence of Cor a 15 gene sequence was confirmed in *C. avellana cv.* Jefferson as well as in *C. avellana cv.* TGL, suggesting the potential relevance of the new allergen, not only in Italy but also in Europe and USA.

Thus, the present study confirms the relevance of including lipophilic allergens, such as oleosins, in the routine protein extracts used for diagnostic tests, as has already been found for peanuts. Future studies are needed to confirm our findings on a larger numbers of patients, and

to demonstrate whether the same observations can be extended to adult populations.

2.4 Bibliography

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CHAPTER 3

EFFECT OF HOT-AIR AND INFRARED-ROASTING ON HAZELNUT ALLERGENICITY

Aim of the Work

It is known that thermal processing, commonly used during food production, may affect food allergenicity, by inducing physical and/or chemical changes in macronutrients. However, due to the lack of detailed information on processing procedures, available evidences on this topic are presently contradictory. This study aims to evaluate the effects of different types of roasting on hazelnut protein solubility, immune reactivity and structure.

Abstract

We investigated the IgE-binding capacities of both water-soluble and water-insoluble hazelnut protein fractions from *C. avellana* (*cv.* Tonda Gentile delle Langhe), before and after roasting, using two different roasting techniques: hot air (HA) as a ‘‘traditional method,” and infrared (IR), as an ‘‘innovative method”. Two combinations of time and temperature for each oven were considered: 140°C for 25min (LT) and 170°C for 17 min (HT) in HA; 140°C for 12 min (LT) and 170°C for 10 min (HT) in IR. Total protein and oil body associated protein extracts were used for immunoblotting experiments with allergic patient sera (n=16). Moreover, cell structural modification induced by processing were analyzed by applying differential staining microscopy techniques (Coomassie blue, Nile red and PAS) and by electron microscopy (TEM).

A decrease in hazelnut protein solubility was detectable following LT-IR roasting and it became more evident after HT processing, for both roasting types. Concerning allergenicity, the raw hazelnut proteins extract was the most immune reactive, followed by LT-HA and LT-IR processed hazelnut, that showed a very similar immune reactive pattern. The HA-HT treatment resulted in a low, although detectable, immune reactivity, while the IR-HT processing caused a quite complete disappearance of immune reactivity of hazelnut proteins. The different hazelnut allergens (Cor a 8, 9, 11, 12, 13, 14, 15) were differently affected by thermal treatments, in accordance with their thermal stability, as already reported by literature. Microscopical analyses suggested that the cell organization was mainly affected by the level of temperature, regardless the roasting method considered. Roasting caused cytoplasmic network disruption with the loss of the compartmentalization of lipid in oil bodies in the majority of cells.

HA oven resulted to be the most conservative processing, especially at low temperatures, both considering protein solubility and immune-reactivity. Both the HT treatments demonstrated to reduce immune-recognition of hazelnut proteins by allergic patient sera.

3.1 Methods

3.1.1 Thermal processing of hazelnut

A pilot scale experiment of hazelnut roasting was set up at Brovind (Cortemilia, Italy). Two pilot scale ovens, based on different technologies, were used: hot air (HA) and infra-red (IR).

The experiment was designed to obtain roasted hazelnuts with similar moisture contents, by means of applying the two different considered technologies, with the final aim of assessing whether the different heat transfer method provided by the two technologies had an effect on the overall hazelnut quality, and, in particular, on protein quality.

Preliminary experiments were conducted to identify the temperature/time conditions to set for each oven, in order to obtain comparable products, in terms of residual moisture. For each oven, two different temperature conditions were then chosen: low temperature (140°C, LT) and high temperature (170°C, HT). For the HA oven each drying cycle was set on 25, 30 and 35 min at LT and 10, 13 and 17 min at HT. For the IR oven the cycle times were set on 10, 12 and 14 min at LT and 6, 8 and 10 min at HT. The temperature of the ovens was monitored with a set of calibrated Pt100 temperature sensors placed in strategic positions inside the thermal process volume of each oven. For HA and IR oven, 1.5 and 4.0 Kg of hazelnuts were roasted for each cycle, respectively, and the roasting experiments were performed in three separate replicates (see experimental plan in Fig 1). The moisture content of raw and roasted hazelnut was assessed immediately after the roasting cycle by means of an Infrared Moisture Analyser (Sartorius, Gottinga, Germany).

3.1.2 Hazelnut soluble and insoluble protein extraction

Raw and roasted hazelnuts were extracted in triplicate for each biological replicate. One g of chopped hazelnuts was defatted three times with 10 ml of hexane; for each step, the hexane phase was removed after 30 minutes of shaking in ice. Samples were dried for 30 min in Speedvac at RT. Fifty mg of defatted hazelnut powder were resuspended in 1.6 ml of 25mM Na_2HPO_4, 1.5M NaCl (pH7.5) containing 1 tablet of protease inhibitors (Complete, Roche, Basel, Switzerland). After sonication (4 cycles of 10 sec ON and 10 sec OFF), samples were shacked for one hour and then centrifuged at 21460 x g for 20 min at 4°C. The supernatants were filtrated with a 0.45 µm filter and collected as Water-Soluble (S) sample. The pellets were extracted o.n. with 0.8 ml of 7M urea, 2M thiourea, 20mM Tris-HCl, pH 8.8 (Urea buffer). After centrifugation at 21460xg, for 20 min at 4°C, the supernatants were filtrated with a 45 µm filter and collected as Water-Insoluble (I) sample.

The S and I samples were quantified by Bradford assay (Biorad, Hercules, California).

3.1.3 Oil bodies associated proteins extraction

The oil bodies (OB) associated proteins were extracted as described in paragraph 2.1.3.

3.1.4 LDS-PAGE

LDS-PAGE were performed using precast gels (NuPAGE 4-12% Bis–Tris, Invitrogen Life Technologies Ltd., Paisley, UK) in XCell SureLock Mini-Cell System (Invitrogen) according to manufacturer procedures. Each sample was diluted in NuPage LDS Sample Buffer (Invitrogen), under non-reducing condition and loaded in equal amount (5 µl). Gels were stained with Colloidal Coomassie Blue and scanned with ChemiDoc MP System densitometer (Bio-Rad).

3.1.5 Hazelnut allergic patients

Sera from 16 hazelnut allergic pediatric patients were collected from "Food Allergy Division" of the "Regina Margherita Childrens' Hospital", Turin (Italy). Patients with convincing clinical history of hazelnut allergy were selected. According to RAST against hazelnut major allergens (ThermoFisher Scientific, Waltham, Massachusetts), sera were grouped in five pools (Table 1):

-**pool A**, 4 patients (ID1 to ID4), with positive RAST to Cor a 1, Cor a 8, Cor a 9 and Cor a 14;

-**pool B**, 1 patient (ID 5) with positive RAST to Cor a 8, Cor a 9 and Cor a 14;

-**pool C**, 8 patients (ID 6 to ID13) with positive RAST to Cor a 9 and Cor a 14;

-**pool D**, 3 patients (ID 14 to ID16) with positive RAST to Cor a 8;

-**pool E**, all the patients (ID 1 to ID16)

Three sera of non-nut sensitized non-allergic hazelnut consumers were pooled and used as control. The study was reviewed and approved by the hospital ethical committee. All patients gave written informed consent before to be enrolled in the study.

3.1.6 Immunoblotting

For immunoblotting, S and I hazelnut extracts were mixed (1:1). The same protein amount (5µg) was loaded from each sample. Immunoblotting was performed as described in paragraph 2.1.5.

Pool ID		Subject ID	Sex	Age	IgE CAP-RAST (KU/l)				
					Cor a 1	Cor a 8	Cor a 9	Cor a 14	Total hazelnut IgE
E	A	1	M	13	49,70	0,50	1,71	15,70	50,70
		2	M	18	40,40	0,73	11,70	31,20	48,10
		3	M	6	74,30	13,90	6,67	21,90	n.d.
		4	F	3	0,26	0,16	8,89	1,75	7,76
	B	5	M	9	<0,1	>100	18,40	68,00	46,70
	C	6	F	3	<0,1	<0,1	2,43	1,53	2,34
		7	M	6	<0,1	<0,1	1,13	1,70	n.d.
		8	F	11	<0,1	<0,1	>100	>100	>100
		9	F	12	<0,1	<0,1	>100	29,30	>100
		10	M	8	<0,1	<0,1	6,00	6,34	13,50
		11	M	3	<0,1	<0,1	27,20	7,42	11,00
		12	M	6	<0,1	<0,1	5,17	1,42	2,93
		13	F	6	<0,1	0,58	4,01	2,35	2,52
	D	14	F	5	n.d.	2,46	n.d.	<0,1	7,43
		15	F	4	n.d.	1,85	<0,1	<0,1	n.d.
		16	M	9	n.d.	5,64	<0,1	<0,1	n.d.

Table 1. Hazelnut allergic patients characterization by RAST analysis. Pool A: 4 patients (ID from 1 to 4) with positive RAST to Cor a 1, Cor a 8, Cor a 9 and Cor a 14. Pool B: 1 patient (ID 5) with positive RAST to Cor a 8, Cor a 9 and Cor a 14. Pool C: 8 patients (ID from 6 to 13) with positive RAST to Cor a 9 and Cor a 14. Pool D: 3 patients (ID from 14 to 16) with positive RAST to Cor a 8. Pool E: pool of all the patients in the study (ID from 1 to 16).

3.1.7 Mass spectrometry analysis (ESI-Q-TOF) and protein identification

In gel digestion and mass spectrometry (ESI-Q-TOF) analysis were performed as described in paragraph 2.1.6.

The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston) with UniProt *Corylus avellana* database. The following parameters were set for searching: S-carbamidomethyl derivate on cysteine as fixed modification, oxidation on methionine, Acetyl (N-term), Met-loss (Protein N-term M), as variable modifications and three missed cleavage sites for trypsin digestion. Peptide mass tolerance were set at 50 ppm and MS/MS tolerance at 0.1 Da. Only proteins with at least three peptides and with a peptide score > peptide identity were considered for identification purposes.

3.1.8 Microscopical analyses

Hazelnut small pieces were fixed in 2.5 % (v/v) glutaraldehyde in 10 mM phosphate buffer (PB) pH 7.2, O.N. at 4°C; then rinsed in the same buffer, and post fixed in 1% OsO4 in PB for 1 h, at RT. After rinsing in PB, they were dehydrated in an ethanol series (30, 50, 70, 90, and 100%; 10 min each step) at RT. The samples were infiltrated in 2:1 (v/v) ethanol/London Resin White (EMS, PA _ USA) for 1 h, 1:2 (v/v) ethanol/LRW for 2 h, and 100% LRW overnight at 4°C according to Moore *et al*. (1991)

Semi-thin sections (1µm) were stained with 1% toluidine blue to check the sample quality using an optical microscopy. Based on these observations, ultra-thin sections (70 nm) were then cut and stained with Uranyl Acetate Substituted (Agar Scientific, Stansted UK) and lead citrate before observation with a Philips CM10 transmission electron microscope.

3.1.9 Histochemistry

Proteins staining with Coomassie Brilliant Blue (CBB): semi-thin sections (1µm) were staining at RT for 30 min in 0.25% (w/v) Coomassie Brilliant Blue R-250 dye in a solution of methanol : acetic acid : water (MAW, 5:1:4). Semi-thin sections were rinsing in the MAW solution for 15 min at RT and again rinsed in water for 10 min.

Carbohydrates staining with Schiff's reagent (PAS): semi-thin sections (1µm), obtained from the LR White embedded samples, were dipped in a 1% (w/v) periodic acid for 30 min, rinsed in water for 5 min and stained in Schiff's reagent for 10 min, in the dark and again rinsed in water for 10 min.

Lipids staining by Nile Red: hazelnut sections (25 µm) were prepared using a Balzer Vibratome series 1.000 apparatus. Sections were incubated with Nile Red 1 mg/ml in acetone and diluted 1:100 in PBS for 10 min at RT in the dark. Sections were mounted in 50% of glycerol. Observations were carried out using a confocal laser-scanning microscope (Leica TCS SP2). The yellow-gold fluorescence that is specific of lipids was measured at 488/550 nm.

3.2 Results and Discussion

3.2.1 Evaluation of roasting parameters

The control of temperature and moisture distribution is crucial for food process design, quality control, choice of appropriate storage and handling practices. The most widely used method for nut roasting is the convective heat transfer process, performed in HA oven (Perren R and Escher F, 2007). Another innovative possibility is IR heating, successfully used in the dry-roasting and pasteurization of almonds (Yang *et al*., 2010) and hazelnut roasting (Belviso *et al*., 2017). However, most of the IR roasting effects on nuts quality have only been partially studied. Belviso and coworkers (2017), monitoring physical, chemical and sensory changes of hazelnut roasted either in an HA or in IR oven, established that HA processing resulted in hazelnuts with lower rupture force and improved oxidative stability. Binello and coworkers (2018) stated that it was not possible to define an optimal roasting procedure because a key factor is the intended use of the final product (whole nuts, pastes, etc). Differently from Belviso and coworkers (2017), they found out that IR oven better preserves the antioxidant compounds content of whole hazelnut. As far as pastes, hazelnuts treated with an IR oven at highest temperature showed higher viscosity and density, in addition to a stronger aroma.

In our study, roasting experiments were performed using two ovens, based on different heat transmission mechanisms: HA and IR ovens. Two temperatures (140°C and 170°C) and 3 cycle times for each temperature were considered (Fig 1). The LT protocol set up for simulating the production of soft aroma and light colored nuts, while the HT protocol was set up, to simulate the production of roasted hazelnuts for the production of pastes with strong flavour, colour and texture.

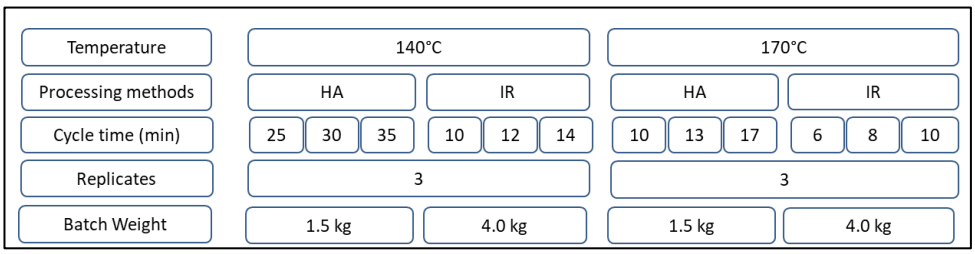

Fig 1. Experimental design. Hot Air (HA) and Infrared (IR) hazelnut roasting at two temperature levels (140°C and 170°C) and 3 cycle times for each temperature.

To evaluate process performance, water loss during roasting was measured in real-time, by means of a chilled mirror hygrometer connected to each oven chimney. Hazelnut moisture content was also measured before and after roasting. A longer roasting time was needed for obtaining the same water loss at the same temperature for HA compared to IR: 25 min vs 12 min for 140°C and 17 min vs 10 min for 170°C (Fig 2).

The two combinations of time and temperature ensuring comparable end-products in term of water content between the two ovens (HA and IR) were: 140°C for 25min (HA-LT) and 140°C for 12 min (IR-LT); 170°C for 17 min (HA-HT) and 170°C for 10 min (IR-HT).

These four experimental conditions (HA-LT, IR-LT, HA-HT, and IR-HT) were considered for all further determination of protein aggregation, solubility and allergenicity.

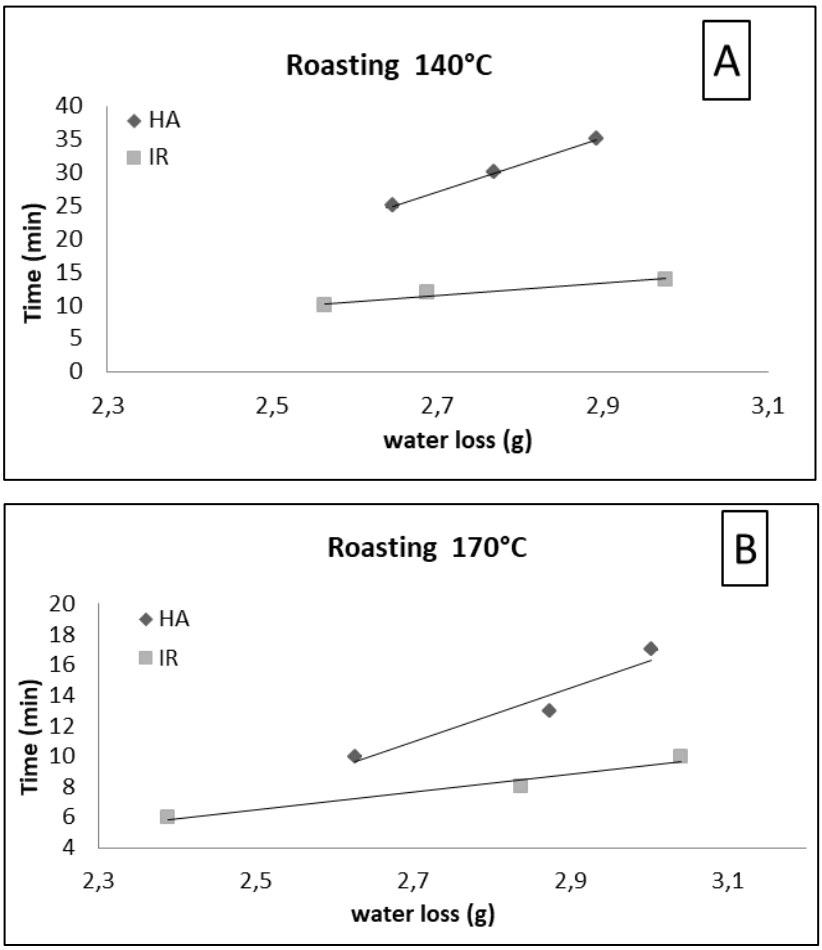

Fig 2. Water loss under Hot Air (HA) and Infrared (IR) roasting, at two different temperature (**A:** 140°C and **B**: 170°C) and 3 times for each temperature. The experimental conditions chosen for the further analysis are marked by circles: HA 140°C for 25 min and 170°C for17 min; IR 140°C for 12 min and 170°C for 10 min.

3.2.2 Thermal treatment effects on hazelnut protein extraction

Hazelnuts roasted according to the two methods and containing the same moisture content, showed an overall decrease in protein content (Fig 3 panel A). The decrease in protein concentration was more pronounced for the IR oven and for the HA oven at high temperature. Moreover, roasted hazelnuts showed a decrease of protein solubility when the processing temperature increased (Fig 3 panel B). We set up two protocols for protein extraction: the first based on an aqueous buffer with the aim of extracting the water-soluble proteins (S), the second based on urea buffer in order to solubilize water-insoluble proteins (I). The roasting processes at low temperature (especially HA-LT) showed a similar relative aboundance of S and I protein fraction content in comparison to the raw sample. On the countrary, for the samples processed at high temperature (HA-HT and IR-HT), the ratio S/I was reverted. At high temperature (HA-HT and IR-HT), the decrease in the S fraction corresponded to an increase in I fraction. In raw, HA-LT and IR-LT samples, the S protein component accounted for 63-73% of the total proteins content, while in the HA-HT and IR-HT the S proteins represented only 19-26% (Fig 3 panel C).

LDS-PAGE of each sample was performed under non-reducing condition, in order to highlight any possible protein aggregation phenomenon. Protein samples were extracted from the same hazelnut weight and analyzed keeping constant the loading volume, in order to point out the differences in protein profile between S and I fractions and among the different processing protocols considered. The HA-LT treatment showed a protein profile, for both S and I samples, more similar to raw in comparison to IR-HT, that was the processing causing the highest protein shift from S to I (Fig 3 panel D). For HA-HT and for both the IR treatments (LT and HT) the disappearance of protein bands in S lane is balanced by the appearance of some smearing at high molecular weight of I lane, probably indicating the presence of aggregated, insoluble protein complexes, difficult to resolve (Fig 3 panel D).

As already demonstrated by Downs *et al* (2016) on walnut allergens, protein solubility may have a strong impact on the evaluation of the processing effect on food allergenicity. The decrease in protein solubility could affect the ability of most analytical methods to detect and quantify the presence of allergenic food proteins in thermally processed food matrices. Both immunochemical and MS methods, two of the most employed detection strategies for food allergens evaluation, require effective protein solubilization, in order to perform protein detection and identification. If protein solubility decreases, quantification of allergens may be underestimated, due to epitope misrecognition, following structural modifications induced by thermal processing.

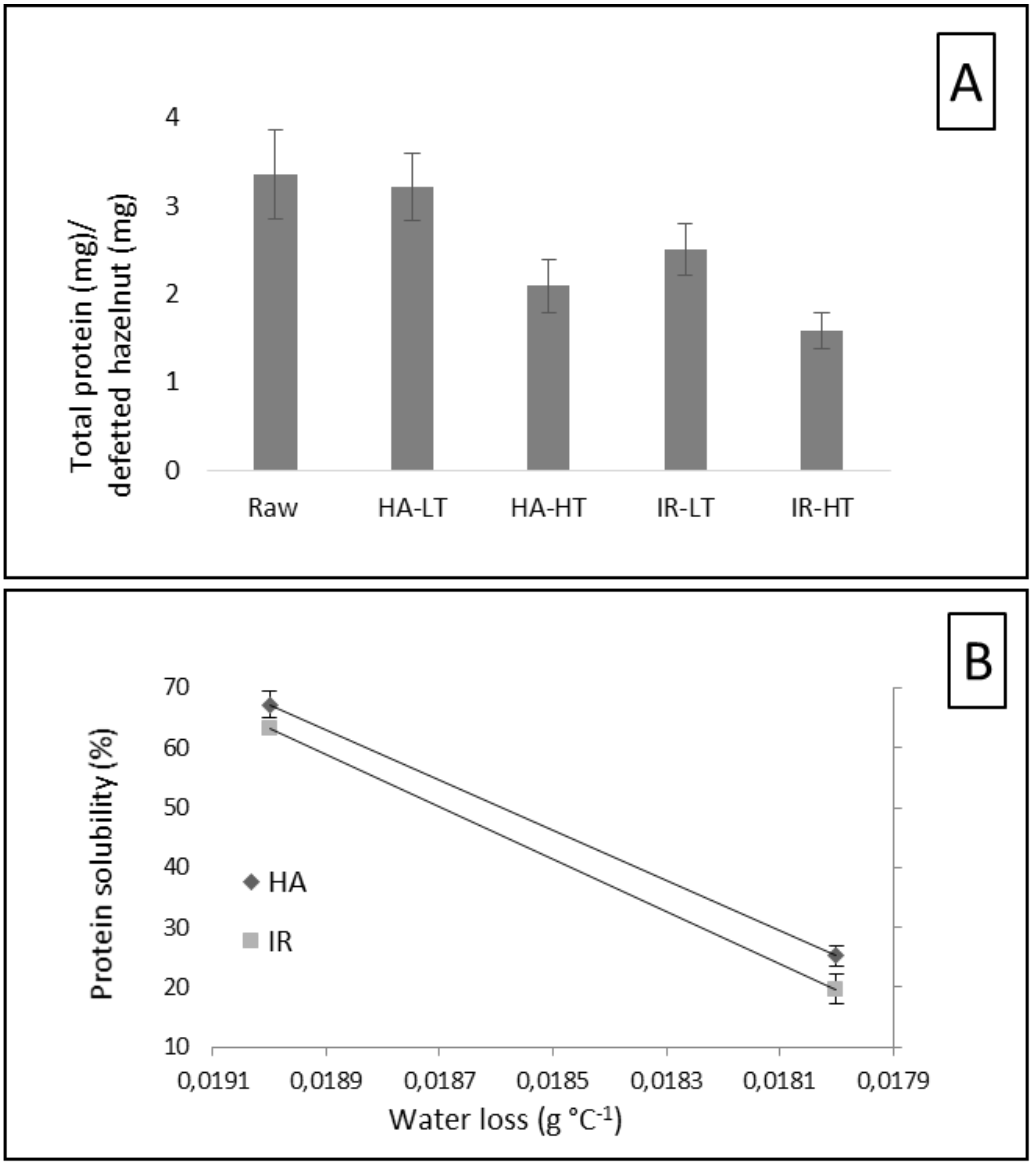

Fig 3. Output of hazelnut proteins extraction. **(A)** Total proteins quantified by Bradford assay calculated by the sommatory of the mg of Water-soluble (S) and Water-insoluble proteins (I) of raw, HA-LT (Low Temperature), HA-HT (High Temperature), IR-LT and IR-HT. **(B)** Correlation between the hazelnuts water loss and the proteins solubility in saline buffer after HA and IR roasting.

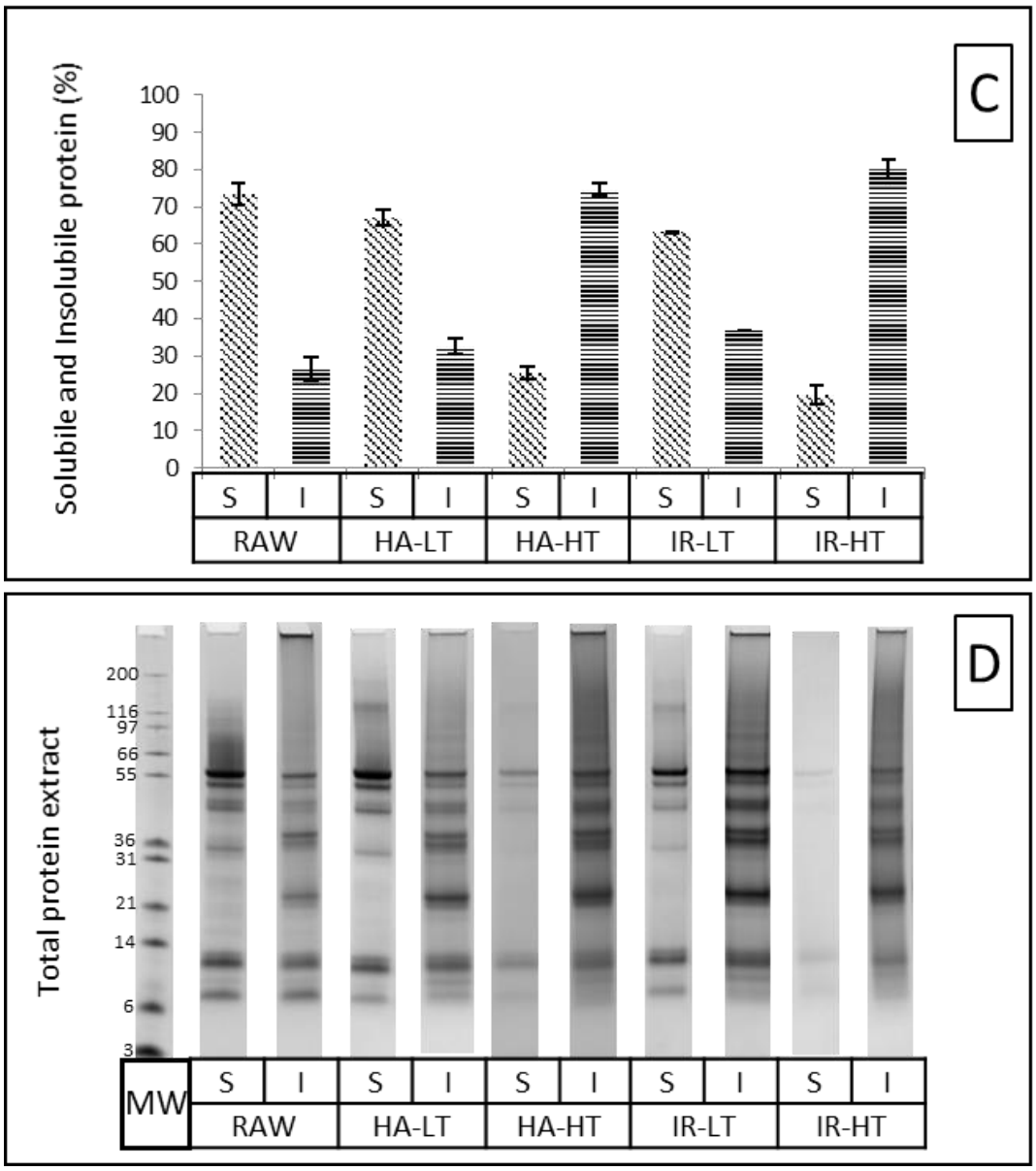

Fig 3. Output of hazelnut proteins extraction. **(C)** Proteins quantification by Bradford assay of raw, HA-LT (Low Temperature), HA-HT (High Temperature), IR-LT and IR-HT. Histograms represent the percentage of Water-Solubile (S) and Water-Insolubile (I) proteins in relation to the total amount of proteins. **(D)** LDS-PAGE of S and I fraction for each proteins extract. MW: molecular weight.

3.2.3 Thermal treatment effects on immunorecognition

As summarized in Figure 4 panel A, the immunoblotting experiments were performed, for each treatment (raw, HA-LT, HA-HT, IR-LT, and IR-HT) on soluble and insoluble proteins (sample T, obtained mixing 1:1 the S and I fraction) and on OB enriched protein fractions (sample O). For T protein fractions, the higher was the processing temperature, the lower the overall intensity of the bands on the gels, and, consequently, the lower the immunoreactivity. As far as OB enriched proteins are concerned, they seemed to be more affected by IR treatment than by HA treatment, at both temperatures considered.

T samples were incubated with 4 pools of allergic patient's sera (A, B, C and D), while O samples were incubated with a single pool (E) contained the sera of all the patients included in the study (Fig 4 panel A, Table 1).

The raw sample was the most immunoreactive for both T and O protein samples (Fig 4 panel B). Both the LTs processing resulted in a very similar immunoreactive pattern, less marked compared to raw (Fig. 4 panel C and E). HA-HT processed samples, although less immunoreactive, preserved a detectable reactivity, especially in the range of molecular mass from 30 to 65 kDa (Fig 4 panel D). Only IR-HT treatment showed a quite complete disappearance of immunoreactivity (Fig 4 panel F).

An important issue to take into account is that, it has been reported that different allergens show different responses to heat processing in term of immunoreactivity (Costa *et al*., 2016). The extent of single band immunoreactivity reduction, caused by the processing, depends on which allergen is present in the band and what its level of heat resistance is.

Currently, eleven allergens from hazelnut are reported in the database of the International Union of Immunological Societies (WHO/IUIS; www.allergen.org), seven of them are demonstrated to be involved in food allergy (Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12, Cor a 13, Cor a 14).

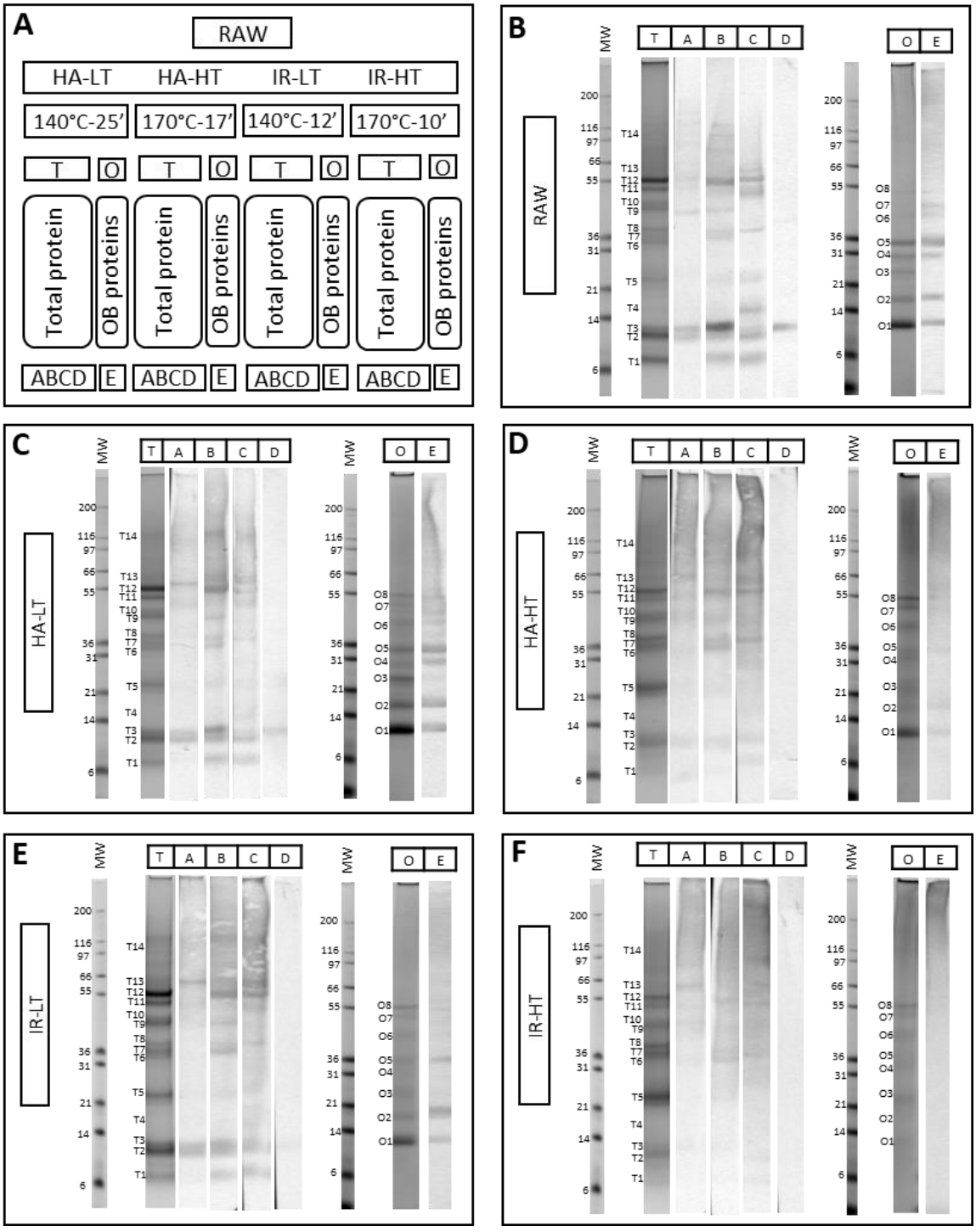

Fig 4. For each samples (RAW, HA-LT, HA-HT, IR-LT and IR-HT) the LDS-PAGE of soluble and insoluble proteins extract (mixing Soluble and Insoluble fraction (1:1) (T) and of oil bodies associated proteins (O) were performed. T samples were tested in immunoblotting with four patient pools (pool A, B, C and D) characterized by different RAST pattern (Tab 1). O samples were tested in immunoblotting with a pool containing the sera of all the patients in the study (E). Bands identified by LC-MS/MS from T and from O samples were indicated with letters from T1 to T14 and from O1 to O8, respectively. MW: molecular weight.

N° band	Entry (NCBI)	Protein name	MW experimental/ MW theoretical (Da)	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
Hazelnut total proteins extract (T)							
T1	AHA36627.1	Cor a 9	8000/59200	465	7	20,4	100
T2	AHA36627.1	Cor a 9	10000/59200	702	8	26,7	32,6
	ACO56333.1	Cor a 14	100000/12600	173	4	23,1	67,3
T3	AHA36627.1	Cor a 9	11000/59200	297	4	8,9	20,60
	4XUW_A	Cor a 8	11000/9926	84	2	29,3	79,4
T4	AHA36627.1	Cor a 9	15000/59200	411	6	19,3	100
T5	AHA36627.1	Cor a 9	22000/59200	4667	14	32,5	95,62
	AAL86739.1	Cor a 11	22000/51110	69	2	11,6	4,38
T6	AHA36627.1	Cor a 9	34000/59200	1248	10	22	100
T7	AHA36627.1	Cor a 9	36000/59200	3242	18	44,4	100
T8	AHA36627.1	Cor a 9	38000/59200	1251	12	37,2	100
T9	AHA36627.1	Cor a 9	45000/59200	2684	13	31,9	66,17
	AAL86739.1	Cor a 11	45000/51110	842	10	27,5	33,83
T10	AHA36627.1	Cor a 9	47000/59200	2871	12	26,8	49,25
	AAL86739.1	Cor a 11	47000/51110	1984	15	35,5	50,75
T11	AHA36627.1	Cor a 9	50000/59200	2490	14	42,2	80,54
	AAL86739.1	Cor a 11	50000/51110	570	7	18,8	19,46
T12	AHA36627.1	Cor a 9	55000/59200	8359	22	56,4	95,17
	AAL86739.1	Cor a 11	55000/51110	319	6	15	4,83
T13	AHA36627.1	Cor a 9	60000/59200	4154	18	47,9	85,48
	AAL86739.1	Cor a 11	60000/5110	310	7	18,8	14,52
T14	AHA36627.1	Cor a 9	130000/59200	2429	14	37	79,6
	AAL86739.1	Cor a 11	130000/51110	623	7	19	20,40

Table 2. Hazelnut total extract proteins identification.

N° band	Entry (NCBI)	Protein name	MW experimental/ MW theoretical (Da)	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
Oil bodies associeted proteins (O)							
O1	AAO65960.1	Cor a 13	12000/14723	2822	4	27,9	55,1
	AAO67349.2	Cor a 12	12000/16745	441	3	20,8	44,9
O2	MK737923	Cor a 15	17000/17741	935	7	42,6	70,3
	AAO67349.2	Cor a 12	17000/16745	172	2	23,3	20,7
	AAO65960.1	Cor a 13	17000/14723	179	2	17,9	9
O3	AAO65960.1	Cor a 13	27000/14723	561	3	26,4	24,3
	AAO67349.2	Cor a 12	27000/16745	515	4	25,8	63,7
	AHA36627.1	Cor a 9	27000/59200	481	7	17,1	12
O4	AAO67349.2	Cor a 12	31000/16745	531	4	25,8	54,6
	MK737923	Cor a 15	31000/17741	175	3	32	24,6
	AAO65960.1	Cor a 13	31000/14723	114	3	26,4	20,8
O5	MK737923	Cor a 15	36000/17741	704	6	42,6	78
	AHA36627.1	Cor a 9	36000/59200	315	6	15,6	10,1
	AAO67349.2	Cor a 12	36000/16745	142	2	12,6	11,9
O6	AHA36627.1	Cor a 9	45000/59200	1843	9	20,2	44
	AAL86739.1	Cor a 11	45000/51110	174	5	13,2	24,9
	AAO67349.2	Cor a 12	45000/16745	90	2	12,6	31,1
O7	AHA36627.1	Cor a 9	50000/59200	765	8	17,7	42,9
	AAL86739.1	Cor a 11	50000/51110	166	4	8	21,7
	AAO67349.2	Cor a 12	50000/16745	60	2	15,1	35,5
O8	AHA36627.1	Cor a 9	55000/59200	2701	12	28,6	77,8
	AAL86739.1	Cor a 11	55000/51110	145	4	10,3	22,2

Table 3. Hazelnut oil bodies associated proteins identification.

With regards to T extract, we found, as already demonstrated by Rigby *et al.* (2008) that allergens Cor a 9 and Cor a 11, when subjected to electrophoretic separation in not-reducing condition, were distributed in different subunits, corresponding to mass from 10 to 65 kDa. Cor a 9 was identified in all the bands considered (from T1 to T14), together with Cor a 11 in bands T5, 9,10,11,12,13,14, with Cor a 14 in band T2 and with Cor a 8 in band T3 (Table 2).

Cor a 9 is a not glycosilated seed storage globulin, belonging to the cupin superfamily. It is formed by an alkaline and an acidic chain, which are contained in bands T11 and T12, respectively. The polypeptides at lower mass (from 10 to 40 kDa, from bands T1 to T10) are probably highly proteolyzed Cor a 9 subunits, as already found by Rigby *et al*. (2008).

Cor a 11, the vicillin-like protein, is a glycosylated storage globulin, also belonging to the cupin superfamily. Its mature subunit is contained in bands T9 and T10 (around 48 kDa), while the smaller polypeptide around 25 kDa (band T5) could correspond to the subunit proteolitically processed in the seed. The higher bands (from T11 to T14) probably contained the glycosylated form of unprocessed subunits (Rigby *et al*., 2008).

Cor a 14, the 2s albumin, belonging to the prolamin superfamily, is a small globular protein characterized by 4 disulphides bond. It was identified in T2 band along with Cor a 9, but it contributed to 67% of the band, according to molar fraction (Table 2).

Cor a 9, Cor a 11 and Cor a 14 immunoreactivity was demonstrated to be stable after roasting treatment, in different studies (de Leon *et al*., 2003; Dooper *et al*., 2008; Müller *et al*., 2000; Pastorello *et al*., 2002; Pfeifer *et al*., 2015; Schocker *et al*., 2000; Wigotzki, Steinhart, & Paschke, 2000). Concerning specifically Cor a 9, it has been demonstrated that its beta-barrel-motif play a key role in stability retention after thermal treatment as well as to digestion (Moreno & Clemente, 2008).

Band T3, one of the most immunoreactive bands, identified as a mix of Cor a 8 and Cor a 9 seemed to be also one of the most temperature-sensitive. In raw samples, it was recognized by three pools of sera (A, B and D) and not from pool C (formed by patient sera not containing IgE towards Cor a 8, Table 1). Band T3 lost immunoreactivity after processing, when moving from LT to HT treatments. Cor a 8 (non-specific lipid transfer protein, nsLTP) is probably the responsible for this behavior, since it accounts for 80% of the band, according to molar fraction, and being nsLTP allergenicity significantly affected by high temperature treatments. López *et al*. (2012) demonstrated that the IgE binding to Cor a 8 was strongly affected by high temperatures and wet processing (121°C and 138°C in autoclave, for 15 and 30 min, respectively), since autoclaving induces the disorganization of protein epitopes.

As described previously in Chapter 2, also OB protein profile was affected by processing. Differently from T protein extract, in O protein

extract for both LT treatments, the number of protein bands increased compared to the raw sample; three new bands, hardly visible in raw sample, appeared between 45 and 60 kDa (O6-O8). These bands, resulting from post-processing aggregation were weakly immunoreactive, since they did not contain Cor a 15, the most immunoreactive protein in O fraction, as demonstrated in Chapter 2, for an Italian pediatric cohort. The overall most immunoreactive bands in OB extracts were O1, O2, O4, O5, containing mainly Cor a 15, Cor a 12 and 13 (Table 3).

3.2.4 Effect of thermal treatments on hazelnut structure

Microscopical observations have been performed with the aim of verifying the impact of roasting on the internal organization of the cells in hazelnut seeds. CBB, PAS and Nile red stains were used to study the distribution of proteins, polysaccharides and storage lipids, respectively. After staining with CBB, protein bodies of different sizes are clearly evident inside the cells of mature seeds (Fig 5 A). These protein bodies have a high number of protein inclusions also varying in size and shape that appeared as white dots after CBB staining. These inclusions may consist of crystal globoids, protein crystalloids or calcium oxalate crystals (Dourado *et al*., 2003). A broad and faint polysaccharide cytoplasmic staining was found after using PAS, while more intense staining was found in cell walls (Fig 5 B). Nile Red staining perfectly shaped OBs (arrows) (Fig 5 C). The same staining treatment also led to a signal on some big masses that fill the cell only in specific regions of the hazelnut seed, showing their lipidic nature (Fig 5, inset).

Looking at the roasted samples (Fig 5 D-O), the major impact on protein bodies is present after treatment with HA-HT and IR-HT, where the samples appeared untangled with respect to the raw sample (Fig 5 G, M). Additionally, in HA-HT sample, cell walls appeared to be wavy with respect to the other samples, as well as by the staining with PAS. Starch, which is also positive to PAS reaction, was not detected in any sample. Regarding lipid localization, staining with Nile Red is distributed in the cells, suggesting that neutral lipids fill up most of the cell cytoplasm. They seemed to be mixed with protein fraction, suggesting that compartmentalization of the oil was lost. Lipid vesicles attached to the external cell wall of the epidermis are also visible, mainly after HA-LT treatment.

At electron microscopy level, in mature seeds PBs and OBs were occupying most of the cytoplasm, and OBs were found as directly surrounding PBs (Fig 6 A, B). Looking at the cells after the roasting, it is evident a loss of sub-cellular organization with a disappearing of the OBs (Fig 6 C). In some cases, e.g. in the HA-HT sample, OBs can come together to form a big lipid droplet (Fig 6 D), while protein bodies lost their typical features.

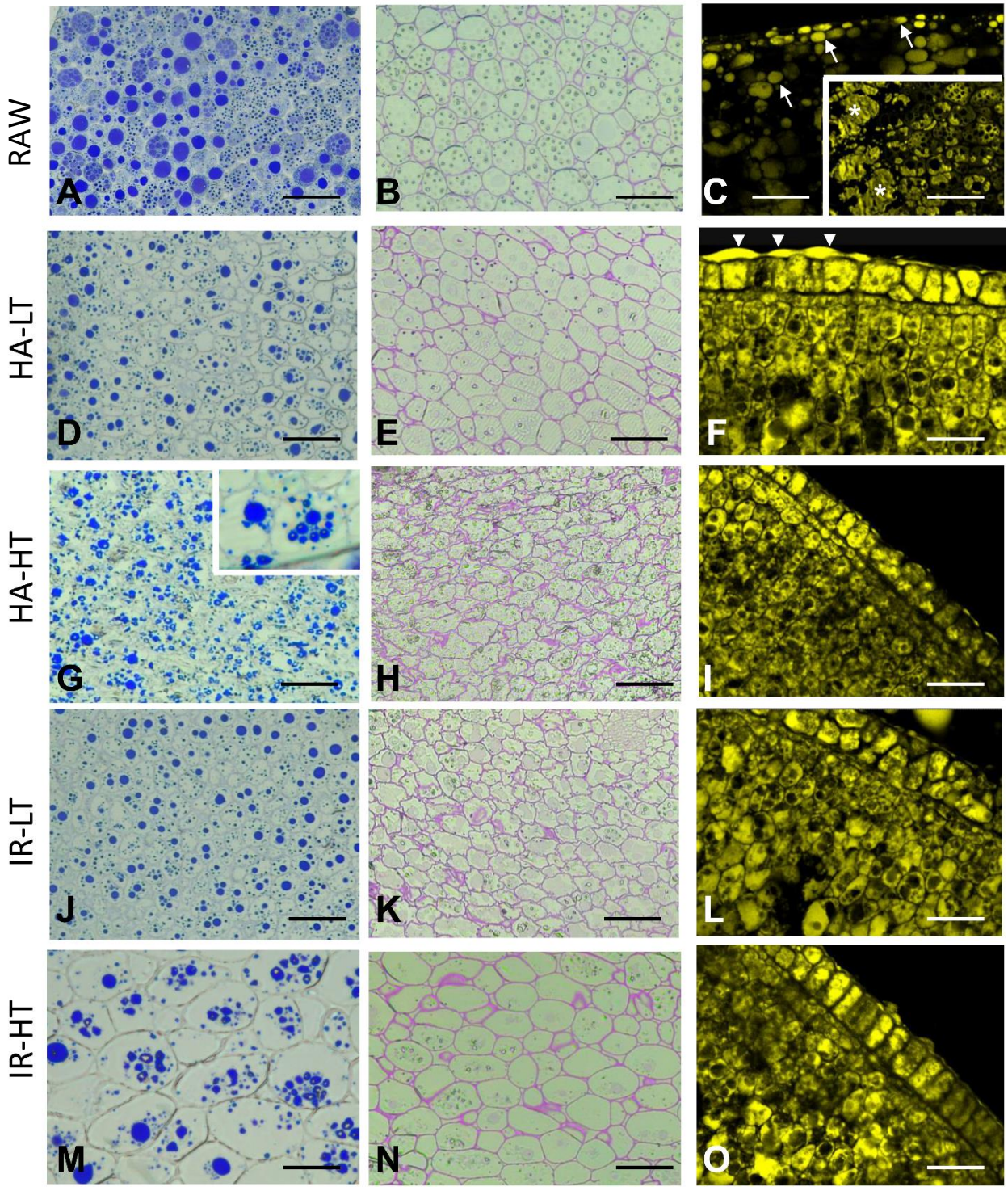

Fig 5. Microscopical observations of raw and roasted (HA-LT, HA-HT, IR-LT and IR-HT) hazelnut seeds. (A-D-G-J-M) Coomassie Brilliant blue (CBB) staining of total proteins; in insert a detail of protein bodies modification after roasting; (B-E-H-K-N) PAS staining: in pink the cell wall material; (C-F-I-L-O) localization of oil bodies (in yellow) by confocal laser scanning microscopy in cells stained with Nile Red; in the insert, a detail of a specific region of the hazelnut seed marked with asterisks; oil bodies are indicated with arrows. Bars = 80 µm in A, B, C, D, E, G, H, I, J, K and 50 µm in F, L, M, O.

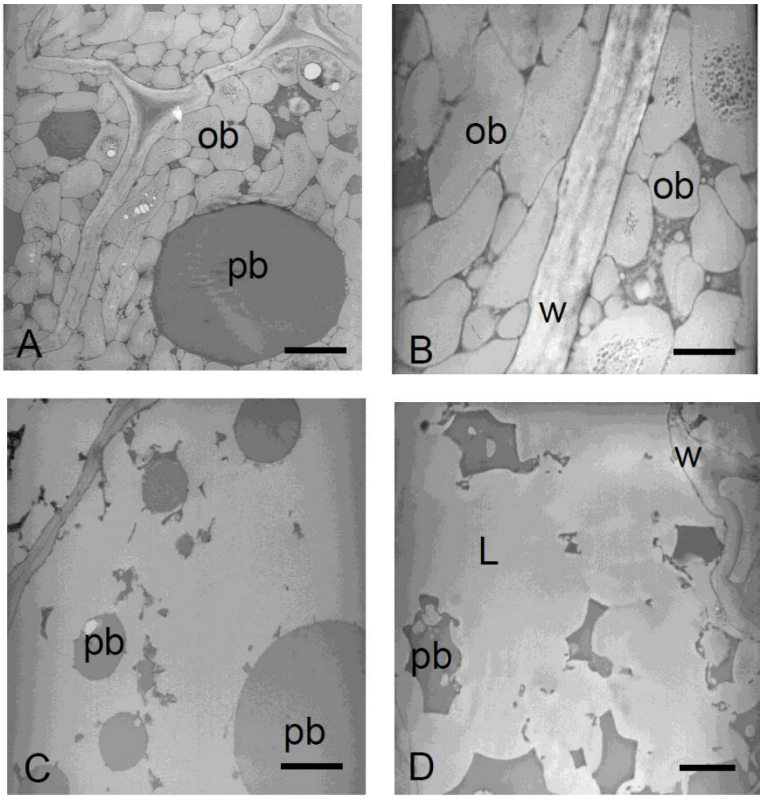

Fig 6. TEM micrographs of the hazelnut cells; A-B: raw sample; C-D: HA-HT sample; pb: protein bodies, ob: oil bodies, L: big lipid droplet, W: cell wall. Bars = 3 µm in A, 1 µm in B, 2 µm in C, D.

3.2.5 Conclusion

Considering the overall effect of processing on hazelnut microstructure, microscopical analyses suggested that cell organization was markedly affected by the heat treatments, regardless the roasting method considered. In our study, roasting caused cytoplasmic network disruption with loss of the compartmentalization of lipid in OBs and alteration of protein bodies and cell wall organization. At molecular level, high temperature roasting affected allergenicity, reducing immune recognition of each allergen to different extent, depending on single protein thermal stability. Overall, hot air roasting is the most conservative processing, while infrared treatment, especially at high temperature, showed to reduce hazelnut immunoreactivity. Thermal processing of hazelnuts showed allergen-, temperature- and time-specific effects.

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CHAPTER 4

IDENTIFICATION OF ALLERGENIC PROTEINS INVOLVED IN PRIMARY RESPIRATORY AND FOOD ALLERGY TO YELLOW MEALWORM (*TENEBRIO MOLITOR*)

Aim of the Work

Mealworm (*Tenebrio molitor*) has great potential as feed and food in many European countries. A better investigation on mealworm proteins role on the development of respiratory allergy among breeders and of food allergy among consumers, is currently necessary.

The aim of this work was to identify the potentially allergenic protein of *T. molitor* larvae and feces involved in primary respiratory and food allergy in two subjects employed in *T. molitor* breeding.

Abstract

Proteins were extracted from both *T. molitor* larvae and feces. BAT and immunoblot analysis were performed with the sera of the two allergic subjects. The immune-reactive bands were identified by means of LC-MS/MS.

BAT was positive for both patients considering both samples. Immunoblotting showed a 15 kDa reactive band in both extracts. In *T. molitor* feces extract the immunoreactive protein was identified as the cockroach allergen-like protein (CAL). In *T. molitor* larvae extract, in addition to CAL, Troponin C and 86 kDa early-staged encapsulation protein were identified.

The patients appeared to be immune-reactive to *T. molitor* larvae because of inhalation/contact with feces. The protein most responsible for the observed allergic reaction is CAL, which has never before demonstrated to be implicated in allergy to *T. molitor*.

4.1 Methods

4.1.1 Clinical patient characterization

We here report the case of two patients (Pt#1 and Pt#2, both male, 24 and 27 years old, respectively) employed in the production of insect flour made of yellow mealworm larvae (*Tenebrio molitor* larvae, TML) and black soldier fly larvae (*Hermetia illucens* larvae, HIL). The patients did not show previous history of food or respiratory allergies. In the same factory, a total of ten workers handled TML, eight out of ten without developing any symptom. The first symptoms of the two patients (rhinoconjunctivitis, itching and contact erythema) appeared after some weeks of repetitive exposure to TML. They did not report any symptoms when exposed to HIL. The symptoms were particularly severe during the sifting of TML from their feces (TMF), and when the patients entered the rearing room after the sifting operation, but they disappeared after 24 hours. The sensitization therefore presumably occurred through a percutaneous route and/or by inhalation. After developing symptoms, Pt#1 continued to work using adequate protection devices, while Pt#2 left the job. The two patients were used to eat such edible insects as the greater wax moth, crickets and HI. They experienced an oral allergy syndrome (OAS) characterized by oral pruritus and tightness in the throat, as soon as they start eating for the first time a TML hamburger. Symptoms recovered spontaneously in about 40 minutes. Following this episode, they continued to tolerate other edible insects, but never tried to taste TML again and refused to undergo provocation test.

Skin prick tests (SPTs) were performed with standard inhalant allergens and food allergens. Levels of sIgE to mealworm, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, Der p10 and prawn were determined by ImmunoCap (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions wit positive cut-off at 0.10 KUA/L. Blood and sera from the two patients and also from a negative control were collected by Mauriziano Hospital of Turin.

4.1.2 *Tenebrio molitor* larvae and fecal extract preparation

The *Tenebrio molitor* larvae were purchased from Gaobeidian Shannong Biology CO., LTD (Gaobeidian, Hebei province, China). One gram of both samples (TML and TMF) were extracted for 3 hours with 5 ml of 0.1M PBS pH 7.4 with sonication each 30 min. After centrifugation at 16300xg, for 30 min at 4°C, the supernatant was collected and again centrifuged. After centrifugation, the supernatant was subsequently cleaned using a 2-D Cleanup Kit (Biorad, Hercules, USA) and the protein concentration was determined by means of a 2-D Quant-Kit (GE, Boston, USA).

4.1.3 Basophil Activation Test (BAT)

BAT was performed according to a previously reported technique (Giorgis *et al*., 2018). Briefly, endotoxin-free heparinized whole-blood samples were obtained from the allergic patients and from a healthy control. Cells were challenged with 100 µl of TML, a TMF protein extract and an anti-IgE (10µg/ml; clone G7-18; BD Bioscience, USA) for 20 min at 37°C in a water bath. fMLP (0.5µg/ml; Sigma Aldrich, Italy) and Tyrode (Sigma Aldrich), 20µM HEPES in 7.5% NaHCO3 (pH 7.4) were used as positive and negative controls, respectively.

Basophils were stained with 5 µl of anti-human CD3 Pacific Blue (Beckman Coulter, USA), 10 µl of anti-human CD63-FITC (clone H5C6; BD Biosciences), 10 µl of anti-human CD203c-APC (clone NP4D6; BioLegend) and 10 µl of monoclonal anti-human CD294 (CRTH2) PE (Beckman Coulter, USA) for 15 min on RT. Flow-cytometric analyses were performed in a NAVIOS flow cytometer (Beckman Coulter, USA). We gated on physical parameters forward (FS) and side (SS) scatter to exclude debris. We then gated on CRTH2 (CD294) positive/CD3 negative cells to isolate basophils.

4.1.4 LDS-PAGE

Five µg of protein sample were diluted in LDS Sample Buffer (Invtrogen, Life Technologies Ltd., Paisley, UK) under reducing conditions (with 2% of a NuPAGE Sample Reducing Agent), and separated with 10% NuPAGE mini gels in MES Running Buffer (Invitrogen), according to the manufacturer's protocol. The gel was then fixed in 30% ethanol and 10% ortophosphoric acid, for 2 hours, stained in Colloidal Coomassie Blue and scanned using a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

4.1.5 Immunoblotting

Immunoblotting was performed as described in paragraph 2.1.5.

4.1.6 Mass spectrometry analysis (ESI-Q-TOF) and protein identification

In gel digestion and mass spectrometry (ESI-Q-TOF) analysis were performed as described in paragraph 2.1.6.

The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston) using the NCBI Coleoptera database. Trypsin was specified as a digestion enzyme with three missed cleavages. The following parameters were set for the searches: an S-carbamidomethyl derivate on cysteine as a fixed modification and oxidation on methionine, Acetyl (N-term) and Met-loss (Protein N-term M) as variable modifications. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance was set

at 0.1 Da. Only proteins with at least three peptides and with a peptide score > peptide identity were considered for identification purposes.

4.2 Results and Discussion

The SPTs with inhalant and food allergens were negative in both patients, except for Grass in Pt#1 and Alternaria in Pt#2. Specific TM IgEs were present in both patients (21.00 and 10.63 kUA/L, respectively), while no specific IgEs were found for house dust mites, Prawn and Der p10. BAT was positive in both subjects for TMF (CD63+ cell: 67.9% in Pt #1, 79.5% in Pt #2) and TML (CD63+ cell: 74.2% in Pt #1, 79.3% in Pt #2) and negative in a healthy control subjec (Fig 1).

Immunoblotting showed a 15 kDa reactive band in both the TML and TMF extracts, when incubated with both patients' sera (Fig 2, panel A). Three additional aspecific bands were also detected in the healthy control patients' serum pool, but only in the TML extract (Fig 2). The cockroach allergen-like protein (CAL) was identified in the TMF sample, while, in addition to CAL, Troponin C (TnC, from *Tribolium castaneum*) and the 86 kDa early-staged encapsulation protein (ESE) were identified in the TML extract (Fig 2, panel B).

Occupational allergies following inhalation and/or contact exposure to TM have been reported in several case reports (De Gier *et al*., 2018, Broekman *et al*., 2017). Broekman *et al*. (2017) described the case of four breeders who developed respiratory allergy to TML, two of whom had a mild allergic reaction after eating TML. IgE immunoblot showed that all the subjects recognized several panallergens (e.g. tropomyosin, arginine kinase) but also a larval cuticle protein, which seemed to be the most probable dominant allergen, as the 4 subjects had a primary sensitization to TML and tolerated crustaceans.

In our two patients, IgE binding was shown toward TnC and ESE in TML, and toward CAL in both TML and TMF. TnC is an Arthopoda panallergen and has been characterized as a minor allergen in shrimps (Ruethers *et al*., 2018), whereas it is an important cockroach allergen (Bla g 6, Per a 6) (Glesner *et al*., 2018). Bla g 6 from *Blattella germanica* showed a 78.84% identity with TnC from TM (https://www.uniprot.org/blast).

ESE is a defense response protein against invading parasites and pathogens, and it requires subsequent cutting steps to be activated (Cho *et al*., 1999). We found a degraded fragment of this molecule with a mass of about 15 KDa, corresponding to the C-terminal Hemocyanin domain. Hemocyanin has already been reported to be a minor allergen in crustacean allergies (Giuffrida *et al*., 2014).

CAL is a nitrile-specifier protein (NPS) with a detoxifying function localized in the midgut microvillar part of the insect (Ferreira *et al*., 2007). During digestion, CAL is released into the gut lumen, cut into fragments by trypsine-like enzymes and secreted in the stools. The most similar NPS to CAL is Bla g1 (37.6% identity, https://www.uniprot.org/blast), one of the major allergens of *Blattella germanica*. Bla g 1 is labile to heat (Teifoori *et al*., 2017) and its genes

consist of a duplex that is multiplied to create a repeated motif. A great genetic variability, in terms of the number of repeats (4-14), has been reported. The molecular weight of this allergen varies from 6 to 37 KDa, because of the action of the trypsin-like enzymes in the gut, and it is therefore considered as an "isoallergen" (Mueller *et al*., 2013). The sensitization in our two patients could have occurred by inhalation/contact with stools, as happens to patients allergic to cockroaches and house dust mites.

In light of the clinical history and the results of the SPTs and specific IgEs, we can assume a primary sensitization to TM in our subjects, given the lack of sensitization to food allergens (crustaceans) and inhalants (mites). We consider that CAL could be the allergen that was responsible for the respiratory and food allergy in our patients, since it is present in both body and insect feces and its heat-lability could explain the type of food allergic reaction (OAS) that occurred. Moreover, the ability of patients to tolerate other edible insects and crustaceans makes the involvement of such panallergens as TnC and ESE less likely. However, we cannot exclude a possible involvement of these proteins in sensitization, perhaps after mealworm intake. Interestingly, the proteins we identified are different from those identified by Broekman and coworkers (2017), probably because different routes of sensitization are involved in TM food allergies.

In conclusion, our patients probably developed allergy to TML because of inhalation/contact with feces. The allergen candidate considered most responsible for the observed food allergic reaction could be CAL, which, to the best of our knowledge, has never before been implicated in allergy to TM.

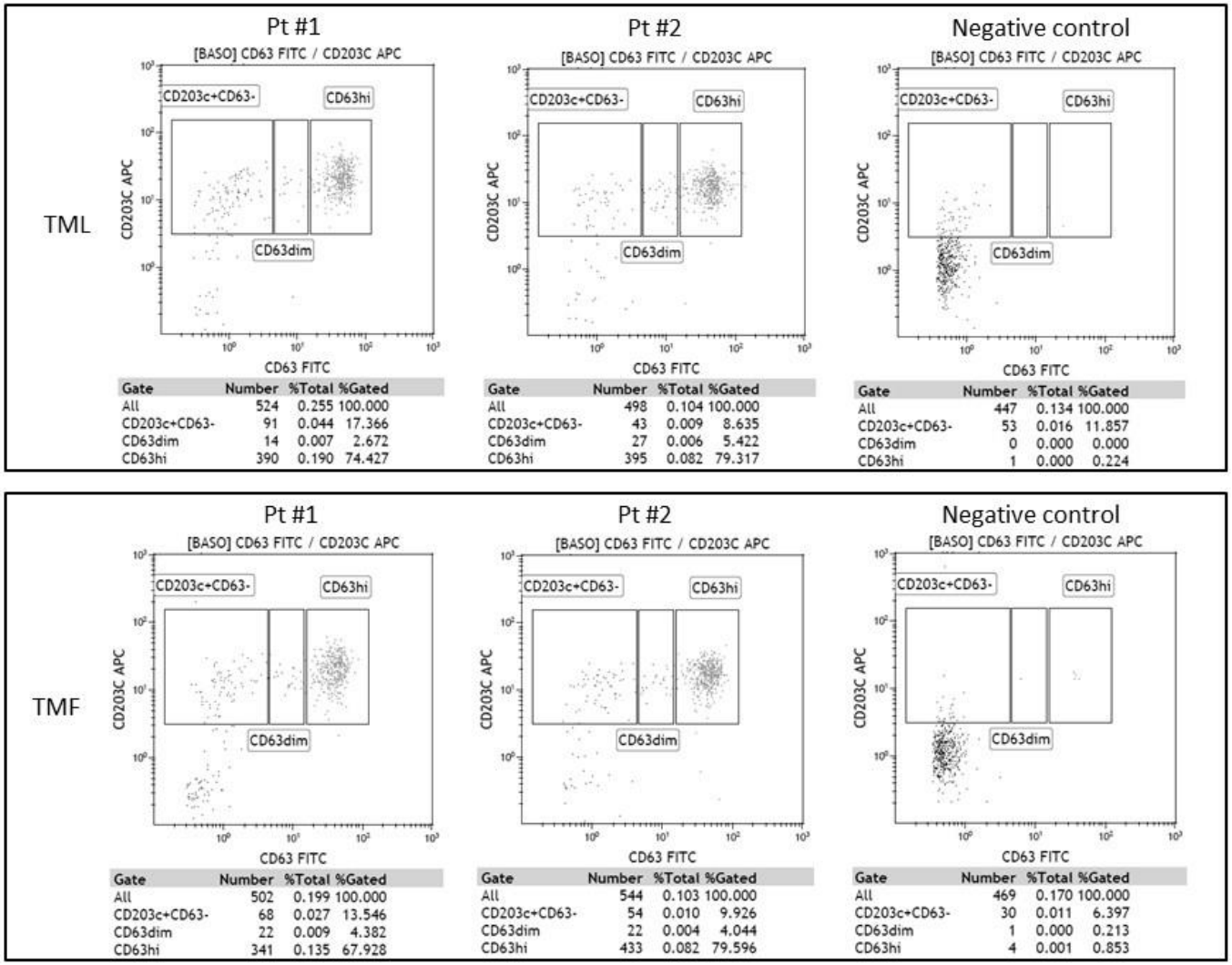
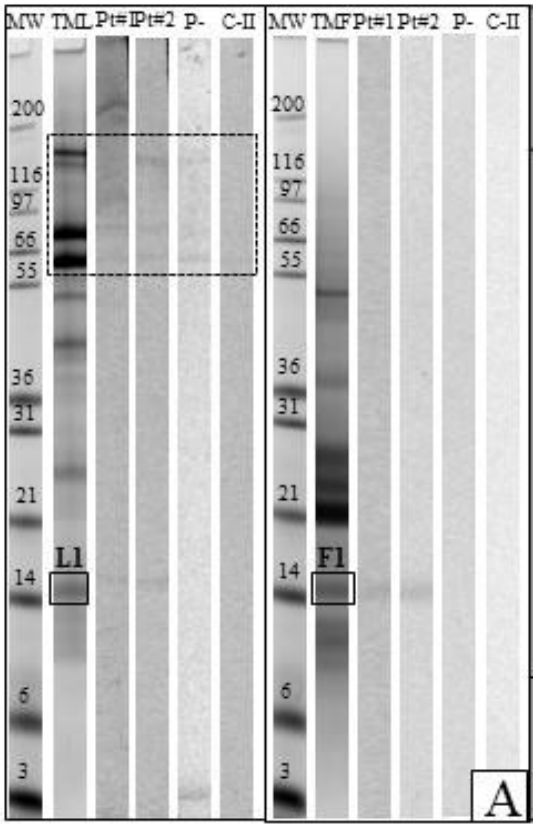


Fig 1. BAT experiment with TML and TMF protein extract for Pt#1 and Pt#2, compared with a negative control (not insect –sensitized not allergic patient). Boxes and tables reports the cytofluorimetric quantification of the activation (CD203c) and degranulation (CD63) markers of basophils from patients sera, after the contact with the allergens (TML and TMF). In Pt#1 and Pt#2, the CD63hi degranulation marker is highly activated (more than 50% of total basophils), while it is almost undetectable in the control patient, proving the allergenicity of the TMF and TML protein extracts for patients Pt#1 and Pt#2.



Band	Entry (UniProt)	Name	MW exp/ MW theor (Da)	Protein Score	N° of matching peptides	Protein coverage (%)
L1	AAP92419.1	Cockroach allergen-like protein (*Tenebrio molitor*)	15000/65441	1011	9	20.8
	BAA81665.2	86 kDa early-staged encapsulation inducing protein (*Tenebrio molitor*)	15000/90680	278	4	9.4
	EFA10458.1	Troponin C, isoform 2-like Protein (*Tribolium castaneum*)	15000/17593	255	5	30.9
F1	AAP92419	Cockroach allergen-like protein (*Tenebrio molitor*)	15000/65441	473	7	14.3

B

Fig 2. Panel A: TML and TMF protein immunoblotting with Pt#1 and Pt#2 sera. MW: molecular weight; P-: negative patient's sera; C-II: negative control without serum; the identified bands are in the continuous box; the aspecific bands are in the dotted box. **Panel B:** L1 and F1 protein bands identification.

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CHAPTER 5

INFLUENCE OF PROCESSING ON THE ALLERGIC CROSS-REACTIVITY OF FIVE EDIBLE INSECTS

Aim of the Work

House dust mites and shrimps share some ubiquitous proteins that are widely distributed among arthropods (including insects) and which could be responsible for cross-reaction phenomena in allergic patients. The current work was aimed at investigating the cross-allergenicity of Italian patients, primarily allergic to shrimp, house dust mites and mealworm, towards five edible insects (mealworm, buffalo worm, silkworm, cricket and grasshopper), with particular emphasis on the effect of thermal processing (boiling and frying) on cross-allergenicity.

Abstract

Five edible insects were investigated: buffalo worm (*Alphitobius diaperinus*), mealworm (*Tenebrio molitor*), silkworm (*Bombyx mori*); cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria*). The water-soluble and -insoluble proteins were extracted from raw and processed (boiled and fried) insects and the protein profiles were analyzed by LDS-PAGE. The IgE cross-reactivity of shrimp (n=8), house dust mite (n=28) and primary mealworm sensitized (n=2) allergic patients towards the five raw and processed edible insects were screened by means of dot-blot. Cross-reactive proteins were identified by immunoblotting followed by LC-MS/MS analysis for immune-reactive proteins identification.

Buffalo worm, mealworm and cricket showed similar protein profiles for raw and boiled extracts, while grasshopper and silkworm differed for both processing methods. For all insects, proteins aggregation increased with increasing processing temperatures, causing an enrichment of the insoluble protein fraction. An overall decrease in allergic patient positivity to dot-blot was found after processing. In the considered cohort of allergic patients, the most cross-reactivity proteins were tropomyosin and myosin.

Based on our results, house dust mites, shrimp and mealworm allergic patients should consume insects with caution, since different proteins, some of them thermostable, are involved in insect's cross-sensitization.

5.1 Methods

5.1.1 Materials

Buffalo worms (*Alphitobius diaperinus*) and mealworms (*Tenebrio molitor*) larvae and adults cricket (*Gryllodes sigillatus*) and grasshopper *(Locusta migratoria*) were purchased from a company that produces edible insects containing foods (Eat Grub, UK). Silkworm larvae (*Bombyx mori*) from the germplasm collection of the CREA Research Centre for Agriculture and Environment (Padova, Italy) were grown on an artificial diet as described previously by Lamberti *et al*. (2019). Insects were considered: a) raw, b) boiled for 5 min at 100°C and c) fried for 3 min a 180°C in sunflower oil.

5.1.2 Sera patients

Patients with convincing sign of house dust mites (HDM) allergy and positive IgE RAST to HDM (HDM patients, n=28), and patients with convincing sign of shrimp allergy and positive IgE RAST to shrimp (SH patients, n= 8) were recruited by Ordine Mauriziano Umberto I Hospital in Torino and included in this study.

Two additional subjects with primary respiratory and food sensitization to mealworm (PM patients) previously described in Chapter 4, were also included in this study. All subjects gave a written informed consent.

5.1.3 Insects protein extraction

One gram of lyophilized and boiled chopped insects were extracted with 5 ml of PBS (0.1M pH 7.4) and a table of CompleteTM per 50 ml of buffer as protease inhibitor (Sigma-Aldrich S.r.l., St. Louis, MO, USA). Each sample was sonicated (40 MHz for 30s) on ice for seven cycles, with 30 min of break under agitation on ice after each cycle.

One gram of fried chopped insects was mixed with 10 ml of hexane in order to remove the residual frying oil. After 30 min of shacking in ice, hexane was removed. This step was repeated three time and then sample was dried in Speedvac for 30 min at room temperature. One gram of defatted fried insect was extracted with 5 ml of PBS (0.1M pH 7.4, with protease inhibitor) using the Polytron tissue homogenizer (Type PT 10-35; Kinematica GmbH, Luzern, Switzerland) (4 step on ice, each steps: 10 sec on and 10 sec off).

After the sonication step for raw and boiled samples and the Polytron step for fried samples, all the extracts were centrifuged at 16300xg for 30 min at 4°C. The upper phase was recovered and centrifuged again in order to remove the impurities and obtained the water soluble proteins extract (W). The pellet was washed twice with PBS and extracted over night at 4°C

with Urea (6M). The urea soluble protein extract (U) was collected after centrifugation (16300xg, 30 min, 4°C). W and U fractions of raw cricket and grasshopper were subjected to a precipitation step by using the methanol/chloroform protocol as described previously by Wessel & Flügge, (1984) while W and U fractions of fried insects were cleaned with ReadyPrep™ 2-D Cleanup Kit (Biorad, Hercules, California, USA). Protein contents were determined by means of the 2D-Quant-kit (GE Healthcare, Chicago, IL, USA).

5.1.4 LDS-PAGE

LDS-PAGE was performed as described in paragraph 4.1.4.

5.1.5 Dot-blot screening patients

Dot-blot screening was used to test insect extracts recognition by allergic patient sera. Dot-blot was performed in triplicate. One µl of mixed (1:1) W and U fraction at final protein concentration of 0.5 µg/µl was spotted on Nitrocellulose Membrane (0.2 µm) and let dry for 30 min, at room temperature. Membranes were blocked with TBS with 0.3% Tween 20 for 30 min and incubated o.n., at 4°C, with the patient sera diluted 1:10 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin). Sera were removed and the membranes were washed three times with the washing solution (TBS, 0.05% Tween 20) for 10 min for each step. Membranes were incubated for 1 hour at RT with an anti-Human IgE antibody (SeraCare Life Sciences Inc., Milford, Massachusetts), diluted 1:5000 in the incubation buffer. Then, the membranes were washed three times with the washing solution. Immunoreactivity was developed with an Alkaline Phosphatase Substrate Kit (Bio-Rad).

Membranes were scanned using a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi and the colour intensity of reactive droplets were quantified using Imagelab 4.1 software (Bio-Rad). The value obtained from the quantification of each reactive droplet colour intensity was divided for the value of the corresponding non-allergic volunteers reactive droplet. Patients recognizing at least twice of the three replicates with a colour intensity value higher than 1.8 times the non-allergic volunteers droplet colour intensity were inserted in the pool of patients used for immunoblotting experiment on the corresponding insect protein extract (arbitrary value).

5.1.6 Immunoblotting

For the immunoblotting analysis W and U fractions of each samples were mix 1:1. Immunoblotting was performed as described in paragraph 2.1.5.

5.1.7 Mass spectrometry analysis (ESI-Q-TOF) and protein identification

In gel digestion and mass spectrometry (ESI-Q-TOF) analysis were permormed as described in paragraph 2.1.6.

The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston) using the following NCBI databases: Tenebrionoidea for mealworm and buffalo worm, *Bombyx mori* for silkworm and Polyneoptera for cricket and grasshopper. The following parameters were set for the searches: trypsin as digestion enzyme with three missed cleavages, S-carbamidomethyl derivate on cysteine as a fixed modification and oxidation on methionine, Acetyl (N-term) and Met-loss (Protein N-term M) as variable modifications. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance was set at 0.1 Da. The charges of the peptides (on a monoisotopic mass) were set at 2+, 3+ and 4+. Only proteins with peptide score > peptide identity, with at least three peptide and with a score higher than 15% of the total band's score were considered for the identification purposes.

5.1.8 Determination of the allergenic potential of reactive proteins

The allergic potential of proteins identified in the reactive bands was verified using Allermatch™ (http://www.allermatch.org). Comparison in Allermatch™ was based on UniProt as well as the WHO/IUIS database. Search were performed using the parameters described by van Broekhoven *et al*. (2016): proteins were considered only when more than 35% of the protein identity matched with a known allergen in a window of 80 amino acid. Only the first three matched allergens belonging to HDM, shrimps and insects were taken into account.

5.2.Results

5.2.1 Effect of processing on protein solubility

The protein profiles of raw, boiled and fried mealworm (*Tenebrio molitor*), buffalo worm (*Alphitobius diaperinus*), silkworm (*Bombyx mori*), cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria*) are shown in Figure 1. Two different protocols were applied in order to optimize both the extraction of water-soluble (W) and urea-soluble (U) proteins.

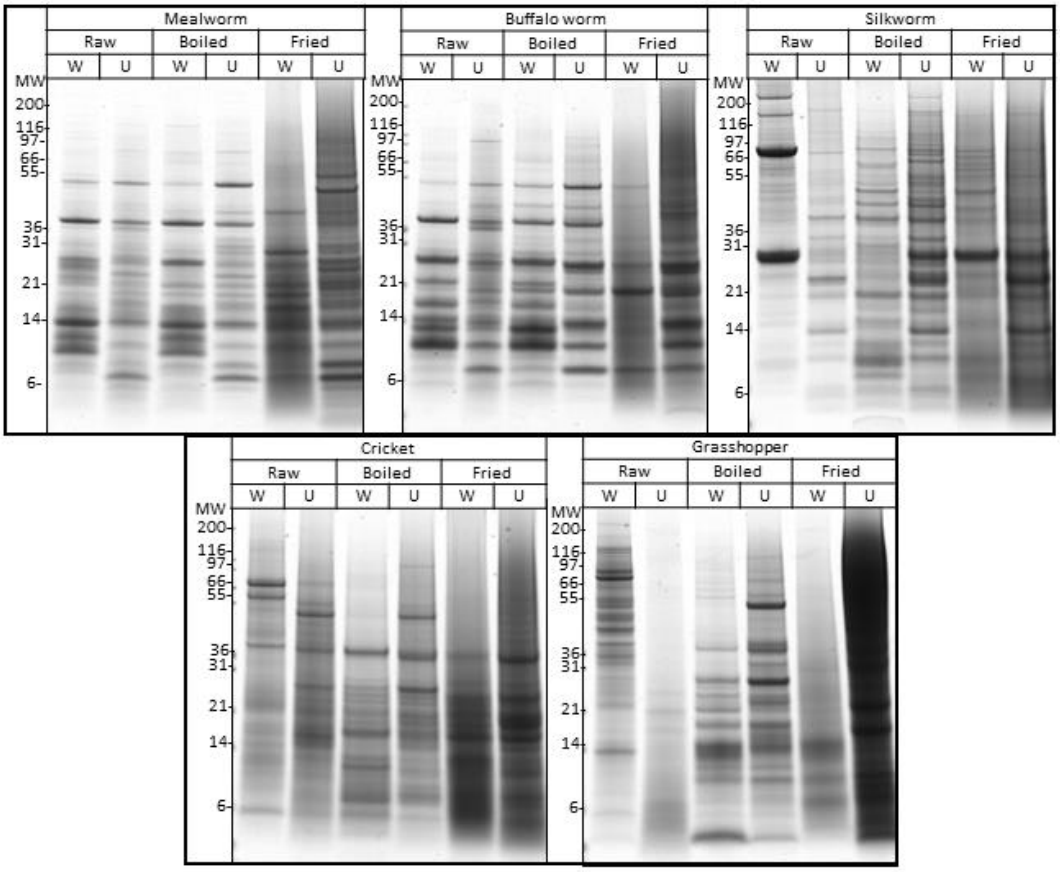

Fig 1. LDS-PAGE of water-soluble (W) and urea-soluble (U) insect proteins extract. MW: molecular weight.

Mealworm and buffalo worms were characterized by similar protein profile, both in raw and boiled samples. Differently, in silkworm and grasshopper, boiling caused an increase in the number of protein bands in U fraction. In cricket, boiling resulted in the disappearance of the two major bands around 55-65 kDa in the W fraction. For all the insects, fried protein profiles differed strongly if compared to raw and boiled extracts. The presence of sunflower oil and the high temperature of processing

(180°C) account for a reduction in protein solubility. The reduction of the number of protein bands in the W fraction was accompanied by the appearance of some smearing in U extracts, likely indicating the presence of insoluble protein complexes, especially in grasshopper and cricket extracts.

5.2.2 Screening for sera by dot-blotting

The patient sera reactive to insects' proteins were selected by dot-blot (Table 1). 71% of HDM allergic patients and 87% of shrimp allergic patients recognized at least one insect extract in dot-blot.

As regards to HDM allergic patients, raw buffalo worm extract was the most immunoreactive (reaching 57% of positivity to dot-blot), while the remaining 4 insects raw extracts showed a positivity, ranging from 18 to 32%. An overall decrease in HDM allergic patient positivity to dot-blot was found after processing (both boiling and frying) for buffalo worm and cricket, even though a percentage of patients remained positive (between 11 to 36%). The same behavior was observed for mealworm and grasshopper, after frying. Comparing mealworm and grasshopper (raw *vs* boiled samples), boiling showed opposite effect: a decrease of cross-reactivity in mealworm and an increase in grasshopper were detected. Processing slightly affect silkworm immunoreactivity, only after frying, while the number of positive patients remained stable after boiling.

As the shrimp, allergic patients are concerned, taking into account the small number of patients that it has been possible to recruit (8 patients), positivity to dot-blot appeared lower than for HDM allergic patients. No shrimp allergic patient recognized raw cricket, fried grasshopper and silkworm either raw or processed, thus these protein extracts were not tested in immunoblotting. Only 1 shrimp allergic patient immunorecognized fried cricket (SH1), raw and processed mealworm (patient SH7) and raw and processed buffalo worm (patient SH3). Only for fried buffalo worm, boiled cricket and raw and boiled grasshopper, it was possible to pool patient sera, with a number of sera between 2 and 4. Grasshopper proteins extracts resulted to be the most cross-reactive with around half the patients positive to dot-blot.

Both the two primary sensitized to mealworm (TM) subjects showed positivity to silkworm and raw mealworm extract and one of them (TM1) lost immunoreactivity to mealworm after processing. Neither patient was immunoreactive to buffalo worm. One of them (TM2) was positive to fried cricket and processed grasshopper extracts, while the other one (TM1) was positive only to raw grasshopper extract.

		Mealworm			Buffalo worm			Silkworm			Cricket			Grasshopper		
	N° of patients	R	B	F	R	B	F	R	B	F	R	B	F	R	B	F
HDM allergic patients	H1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H2	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-
	H3	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+
	H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H5	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	H6	-	-	-	+	-	-	-	-	-	+	+	-	+	+	+
	H7	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+
	H8	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
	H9	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	H10	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	H11	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	H12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H16	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+
	H17	+	+	-	+	+	-	+	+	-	-	-	-	-	+	-
	H18	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-
	H19	-	-	-	+	+	+	-	-	-	+	+	-	+	+	-
	H20	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
	H21	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	H22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H23	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+
	H24	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
	H25	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	H26	-	-	+	+	+	-	+	-	+	-	-	-	-	+	+
	H27	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+
	H28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	6	3	5	16	10	5	5	5	4	7	6	3	9	12	8
		21%	11%	18%	57%	36%	18%	18%	18%	14%	25%	21%	11%	32%	43%	29%
SH allergic patients	SH1	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
	SH2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	SH3	-	-	-	+	+	+	-	-	-	-	+	-	+	+	-
	SH4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SH5	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
	SH6	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
	SH7	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	SH8	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Total	1	1	1	1	1	2	0	0	0	0	3	1	4	3	0
		12.5%	12.5%	12.5%	12.5%	12.5%	25%	0%	0%	0%	0%	37.5%	12.5%	50%	37.5%	0%
TM allergic patients	TM1	+	-	-	-	-	-	+	+	+	-	-	-	+	-	-
	TM2	+	+	+	-	-	-	+	+	+	-	-	+	-	+	+
	Total	2	1	1	0	0	0	2	2	2	0	0	1	1	1	1
		100%	50%	50%	0%	0%	0%	100%	100%	100%	0%	0%	50%	50%	50%	50%

Table 1. Screening of HDM (H1-H28), shrimp (SH1-SH8) and primary sensitized mealworm (TM1-TM2) allergic patients using dot-blot. R: raw; B: boiled; F: fried.

5.2.3 Effect of processing on cross-reactive proteins

Patients with the same primary allergy and positivity to dot-blot screening were pooled in three groups: house dust mite (HDM) allergic patients, shrimp (SH) allergic patients and mealworm (TM) allergic patients. Immunoblotting was performed incubating the three groups of pooled sera with the protein extracts from the 5 insects, each of them considered as raw, boiled and fried. Immunoreactive proteins were identified by mass spectrometry (Fig 2, Table 2 and 3). The cross-reactivity and the effect of processing on cross-reactivity appeared to be variable among the insect species, among reactive proteins and for each of the three groups of considered patients.

Tropomyosin and larval cuticle protein resulted to be the most cross-reactive proteins, being recognized by HDM, SH and TM allergic patients in most of the extracts considered. Tropomyosin was identified only in one band around 36 kDa in mealworm, buffalo worm, silkworm and cricket (bands M3, B3, S4 and C3). Larval cuticle protein was identified in mealworm, buffalo worm, silkworm and grasshopper in multiple bands from 20 to 70 kDa (M1, M2, M4, M5, B1, B4, B5, B6, S2, S3, S5 and G6). It was identified as unique protein in the band or with other potentially cross-reactive proteins (myosin light chain, troponin, 56 kDa early-stage encapsulation-inducing protein, serpin 5 and muscle-specific protein 20). Considering mainly HDM patient sera (larger pool) towards these two proteins, we found that their cross-reactivity was not affected by processing, neither boiling nor frying.

Differently from tropomyosin and cuticle protein, there were some insect-specific cross-reactive proteins like the cockroach allergen-like protein (identified in mealworm), or the vitellogenin precursor and the 30 kDa protein (identified in silkworm). These three proteins were found in single bands: respectively at 14, 190 and 27 kDa. Cockroach allergen-like protein was a cross-reactive protein only for mealworm primary sensitized patients, and its cross-reactivity disappeared after both boiling and frying. Vitellogenin was a cross-reactive protein for HDM and mealworm primary sensitized patients, and its cross-reactivity was not affected by thermal treatments. The 30 kDa protein was cross-reactive only for HDM allergic patients retaining the cross-reactivity even after processing.

Mealworm, buffalo worm and grasshopper myosin was a cross-reactive protein, but it was identified as unique protein only in grasshopper while in the other extracts it was co-migrating with the cuticle protein. When a cross-reactive band contains more than one protein it was not possible to clarify to which protein cross-reactivity was due. As regards to grasshopper myosin cross-reactivity (bands G1, G3 and G5), it appeared resistant to boiling but not to frying, for both HDM and mealworm allergic patients.

Troponin and β-actin were identified as single protein in cricket (bands C1 and C2) and grasshopper (band G4) respectively. They were both cross-reactive for HDM allergic patients and β-actin even for one of the two mealworm allergic patients. Their cross-reactivity was affected by processing, both boiling and frying.

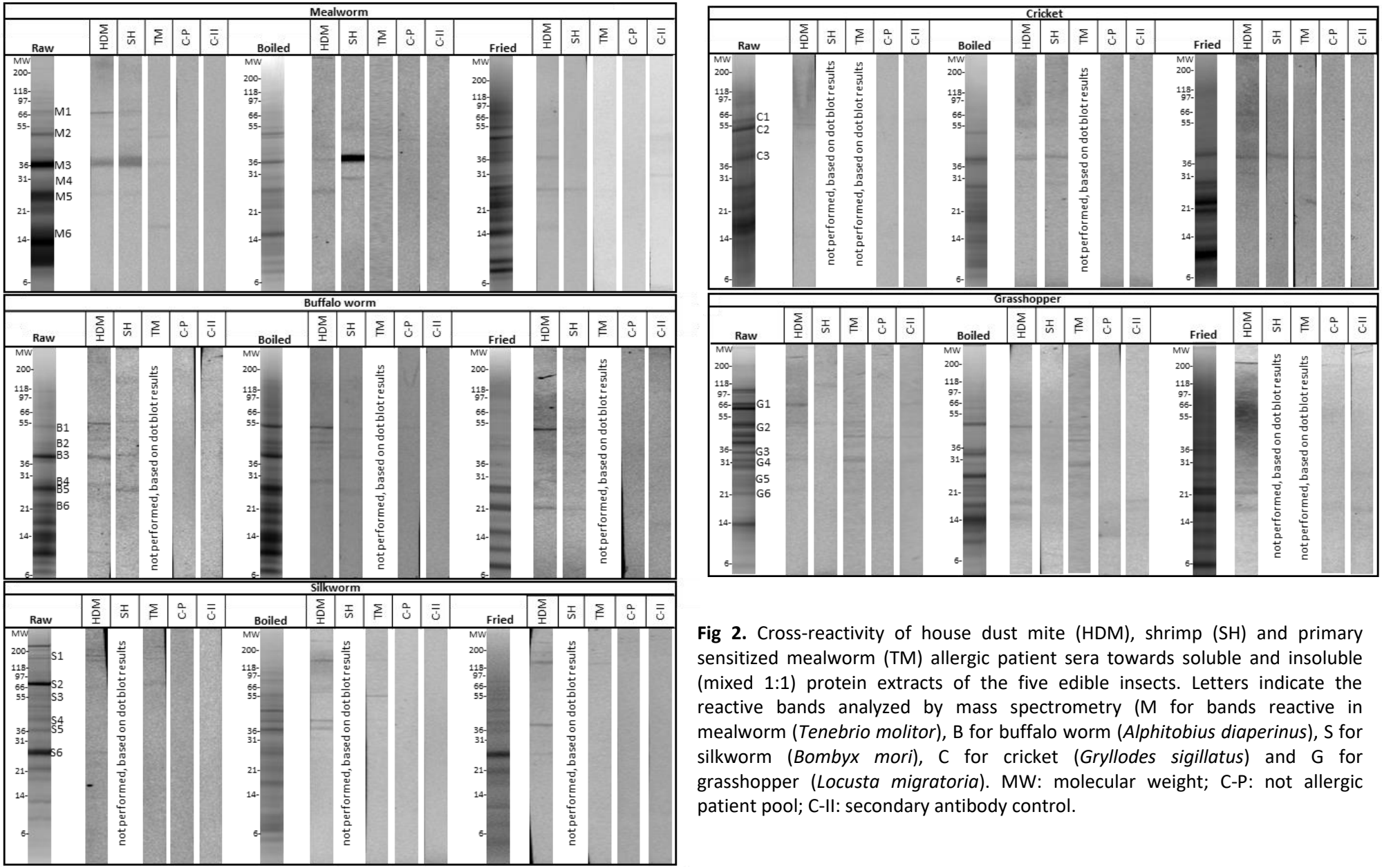

Fig 2. Cross-reactivity of house dust mite (HDM), shrimp (SH) and primary sensitized mealworm (TM) allergic patient sera towards soluble and insoluble (mixed 1:1) protein extracts of the five edible insects. Letters indicate the reactive bands analyzed by mass spectrometry (M for bands reactive in mealworm (*Tenebrio molitor*), B for buffalo worm (*Alphitobius diaperinus*), S for silkworm (*Bombyx mori*), C for cricket (*Gryllodes sigillatus*) and G for grasshopper (*Locusta migratoria*). MW: molecular weight; C-P: not allergic patient pool; C-II: secondary antibody control.

	N° band	Entry (NCBI)	Protein name	Organism	Protein Score	MW theoretical (Da)	MW experimental (Da)	N° of matching peptides	Protein coverage (%)
MEALWORM	M1	P80681,1	Larval cuticle protein A1A	*Tenebrio molitor*	202	17681	70000	3	24,1
	M2	XP_008201464,1	Troponin T isoform X2	*Tribolium castaneum*	291	45878	50000	9	14,3
		P80681,1	Larval cuticle protein A1A	*Tenebrio molitor*	288	17681	50000	4	24,7
		1TMQ_A	Alpha-amylase	*Tenebrio molitor*	223	51704	50000	5	12,5
	M3	XP_008198924.1	Tropomyosin-2 isoform X6	*Tribolium castaneum*	1376	32614	36000	17	57,6
		XP_015839642.1	Tropomyosin-1, isoforms 9A/A/B isoform X13	*Tribolium castaneum*	1091	29366	36000	16	51,8
	M4	BAA78480,1	56 kDa early-staged encapsulation-inducing protein	*Tenebrio molitor*	631	62465	30000	6	13,8
		P80681,1	Larval cuticle protein A1A	*Tenebrio molitor*	465	17681	30000	6	35
	M5	XP_008198303,1	Myosin regulatory light chain 2	*Tribolium castaneum*	235	22231	25000	5	16,5
		BAA78480,1	56 kDa early-staged encapsulation-inducing protein	*Tenebrio molitor*	204	62465	25000	4	7
		P80681,1	Larval cuticle protein A1A	*Tenebrio molitor*	196	17681	25000	6	24,7
	M6	AAP92419	Cockroach allergen-like protein	*Tenebrio molitor*	1011	65441	16000	10	20,8
BUFFULO WORM	B1	EEZ98281,1	Larval cuticle protein A3A-like Protein	*Tribolium castaneum*	334	21875	54000	3	20,5
	B2	Not identified							
	B3	XP_008198924,1	Tropomyosin-2 isoform X6	*Tribolium castaneum*	1606	32614	40000	24	66,8
		XP_015839642,1	Tropomyosin-1, isoforms 9A/A/B isoform X13	*Tribolium castaneum*	1219	29366	40000	22	52,2
	B4	XP_015837065.1	Uncharacterized protein LOC664580	*Tribolium castaneum*	640	73911	26000	4	8,1
		EEZ98281,1	Larval cuticle protein A3A-like Protein	*Tribolium castaneum*	391	21875	26000	4	26,6
		XP_015837064.1	Uncharacterized protein LOC664584	*Tribolium castaneum*	385	55463	26000	3	9,6
	B5	XP_008198303,1	Myosin regulatory light chain 2	*Tribolium castaneum*	938	21719	25000	10	45,7
		EEZ98281,1	Larval cuticle protein A3A-like Protein	*Tribolium castaneum*	318	21875	25000	3	20,5
		EEZ98387,1	Hypothetical protein TcasGA2_TC000851	*Tribolium castaneum*	300	22834	25000	3	15,5
	B6	RZC33111,1	Larval cuticle protein A3A-like	*Asbolus verrucosus*	804	49477	21000	4	9,9
		XP_970301,1	Pupal cuticle protein C1B	*Tribolium castaneum*	566	19583	21000	3	16,8
		EEZ98387,1	Hypothetical protein TcasGA2_TC000851	*Tribolium castaneum*	424	22834	21000	3	15,5
SILKWORM	S1	BAA02444,1	Vitellogenin precursor	*Bombyx mori*	2215	203725	190000	40	26
	S2	FAA00462,1	Putative cuticle protein	*Bombyx mori*	391	44670	75000	8	20
		BAE06190,1	Glycine rich protein	*Bombyx mori*	286	39833	75000	3	3,6
	S3	FAA00450,1	TPA: putative cuticle protein	*Bombyx mori*	771	39434	55000	12	33,3
		AAS68506,1	Serpin-5	*Bombyx mori*	463	44534	55000	9	27,6
	S4	P80034,1	Antichymotrypsin-2	*Bombyx mori*	883	41432	45000	12	33,8
		ABF51441,1	Tropomyosin isoform 1	*Bombyx mori*	417	32823	45000	10	35,4
	S5	FAA00470,1	Putative cuticle protein	*Bombyx mori*	1594	28335	40000	11	56
	S6	CAA38531	30 kDa protein	*Bombyx mori*	2997	30335	27000	17	62,7

Table 2. Proteins identified in the cross-reactive bands of mealworm, buffalo worm and silkworm.

	N° band	Entry (NCBI)	Protein name	Organism	Protein Score	MW theoretical (Da)	MW experimental (Da)	N° of matching peptides	Protein coverage (%)
CRIKET	C1	AVI26881,1	Troponin T	*Teleogryllus emma*	146	46681	60000	4	10,3
	C2	AVI26881,1	Troponin T	*Teleogryllus emma*	558	46681	53000	8	22,9
	C3	AVI26879,1	Tropomyosin isoform 1	*Teleogryllus emma*	571	32821	40000	12	38
		QCI56569,1	Tropomyosin 2, partial	*Acheta domesticus*	553	23083	40000	8	42
GRASSHOPPER	G1	ANS83649,1	Myosin heavy chain isoform E	*Locusta migratoria*	1107	223474	66000	23	13,4
		ANS83645,1	Myosin heavy chain isoform A	*Locusta migratoria*	1077	223453	66000	23	13,5
		BBE27867,1	C-type lysozyme	*Locusta migratoria*	937	81475	66000	16	28,2
	G2	AQE30075,1	Mitochondrial F0F1-ATP synthase subunit beta	*Locusta migratoria*	376	56139	50000	9	24
		AVI26881,1	Troponin T	*Teleogryllus emma*	247	46681	50000	6	11,6
	G3	ANS83649,1	Myosin heavy chain isoform E	*Locusta migratoria*	715	223474	35000	18	9,5
		ANS83645,1	Myosin heavy chain isoform A	*Locusta migratoria*	694	223453	35000	18	10
	G4	ACV32627,1	Beta-actin	*Diabolocatantops pinguis*	1196	42141	31000	16	50
	G5	AAW22542,1	Myosin light chain	*Gryllotalpa orientalis*	714	22580	26000	5	23,9
	G6	P82167,1	Cuticle protein 21.3	*Blaberus craniifer*	802	18918	21000	4	18
		PNF35287,1	Muscle-specific protein 20	*Cryptotermes secundus*	672	20570	21000	3	13

Table 3. Proteins identified in the cross-reactive bands of cricket and grasshopper.

5.2.4 Protein allergenicity potential

The allergenic potential of cross-reactive proteins were predicted using Allermatch™. A protein can be considered potentially allergenic when it shows more than 35% identity with a known allergen, within a window of 80 amino acids or more (Verhoeckx *et al*., 2014). Potentially allergenic proteins based on above-mentioned criteria are listed in Table 4.

Tropomyosin, one of the most cross-reactive protein, matches with the corresponding HDM and shrimp tropomyosin isoallergen. In Table 4, we reported the three best hits with sequence identity higher than 70% for HDM allergic patients and higher than 75% for shrimp allergic patients.

Myosin heavy chain E and A, myosin light chain and myosin regulatory light chain 2 match with paramyosin and myosin allergens found in three HDM species, in two shrimp species and in *Blatella germanica* with sequence identity between 40% to 80%.

For the other cross-reactive proteins no isoallergen with identity percentage higher than 35% were found using Allermatch™.

Identified proteins			Cross-allergen by Allermatch™	
	Proteins	**ID (NCBI)**	**Allergen**	**Allergen code**
Bands recognized by HDM patients				
M3 /B3	Tropomyosin-2 isoform X6 Tropomyosin-1, isoforms 9A/A/B isoform X13	XP_008198924.1 XP_015839642.1	Tropomyosin Tropomyosin Tropomyosin	Tyr p 10 Der f 10 Cho a 10
S4	Tropomyosin isoform 1	ABF51441,1		
C3	Tropomyosin isoform 1 Tropomyosin 2, partial	AVI26879,1 QCI56569,1		
G1 /G3	Myosin heavy chain isoform E Myosin heavy chain isoform A	ANS83649,1 ANS83645,1	Paramyosin Paramyosin Myosin	Blo t 11 Der f 11 Der p 11
Bands recognized by shrimp allergic patients				
M3 /B3	Tropomyosin-2 isoform X6 Tropomyosin-1, isoforms 9A/A/B isoform X13	XP_008198924.1 XP_015839642.1	Tropomyosin Tropomyosin Tropomyosin	Pen m 1 Pen a 1 Pan b 1
C3	Tropomyosin isoform 1 Tropomyosin 2, partial	AVI26879,1 QCI56569,1		
M5 /B5	Myosin regulatory light chain 2	XP_008198303,1	Myosin light chain Myosin light chain	Pen m 3 Lit v 3
Bands recognized by primary sensitized mealworm patients				
M3	Tropomyosin-2 isoform X6 Tropomyosin-1, isoforms 9A/A/B isoform X13	XP_008198924.1 XP_015839642.1	Tropomyosin Tropomyosin Tropomyosin	Chi k 10 Lep s 1 Per f 7
C3	Tropomyosin isoform 1 Tropomyosin 2, partial	AVI26879,1 QCI56569,1	Tropomyosin Tropomyosin	Per f 7 Bla g 7
M5	Myosin regulatory light chain 2	XP_008198303,1	Allergen Bla g 8	Bla g 8
G5	Myosin light chain	AAW22542,1		

Table 4. Cross-allergens analyzed by Allermatch™. Tyr p (*Tyrophagus putrescentiae*); Der f (*Dermatofagoide farina*); Cho a (*Chortoglyphus arcuatus*); Blo t (*Blomia tropicalis*); Der p (*Dermatophagoides pteronyssinus*); Pen m (*Penaeus monodon*); Pen a (*Penaeus aztecus*); Pan b (*Pandalus borealis*); Lit v (*Litopenaeus vannamei*); Chi k (*Chironomus kiiensis*); Lep s (*Lepisma saccharina*); Per f (*Periplaneta fuliginosa*); Bla g (*Blattella germanica*).

5.3 Discussion

In this study, the HDM, shrimps and *T. molitor* allergic patient IgEs cross-reactivity towards five differently processed edible insect proteins, were evaluated. IgE cross-reactivity occurs when IgE antibodies originally raised against a specific allergen can bind homologous molecules originating from a different allergen source (Hauser, Roulias, Ferreira, & Egger, 2010). In our study, the patient IgEs directed against HDM or shrimps or *T. molitor* have been demonstrated to be able to bind proteins from mealworm (*Tenebrio molitor*), buffalo worm (*Alphitobius diaperinus*), silkworm (*Bombyx mori*), cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria*). Depending on the nature of cross-reactive protein and on thermal treatment considered, cross-reactivity resulted to be differently affected by thermal processing.

Primarily, our data suggest that processing may cause a variation in protein solubility, resulting in a protein shifting from water-soluble (W) to urea-soluble (U) fraction, more marked after frying than after boiling. The alteration of protein solubility after heat processing is demonstrated to be caused by protein unfolding, with the consequent exposure of hydrophobic residues, that may lead to the formation of insoluble aggregates (Lasekan & Nayak, 2016). This alteration in protein solubility, may influence the digestibility, the analytical detection and quantification as well as the immunoreactivity of allergenic proteins (Sharma, Khuda, Parker, Eischeid, & Pereira, 2016). Broekman *et al*. (2015) investigating the effect of thermal processing on mealworm allergenicity, found that the effect was protein-selective: arginine kinase was affected by a shift from water buffer to urea buffer, while a reverse shift from urea to water buffer was found for tropomyosin.

In our study, we used dot-blot screening in order to group the allergic patients according to their dot-blot positivity. From our data, most of the patients recognized at least one insect extract according to those found by van Broekhoven and co-workers (2016) on three mealworm species recognized by HDM and shrimp allergic patients. In our study, HDM allergic patients showed a higher overall percentage of positivity to dot blot with respect to shrimp allergic patient. Raw buffalo worm and processed grasshopper (both boiled and fried) were the most cross-reactive matrices for HDM allergic patients. Grasshopper protein extracts was even the most cross-reactive for shrimp allergic patients, its allergenicity decreased after boiling, and it completely disappeared after frying. The cross-reactivity of shrimp allergic patients towards other insect extracts was often ascribable only to one patient; therefore, these data are not readily usable to formulate general considerations. Differently from our data, Broekman and co-workers (2017b) found a strongly higher percentage of cross-reactive sera of shrimp allergic patients (from 73 to 100%) tested on seven different insect extracts.

As far as the two mealworm sensitized patients are concerned, they showed positivity towards silkworm (both of them), mealworm (both for raw and only one for processed extracts), and grasshopper (one of them). No reactivity was found towards buffalo worm and only one of them was reactive to fried cricket. As already described in the chapter 4, the two patients did not report any symptoms after ingestion of other insects as cricket and greater wax moth. This indicates that primary mealworm sensitization is not predictive for the developing of another insect allergy, suggesting that insect allergenicity was insect species-specific. Species-specific insect allergy was already reported by Focke *et al*. (2003) for housefly (*Musca domestica*) and by Siracusa *et al*. (1994) for greenbottle (*Lucilia Caesar*). Broekman *et al*. (2017b) investigating the cross-allergenicity of four primary mealworm allergic subjects to seven different insects, stated that, primary mealworm allergy does not mean that subjects are likely to react to all insects.

Dealing with cross reactivity at the molecular level, we have to consider that different types of cross-reactive proteins may be involved in sensitization. Some of them are specific for a single food, while others are pan-allergen, like tropomyosin for arthropods. Furthermore, sensitization by cross-allergen recognition might occur with or without clinical relevance. Just a minority of individuals sensitized to pan-allergens go onto develop an allergy, depending on the allergen sources exposure, geographic differences, the type of allergen source and age demography (McKenna *et al*., 2016).

Cockroack allergen like (CAL) protein can be used as an example of specific cross-allergen. The two patients, primary sensitized to *T. molitor* by feces manipulation, resulted to be able to recognize CAL only in raw mealworm, losing their reactivity after processing. Differently, larval cuticle protein was identified in the reactive bands of all the three worms, both by HDM and shrimp allergic patients. Its cross-allergenicity for shrimp allergic patients was already reported by Broekman *et al*. (2015 and 2017b) and by Verhoeckx *et al*. (2016).

A 30K protein was identified as single protein in raw and fried silkworm in a cross-reactive band from HDM allergic patients. Zuo and co-workers (2015) already reported the role of the 30 K protein involved in the pathogenesis of asthma in patients with silkworm occupational allergy.

In the cross-reactive bands, different proteins, belonging to the family of muscle proteins, were identified. Troponin T and Beta actin, identified as single proteins only in cricket and grasshopper respectively, were both heat-labile. Troponin T has been already identified in cockroach as minor allergen as recognized by 16.7% of the allergic patients (Khantisitthiporn *et al*., 2007), and it was identified in different insect allergenic extracts by Broekman *et al*. (2017b). Myosin was identified as single proteins only in grasshopper, where the cross-reactivity was resistant to boiling but not to frying. Its cross-allergenicity potential was already described in insects (van Broekhoven et., 2016; Verhoeckx *et al*., 2014; Broekman *et al*., 2015). Tropomyosin is an already demonstrated major pan-allergen among crustaceans, mollusks, mites and also insects, due to their high similarity

amino acids sequence among species (Barre, Simplicien, Cassan, Benoist, & Rougé, 2018). The cross-allergenicity potential of insect tropomyosin for shrimp and HDM allergic patients was already described (van Broekhoven *et al*., 2016; Broekman *et al*., 2015; Broekman *et al*., 2017b; Verhoeckx *et al*., 2014). Our patients were reactive to mealworm, buffalo worm, silkworm and cricket tropomyosin and cross-reactivity seemed to be heat-stable. Considering our findings, shrimp allergic patients recognized only cricket tropomyosin as already demonstrated by Hall *et al*. (2018). As far as the two primary sensitization allergic patients are concerned, they showed always a faint immunoblotting signal on tropomyosin bands, compared to the other two group of patients. This data is in agreement with different studies where tropomyosin was not detected by insects primary sensitization patients (Broekman *et al*., 2017a; Linares, Hernandez, & Bartolome, 2008). These findings suggest that tropomyosin play an important role as cross-allergen for HDM and shrimp allergic patients, while in primary insects sensitization patients other proteins are more involved in the sensitization process.

Considering the overall effect of processing on the cross-allergenicity of HDM, shrimp and mealworm allergic patient sera towards five edibles we can state that the effect is protein-, species- and treatment-specific. Thermal processing, acting differentially on different cross-reactive protein is not able to completely eliminate cross-allergenicity. Only in silkworm, a remarkable decrease of cross-reactivity of shrimp allergic patient sera was found. Based on our results, HDM, shrimp and mealworm allergic patients should consume insects with caution, since different proteins, some of them thermostable, are involved in insect's cross-sensitization. However, sensitization is not a sufficient condition to develop an allergic reaction. Further studies have to be performed in order to verify the clinical relevance of sensitization and how the sensitization process may progress into the development of an allergic disease.

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CHAPTER 6

IMPACT OF HOLDER AND HIGH TEMPERATURE SHORT TIME PASTEURIZATION ON HUMAN MILK *IN VITRO* DIGESTION IN A PRETERM NEWBORN MODEL

Aim of the Work

High Temperature-Short Time (HTST, 72°C-15'') pasteurization of human milk (HM) has been proposed as an alternative to Holder method (HoP, 62.5°C-30'), to increase the preservation of bioactive compounds. The present research was aimed at assessing whether and to which extent different pasteurization methods affect HM proteolysis and lipolysis kinetics digestion, using an *in vitro* dynamic system (DIDGI®), in a preterm newborn digestion model.

Abstract

Raw and pasteurized HM was subjected to gastrointestinal digestion using the bi-compartmental *in vitro* dynamic system DIDGI®. The system was set up to simulate the digestion of a preterm newborn at a postnatal age of four weeks. Samples were collected from the undigested milk and at different digestion times and were characterized for their particle size distribution, confocal microscopy and protein and lipids profiles.

Confocal microscopy revealed an increased protein aggregation, mainly containing Lactoferrin, on the surface of fat globules following pasteurization. This effect was particularly evident after HoP pasteurization. This phenomenon reflected in notable changes in the profile of the milk fat globule associated proteins after processing. The overall degree of lipolysis did not vary between raw and pasteurized HM, while relevant differences were observed in term of proteolysis. During digestion, HM pasteurized according to both methods led to similar proteolytic patterns, while raw HM presented higher native Lactoferrin content throughout digestion. A slight, although significantly, decreased in the aminoacid release following HoP, with respect to HTST and raw HM was found.

This work provides the first evidences on the differential impact of HoP and HTST pasteurization techniques on the bioaccessibility of HM nutrients for preterm newborns.

6.1 Methods

6.1.1 Collection and pasteurization of human milk

Mature HM was obtained from the Donor HM Bank located at Regina Margherita Childrens' Hospital, Turin (Italy). Each milk donor involved in the research signed a written consent form, where mother's and infant's data protection was ensured. Full-term donor milk was obtained from 5 selected healthy donors, from one to three months after term delivery, and stored at -20°C after collection, for a maximum of four months. HM samples were thawed and pooled to achieve a final volume of about 1.7 L. The pool was then divided into 120 mL aliquots (n=12) and 40 ml aliquots (n=6). One third of the aliquots was immediately stored at -20°C (raw human milk, RHM), while the other aliquots underwent pasteurization following HoP (62.5°C for 30 min) and HTST (72°C for 15 s). HoP was performed using the donor HM bank equipment (Metalarredinox, Verdellino, BG, Italy). HTST was performed by a patented proprietary small-scale device (Giribaldi *et al*., 2016).

6.1.2 Human milk characterization

6.1.2.1 Macronutrient composition

Macronutrient composition of raw (RHM) and pasteurized HM samples was assessed by infrared spectrophotometry, using a Human Milk Analyzer (Miris AB, Uppsala, Sweden), previously validated against reference methods (Billard *et al*., 2016). The total lipid composition in HM before digestion was determined by gas chromatography coupled to a flame ionization detector after direct transmethylation, as described by de Oliveira, Bourlieu, *et al*. (2016).

6.1.2.2 Profiling of intact protein by liquid chromatography-mass spectrometry (LC-MS)

Analysis of the intact proteins was performed by means of a reversed-phase high performance liquid chromatography system (RP-HPLC, HPLC 1100 by Agilent Technologies, Santa Clara, California, USA) fitted to a Q-Exactive mass spectrometer (Thermo Scientific Waltham, Massachusetts, USA). Samples were diluted in trifluoracetic acid (TFA) 0.212% before injection. The injection volume was 40 µl and the oven temperature was set at 40°C. Proteins were separated on C4 VYDAC column (214TP5215, 150 x 2.1 mm) (GRACE, Columbia, USA). The flow applied was 0.25 mL/min, using acetonitrile (ACN) gradient of 0.106% (v/v) TFA in water (A) and 0.1% (v/v) TFA in 80% ACN (B). At the end of the column, a fraction of the eluate entered the Q-Exactive MS at a flow rate of 75 mL/min. Electrospray MS operating in positive ion mode, with an optimized voltage of 4.2 kV, was

used for the identification of intact proteins. Spectra were recorded in full MS mode and selected in a mass range 400–3,000 m/z with a resolution of 17,500. The instrument was externally calibrated according to the supplier's instructions. Ions recovered in the sample were analyzed from the MS spectra using Xcalibur 2.2 Software (Thermo Scientific).

6.1.2.3 Characterization of milk fat globule associated proteins

Milk fat globule associated proteins (MFGPs) were extracted as described by Barello *et al*. (2008). Briefly, samples were centrifuged at 5,000*g* for 30 min at 6°C, the pellet was discharged, and the supernatant was ultracentrifuged at 189,000*g* for 70 min at 6°C. The fat globules were washed three times with 0.9% (w/v) NaCl and resuspended for 1 h in 1% (w/v) ASB-14, 7M urea, 2M thiourea, 20mM Tris. The resuspended protein solution was recovered and precipitated with methanol/chloroform, as described in Wessel & Flügge (1984). Proteins were solubilized in NuPAGE® LDS sample buffer (Thermo-Fisher Scientific), and the protein concentration was determined by means of a 2-D Quant-Kit (GE, Boston, USA). Proteins (5 µg) were separated in triplicate from pasteurized and RHM under non-reducing conditions on 4-12% gradient NuPAGE® Novex Bis-Tris precast gels (Thermo-Fisher Scientific), as described previously (de Oliveira, Bourlieu, *et al*., 2016), and gels were stained by Colloidal Coomassie Blue (Candiano *et al*., 2004). Gel images were acquired using a ChemiDoc™ Imaging Systems (Biorad, Hercules, California, USA) and saved at a resolution of 300 dpi. Densitometric analysis was performed using ImageLab 6.0 software package (Biorad). The relative intensity of each protein band in each lane was calculated as symmetrized percentage change (SPC) of band volume with respect to raw HM sample (Berry & Ayers, 2006).

6.1.2.4 Identification of human milk proteins from gel electrophoresis

Bands with statistically significant differences in intensity between the different treatments were cut, digested and identified by MS. Bands were first reduced in 10 mM DL-dithiothreitol (DTT) / 50 mM NH_4HCO_3 for 45 minutes at 56°C, followed by alkylation in 55 mM iodoacetamide / 50 mM NH_4HCO_3 for 30 minutes in the dark, at room temperature. The bands were then sequentially washed with water, 50% (v/v) ACN / 50 mM NH_4HCO_3., 100% ACN and 50% (v/v) ACN / 50 mM NH_4HCO. Gel pieces were dried in a vacuum concentrator (Eppendorf, Hamburg, Germany), and digested overnight at 37°C under shaking, using 75 ng/µl porcine trypsin (Promega, Madison, Wisconsin) in 50 mM NH_4HCO_3. TFA (5%) was used in order to stop the enzymatic reaction.

The resulting peptides were analyzed using a Dionex U3000 nano-RSLC system fitted to a Q-Exactive MS equipped with a nanoelectrospray ion source. The injection volume was 5 µL. Peptides were separated in a C18 PepMap column (150 mm x 75 µm, Thermo Scientific). The flow applied was 0.3 mL/min with a gradient of 2% (v/v) ACN, 0.08% (v/v) formic acid, 0.01% (v/v) TFA in water (A) and 95% (v/v) ACN, 0.08% (v/v) formic acid,

0.01% (v/v) TFA in water (B). Electrospray mass spectrometer operating in positive ion mode with an optimised voltage of 1.9 kV. Mass spectra were recorded in a 250–2000 m/z range, with a resolution of 17,500, and MS/MS fragmentation was performed on the 10 most abundant ions. Spectra were analyzed using X!Tandem (version 2017.02.01) with the UniProt *Homo sapiens* database (version 2017-10-01). Parameters set for database search were: trypsin as digestion enzyme; two possible missed cleavages; mass error of 10 ppm on peptides and 0.05 Da on ions; oxidation of methionine and phosphorylation of serine, threonine and tyrosine, as possible post-translational modifications

6.1.2.5 Analysis of human milk microstructure by confocal microscopy

The microstructure of undigested RHM, HoP and HTST HM samples was observed using Nikon C1Si confocal laser scanning microscopy (CLSM) on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) as previously described by Bourlieu *et al*. (2015). A 40 x oil-immersion objective was used for all images. Three fluorescent dyes, Fast Green®, Rd-DOPE® and Lipidtox®, were used to colour simultaneously protein, amphiphilic compounds and apolar lipids, respectively.

6.1.2.6 Determination of Bile Salt-Stimulated Lipase (BSSL) activity

HM BSSL activity was measured in triplicate according to a previously described protocol (Giribaldi *et al*., 2016).

6.1.3 *In vitro* dynamic digestion

RHM, HoP and HTST HM samples were subjected to gastrointestinal digestion using the bi-compartmental *in vitro* dynamic system DIDGI® (Ménard *et al*., 2014). The system was set up to simulate the digestion of a preterm newborn at a postnatal age of four weeks. The specific gastrointestinal parameters were detailed previously (de Oliveira, Bourlieu, *et al*., 2016) and summarized in Table 1. The transit time of the fluids followed the Elashoff equation with $t_{1/2}$=36 min and β=1.15 for the gastric emptying and $t_{1/2}$= 200 and β= 2.2 for the intestinal emptying. The pH acidification in the gastric compartment followed a polynomial curve based on previous *in vivo* studies as reviewed by Bourlieu *et al*. (2014), and updated with recent data obtained from De Oliveira *et al*. (2017): pH acidification=8E-05time² - 0.031time + 6.80. The intestinal pH was maintained constant at 6.2. Gastric enzymes were added as rabbit gastric extract (pepsin and lipase: 120 and 8.6 U/mL of gastric content, respectively) and intestinal enzymes as porcine pancreatin (trypsin, and lipase: 1.52 and 59 U/mL of intestinal content) in addition to bovine bile salts. Digestion experiments were performed in triplicate for each matrix over three hours. Aliquots were collected before digestion and at 30, 60, and 90 min after the beginning of the digestion in both gastric and

intestinal compartments. Additional samples were collected from the intestinal compartment at 120 and 180 min. Structural analyses (particle size distribution profiling) were immediately performed. Samples for lipid analysis were immediately processed for lipid extraction, after the addition of 5 mg of 4-bromophenylboronic acid per mL of digesta, as lipase inhibitor. Protease inhibitors were also immediately added, namely 10 µL of pepstatin A (0.72 mM) per mL of gastric digesta or 50 µL of pefabloc (0.1 M) per mL of intestinal digesta, before storage at -20°C until analysis.

Gastric conditions (37°C)		
Simulated Gastric Fluid (SGF) (stock solution adjusted at pH 6.5)	Na^+	118 mmol/L
	K+	9.8 mmol/L
	Cl^-	137 mmol/L
Fasted state / initial conditions	SGF	2.6 mL
	pH	3.9
Milk ingested	Total volume	100 mL
	Flow rate	10 mL/min from 0 to 10 min
Gastric pH (acidification curve)	$pH = 8E\text{-}05t^2 - 0.031t + 6.80$ with *t*: time after ingestion in min	
SGF + enzymes (RGE; Rabbit Gastric Extract)	Gastric lipase	8.6 U/mL of gastric content
	Pepsin	120 U/mL of gastric content
	Flow rate	1 mL/min from 0 to 10 min
		0.5 mL/min from 10 to 180 min
Gastric emptying (Elashoff fitting)	$t_{1/2}$	36 min
	β	1.15
Intestinal conditions (37°C)		
Simulated Intestinal Fluid (SIF) (stock solution adjusted at pH 6.2)	Na^+	140 mmol/L
	K^+	4 mmol/L
	Ca^{2+}	1 mmol/L
Intestinal pH	6.2	
SIF + bile	Bile salts	1.6 mmol/L of intestinal content
	Flow rate	0.5 mL/min from 0 to 180 min
SIF + pancreatin	Pancreatic lipase	59 U/mL of intestinal content
	Flow rate	0.25 mL/min from 0 to 180 min
Intestinal emptying (Elashoff fitting)	$t_{1/2}$	200 min
	B	2.2

Table 1. Gastrointestinal parameters for *in vitro* dynamic digestion of HM simulating preterm newborn conditions.

6.1.3.1 Particle size distribution of human milk during gastric digestion

The distribution of the size of the particles before and during gastric digestion of the differently treated HM was profiled using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) laser light scattering equipped with two laser sources, as previously described (Bourlieu *et al*., 2015). The refractive indexes used were 1.458 for lipid at 633 and 466 nm and 1.333

for water (dispersant solution) in the measurement cell. The diameter mode, the diameter mean D [4,3] and the specific surface (SS) were calculated from the size distribution.

6.1.3.2 Determination of the degree of protein hydrolysis (DH) of human milk

The DH was calculated from the measurement of primary amines (-NH_2) released during the *in vitro* digestion using a fluorescent microplate analysis based on the reaction of ortho-phthaldialdehyde (OPA) and DTT, with primary amines resulting in 1-alkylthio-2-alkylisondole and detected at 340 nm, as previously described by Deglaire *et al*. (2019). The total releasable primary amines [NH_2 (total)] were also determined in HM sample before digestion, following total acid hydrolysis in 6 N HCl at 110°C for 24 h. The DH was calculated as follows:

$$\mathrm{DH}\ (\%) = \frac{100 \text{ x } [(\frac{\mathrm{mg}}{\mathrm{L}}\mathrm{NH2(t)}) \text{ x digestion dilution factor} - (\frac{\mathrm{mg}}{\mathrm{L}}\mathrm{NH2\ (t0)})]}{\frac{\mathrm{mg}}{\mathrm{L}}\mathrm{NH2(total)}}$$

where [NH_2(t)] is the concentration of primary amines after t min of digestion, and [NH_2(t0)] is the concentration of primary amines in each starting HM sample before digestion.

6.1.3.3 Profiling of HM protein digestion by gel electrophoresis

Protein electrophoresis was performed on starting HM samples and on gastric and intestinal digests, both in reducing and in non-reducing conditions. After quantification of the protein concentration in the undigested HM samples (RHM, HoP and HTST) using 2-D Quant-Kit, 3 and 30 µg of proteins for the gastric and intestinal digests, respectively, were separated on gels. Total proteins were diluted in NuPAGE® LDS sample buffer, and electrophoresis and image acquisition were performed following the procedure described in section 6.1.2.3.

In order to minimize the variations between the different gels, densitometric analysis was performed on the relative quantity of the gel bands, as calculated by ImageLab software package by standardizing the volume of each band in each gel on the volume of a reference band (in our approach, native Lactoferrin band in undigested RHM sample). The relative abundance of each band at each digestion stage was then calculated with respect to its value in RHM sample before digestion, and expressed as % with respect to undigested milk quantity. Bands showing significant variation between the pasteurization treatments were cut, digested and identified by mass spectrometry as described in section 6.1.2.4.

6.1.3.4 Quantification of amino acid release from human milk during intestinal digestion

The quantity of release of each amino acid (AA) was determined as detailed elsewhere (de Oliveira, Bourlieu, *et al*., 2016). Cysteine, proline and tryptophan were not quantified. The AA bioaccessibility was calculated as follows:

$$\text{AA bioaccessibility (\%)} = \frac{100 \text{ x } (\frac{\text{mg}}{\text{L}} \text{ AA (t) x digestion dilution factor})}{\frac{\text{mg}}{\text{L}} \text{AA (total)}}$$

where AA(t) is the amount of AA after t min digestion, and AA(total) is the amount of each AA that was obtained after total acid hydrolysis in 6 N HCl at 110°C for 24 h of undigested milk.

The total amino acid release was calculated as sum of all AA released at digestion time (t), divided with the total AA in the undigested milk obtained after acid hydrolysis.

6.1.3.5 Lipid analysis

Lipid extraction was performed as described previously (Bourlieu *et al*., 2015). The quantification of the lipid classes was performed by thin layer chromatography. The extracted lipids were spotted on silica gel plates (10 x 20 cm, 0.25 mm, Si G60, Merck) using Automated TLC Sampler III (CAMAG, Muttenz, Switzerland). The lipid classes were separated with a solvent mixture of hexane/diethyl ether/acetic acid (70/30/2 v/v/v), revealed with a solution of bands visible on copper sulfate/orthophosphoric acid and heated 15 min at 150°C. Densitometric analysis of the silica plates were performed and the triacylglycerol (TAG) digestion was estimated as percentage of TAG remaining in the gastric and intestinal digests at each time, as compared to the total TAG in the undigested milk. Standards TAG were loaded on plates in order to check the linearity and allow the TAG quantification.

6.1.4 Statistical analyses

All statistical analyses were performed using PAST software, version 3 (Hammer, Harper, & Ryan, 2001). A probability $p \leq 0.05$ was considered as statistically significant. Results were expressed as mean ± standard deviation (SD), unless otherwise stated.

T he abundance in undigested HM samples of MFGPs bands (expressed as SPC) and of soluble protein bands (expressed as relative abundance) were analyzed using one-way ANOVA. Normality of residuals was assessed by Shapiro-Wilk's test. Where significance was assessed, post hoc tests were conducted using the Tukey's multiple comparison post hoc test. When unequal variances and/or lack of normality were found, Kruskal-Wallis

coupled to Dunn's post hoc test were used. BSSL activity data were analyzed using Kruskal-Wallis test.

Data from the profiling of differently pasteurized HM (RHM, HoP and HTST) during digestion were analyzed for significance at $p \leq 0.05$ by two-way ANOVA, with digestion time and pasteurization treatment as factors. Where significance was assessed, post hoc tests were conducted, using the Tukey's multiple comparison test.

6.2 Results

6.2.1 Human milk characterization before digestion

Raw and pasteurized HM samples (RHM, HoP, HTST) prior to digestion showed similar macronutrient composition (27.7 ± 1.5 g/L of lipids, 8.0 ± 0.1 g/L of proteins and 82.3 ± 1.2 g/L of carbohydrates). RHM lipid fraction contained 40.3% (w/w) of saturated, 46.5% (w/w) of MUFAs and 13.2% (w/w) as PUFAs. The ω3/ω6 PUFA ratio was 9.9. The lipid profile was dominated by six main FAs: oleic (44 ± 0.1%), palmitic (20.4 ± 0.1%), linoleic (10.7 ± 0.1%), stearic (5.9 ± 0.1%), myristic (5.6 ± 0.1%) and lauric (5.1 ± 0.1%) acids.

Confocal microscopy observation of HM samples prior to digestion (Fig 1A) showed that both pasteurization methods led to heat-induced protein aggregates (blue dots), both in the soluble phase and at the interface of the HM fat globule membrane. The protein-fat interaction phenomena, which could be observed also in raw HM, was more remarkable following HoP treatment, with respect to HTST. However, no difference in the particle size distribution in the different milk samples before digestion was seen (Fig 1B – G0), and all the samples showed a bimodal distribution, with two main peaks at size of about 0.12 and 8 µm (Fig 1 table– G0).

In order to gain more information about the nature, quantity and composition of protein aggregates in pasteurized HM, the protein fraction of raw and pasteurized samples was profiled by LC-MS and electrophoresis. LC-MS separation of soluble intact proteins allowed to detect 3 major protein fractions, and 2 minor protein peaks (Fig 2). The peak identified as human Lactoferrin showed a higher area in HTST milk than in HoP milk sample (75% vs 33% with respect to the protein peak area measured in RHM). Native α-lactalbumin was identified in all the samples, whereas its Na+ abduct was detected only in RHM. β-casein was identified in all samples with 2 or 4 sites of serine phosphorylation, while its native form was identified only in the HTST sample.

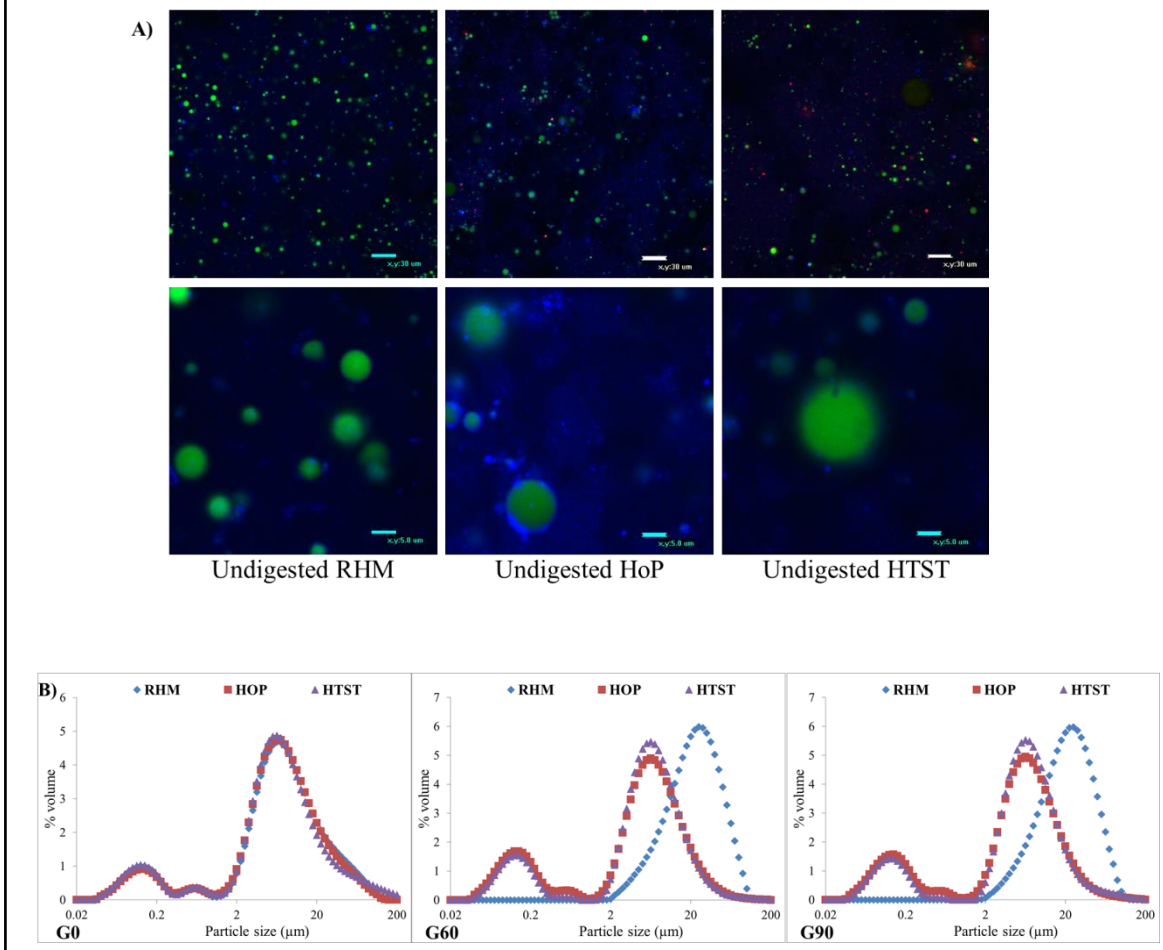
Electrophoretic profile of soluble proteins in reducing (Fig 3A) and non-reducing (Fig 3B) conditions showed significant differences among RHM, HoP and HTST samples in the relative abundance of specific bands. The detailed identifications of the proteins contained in gel bands are reported in Table 2A.

Gels run under non-reducing conditions showed a higher number of bands with significant differences among samples (bands 1-3nr and bands 7-12nr in Fig 3B). Under reducing conditions, only one minor band (band R2 in Fig 3A), containing Macrophage mannose receptor 1, showed a significantly slightly reduced (-15%) abundance in HoP milk.

Lactoferrin band in reducing conditions showed a very similar abundance in pasteurized and raw HM samples; on the opposite, when non reducing conditions were used, native Lactoferrin band abundance (bands 10-12nr in Fig 3B) following HoP was 30% of that of RHM, and 60% following HTST. The decrease in the relative abundance of Lactoferrin is accompanied by an increase (about 2-fold) of high molecular weight aggregates (bands 1-3nr in Fig 3B), mainly composed by Lactoferrin, in both pasteurized milk samples. Other proteins detected in aggregates include Fatty acid synthase, Xanthine dehydrogenase/oxidase, Macrophage mannose receptor and BSSL. The latter protein was also identified in bands 8nr and 9nr, that showed a significantly lower relative abundance with respect to RHM in HoP (-66%) and in HTST (-39%).

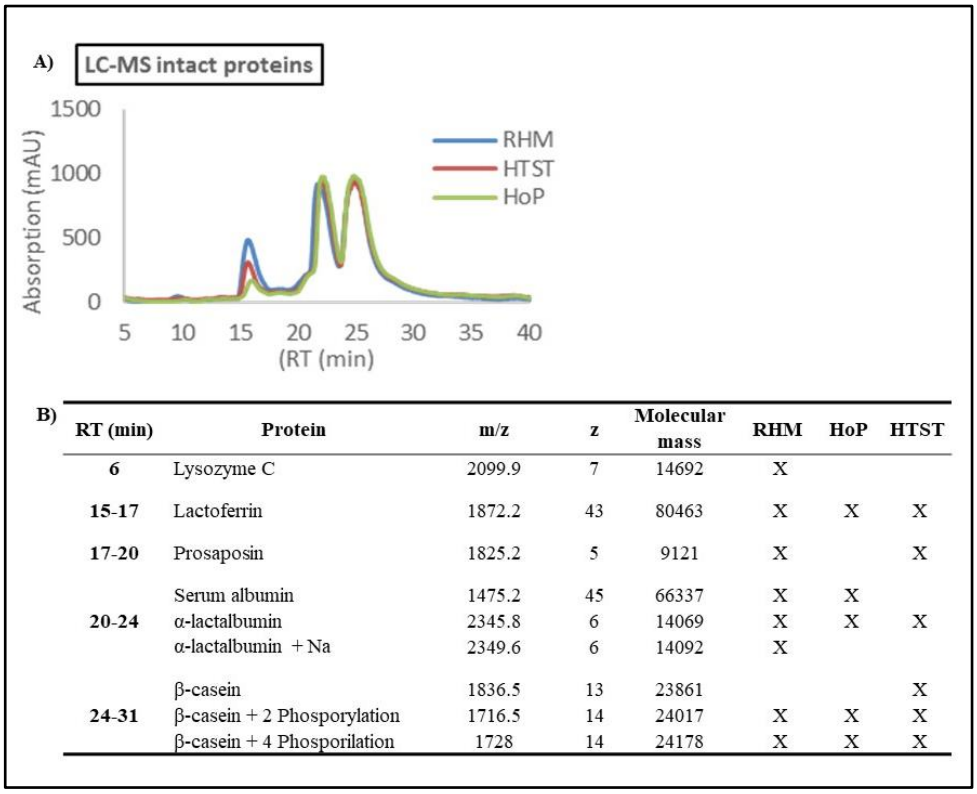
Accordingly, the BSSL activity, in both pasteurization treatments, was almost completely inhibited as compared to that found in RHM (149 ± 5 μmol/mL/min). Nevertheless, HTST treated milk samples displayed a higher, however not significantly, BSSL activity (0.25 ± 0.08 μmol/mL/min) in comparison to that of HoP treated samples (0.08 ± 0.01 μmol/mL/min).

MFGPs were also profiled by electrophoresis under non-reducing condition (Fig 3C), and the resulting bands were identified by LC-MS/MS (Table 2B). At high molecular weight, corresponding to bands A in RHM, band B in HoP, and band C in HTST, an increase in band abundance of 47% for HoP and of 25% for HTST, with respect to RHM, was found. The increased number of identified peptides owing to Lactoferrin and xanthine dehydrogenase indicated probable increased abundance of these proteins in the band. Accordingly, native Lactoferrin band (F) significantly decreased after HTST pasteurization (band H, -38%) and was almost undetectable in HoP undigested sample (-78%). Band D, mainly containing BSSL, was also highly reduced in HTST (band E, -60%), and undetectable in HoP milk. The reduction (about 50%) of β-casein in band P in RHM following both pasteurization methods corresponded to its increase in band Q. The same behavior was observed for Lysozyme containing bands (with decreased abundance in band S and increased abundance in band T) following pasteurization. α-lactalbumin was detected after both pasteurization treatments in band V.



		G0	G60	G90	Treatment		Interaction
Mode (µm)	RHM	7.0 ± 0.1	27.3 ± 8.5	26.0 ± 8.3		*A*	
	HoP	6.9 ± 0.1	6.7 ± 0.0	6.7 ± 0.2	***	*B*	***
	HTST	6.4 ± 0.2	6.6 ± 0.5	6.7 ± 0.9		*B*	
					Time	**	
D [4, 3] (µm)	RHM	13.4 ± 0.2	28.6 ± 7.5	27.6 ± 7.7		*A*	
	HoP	12.5 ± 2.3	8.6 ± 1.1	9.1 ± 2.2	***	*B*	***
	HTST	9.4 ± 1.4	9.0 ± 2.2	10.2 ± 2.3		*B*	
					Time	*ns*	
SS (m^2/g fat)	RHM	7.8 ± 1.1	0.4 ± 0.1	0.4 ± 0.1		*A*	
	HoP	7.3 ± 0.0	12.3 ± 0.1	11.4 ± 1.8	***	*B*	***
	HTST	8.4 ± 0.5	10.6 ± 2.3	10.0 ± 1.6		*B*	
					Time	*ns*	

Fig 1 (A) Confocal laser scanning microscopy images of aw (RHM) and pasteurized (HoP and HTST) undigested HM samples. Proteins are labeled in blue (FastGreen®), and apolar lipids in green (Lipidtox®). **(B)** Particle size distribution (as % volume) of raw and pasteurized in undigested (G0) and digested (G60 and G90) HM samples. Table shows the particle size characteristics (mode, diameter, SS - specific surface) of samples, during *in vitro* gastric digestion. Statistics: two-way ANOVA $p < 0.001$ (***); $p < 0.01$ (**); $p > 0.05$ (ns). Italicized letters: Tukey's post hoc homogeneity classes for treatment.



B)

RT (min)	Protein	m/z	z	Molecular mass	RHM	HoP	HTST
6	Lysozyme C	2099.9	7	14692	X		
15-17	Lactoferrin	1872.2	43	80463	X	X	X
17-20	Prosaposin	1825.2	5	9121	X		X
	Serum albumin	1475.2	45	66337	X	X	
20-24	α-lactalbumin	2345.8	6	14069	X	X	X
	α-lactalbumin + Na	2349.6	6	14092	X		
	β-casein	1836.5	13	23861			X
24-31	β-casein + 2 Phosporylation	1716.5	14	24017	X	X	X
	β-casein + 4 Phosporilation	1728	14	24178	X	X	X

Fig 2. (A) LC-MS profiles of intact proteins of raw (RHM) and pasteurized (HoP and HTST) undigested HM samples; **(B)** Identification of the main protein peaks in Panel A. RT: retention time (minutes). m/z: mass to charge ratio, z: numer of detected ions.

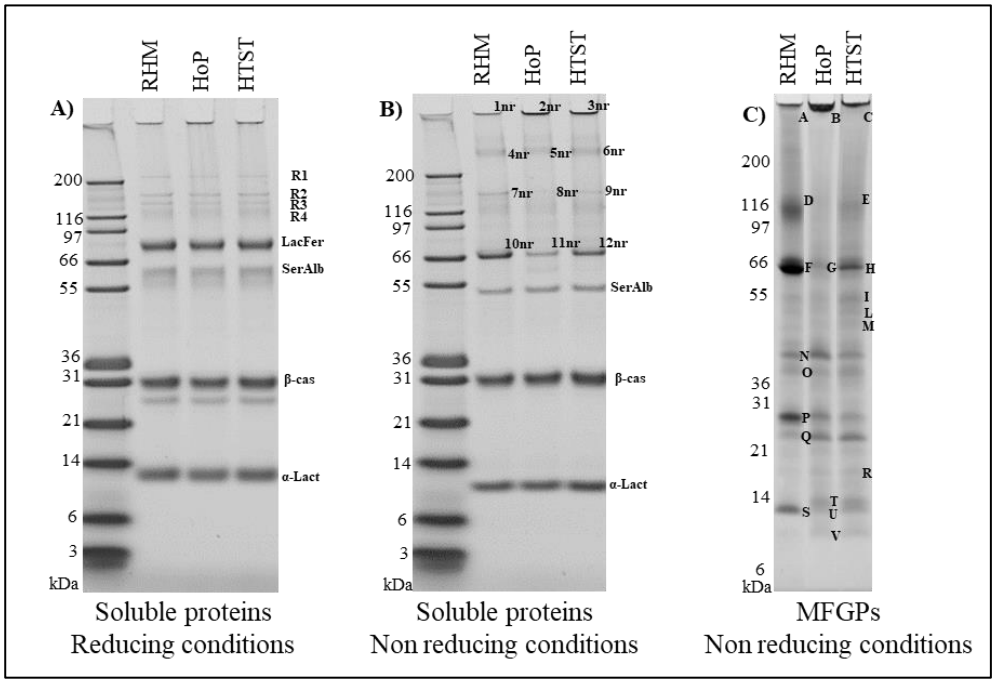


Fig 3. Protein profiles of raw (RHM) and pasteurized (HoP and HTST) undigested HM samples in reducing **(A)** and non-reducing **(B)** conditions, and of milk fat globule (MGF) associated proteins in non-reducing conditions **(C)**. Samples were loaded in the gels with the same amount of proteins. Lactoferrin (LacFer) and α-lactalbumin (α-Lact) were identified by antibody recognition. Serum albumin (SerAlb) and β-casein (β-cas) were identified in previous papers. Differently coded protein bands were identified by LC-MS/M (Table 2).

Band	Protein name	Entry (UniProt)	MW theoretical/ MW experimental (kDa)	Protein Score (E value)	Protein coverage (%)	Matching/ unique peptides (n°)
Identifications in Fig 3A, Solubile proteins Reducing conditions						
R1	Fatty acid synthase	P49327	273/>200	-27.6	2	7/7
R2	Macrophage mannose receptor 1	P22897	166/150	-26.5	6	10/10
R3	Xanthine dehydrogenase/reductase	P47989	146/140	-24.0	6	9/9
R4	Bile salt-activated lipase	P19835	79/120	-18.6	6	6/5
	Lactoferrin	P02788	77/120	-9.1	5	4/4
Identifications in Fig 3B, Solubile proteins Non Reducing conditions						
1nr	Fatty acid synthase	P49327	273/>200	-40.9	5	12/12
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-22.1	12	6/6
	Lactoferrin	P02788	78/>200	-20.9	10	7/7
2nr	Lactoferrin	P02788	78/>200	-388.9	72	178/94
	Fatty acid synthase	P49327	273/>200	-114.1	11	28/28
	Macrophage mannose receptor 1	P22897	166/>200	-88.1	15	22/21
	Xanthine dehydrogenase/oxidase	P47989	146/>200	-77.6	14	19/17
	Bile salt-activated lipase	P19835	79/>200	-66.2	18	17/14
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-53.1	25	19/16
3nr	Lactoferrin	P02788	78/>200	-241.8	62	101/65
	Fatty acid synthase	P49327	273/>200	-67.4	7	17/17
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-50.0	25	14/14
	Xanthine dehydrogenase/oxidase	P47989	146/>200	-44.1	9	13/13
	Macrophage mannose receptor 1	P22897	166/>200	-30.8	8	10/10
	Bile salt-activated lipase	P19835	79/>200	-19.8	8	6/6
4nr	Polymeric immunoglobulin receptor	P01833	83/250	-24.9	7	7/7
	Immunoglobulin heavy constant alpha 1	P01876	37/250	-23.6	20	10/7
	Immunoglobulin heavy constant alpha 2	P01877	36/250	-17.8	12	4/4
5nr	Lactoferrin	P02788	78/>200	-49.3	17	17/16
	Polymeric immunoglobulin receptor	P01833	83/>200	-30.1	9	8/7
	Immunoglobulin heavy constant alpha 1	P01876	37/>200	-23.6	13	14/5
	Immunoglobulin heavy constant alpha 2	P01877	36/>200	-17.2	9	6/3
6nr	Immunoglobulin heavy constant alpha 1	P01876	37/>200	-22.2	16	12/6
	Lactoferrin	P02788	78/>200	-21.8	9	7/7
	Polymeric immunoglobulin receptor	P01833	83/>200	-21.8	6	6/5
	Immunoglobulin heavy constant alpha 2	P01877	37/>200	-13.4	12	5/4
7nr	Not identified					
8nr	Macrophage mannose receptor 1	P22897	166/150	-35.3	7	12/10
	Xanthine dehydrogenase/oxidase	P47989	147/150	-25.6	6	10/10
	Bile salt-activated lipase	P19835	79/150	-8.3	3	3/3
9nr	Bile salt-activated lipase	P19835	79/150	-20.9	9	8/8
10nr	Lactoferrin	P02788	78/66	-198.0	50	126/60
11nr	Lactoferrin	P02788	78/66	-97.9	30	44/28
12nr	Lactoferrin	P02788	78/66	-314.95	66	144/86

Table 2A. Protein identification by LC-MS/MS of the bands in Fig 3A and 3B.

Band	Protein name	Entry (UniProt)	MW theoretical/ MW experimental (kDa)	Protein Score (E value)	Protein coverage (%)	Matching/ unique peptides (n°)
Identifications in Fig 3C, MFGP Non Reducing conditions						
A	Fatty acid synthase	P49327	273/>200	-307.8	28	93/91
	Xanthine dehydrogenase/oxidase	P47989	146/>200	-114.1	18	30/29
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-95.6	32	41/30
	Lactoferrin	P02788	78/>200	-94.2	35	26/26
B	Fatty acid synthase	P49327	273/>200	-268.8	25	81/80
	Xanthine dehydrogenase/oxidase	P47989	146/>200	-184.1	27	71/58
	Lactoferrin	P02788	78/>200	-183.3	58	63/53
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-89.6	34	36/27
C	Fatty acid synthase	P49327	273/>200	-327.2	30	100/94
	Xanthine dehydrogenase/oxidase	P47989	146/>200	-260.2	36	104/75
	Lactoferrin	P02788	78/>200	-211.4	59	81/73
	Butyrophilin subfamily 1 member A1	Q13410	59/>200	-87.8	32	39/30
D	Bile salt-activated lipase	P19835	79/116	-216.2	40	129/73
	Lactoferrin	P02788	78/116	-157.8	53	46/46
	Butyrophilin subfamily 1 member A1	Q13410	59/116	-55.7	27	28/21
E	Lactoferrin	P02788	78/116	-49.0	20	21/19
	Butyrophilin subfamily 1 member A1	Q13410	59/116	-44.1	21	21/16
	Bile salt-activated lipase	P19835	79/116	-37.3	10	15/12
F	Lactoferrin	P02788	78/66	-807.3	86	582/210
G	Lactoferrin	P02788	78/66	-83.6	27	38/28
H	Lactoferrin	P02788	78/66	-246.1	60	126/70
I	Butyrophilin subfamily 1 member	Q13410	59/55	-119.0	34	66/37
L	Butyrophilin subfamily 1 member	Q13410	59/52	-64.8	28	33/23
	Serum albumin	P02768	69/52	-31.5	18	11/11
M	Lactoferrin	P02788	78/50	-18.0	6	6/6
	Lactadherin	Q08431	43/50	-13.2	15	7/7
N	Lactadherin	Q08431	43/43	-74.5	31	25/22
O	Lactadherin	Q08431	43/40	-69.7	34	27/21
P	Beta-casein	P05814	25/28	-24.6	31	15/8
	Apolipoprotein	P02647	31/25	-16.7	19	8/7
Q	Beta-casein	P05814	25/25	-4.6	8	3/2
R	Not identified					
S	Lysozyme	P61626	17/13	-40.1	33	27/11
T	Lysozyme	P61626	17/14	-15.6	21	5/4
	Fatty acid-binding protein	P05413	15/14	-13.6	30	7/4
U	Lysozyme	P61626	17/13	-31.0	34	11/10
	Fatty acid-binding protein	P05413	15/13	-6.6	12	3/2
V	Alpha-lactalbumin	P00709	16/10	-5.8	11	3/2

Table 2B. Protein identification by LC-MS/MS of the bands in Fig 3C.

6.2.2 Structural changes during gastric digestion

Figure 1B shows the profile of particle size distribution in raw and pasteurized HM samples during simulated gastric digestion in preterm newborn conditions, with the corresponding particle size parameters (D[4,3], specific surface area and mode diameter). Although the bimodal distribution of the undigested milk was similar (G0), irrespectively of the treatment, major differences were observed during digestion, with the formation of significantly larger aggregates in RHM (mode diameter 26-27 µm, specific surface area 0.4 m^2/g fat) than HTST and HoP (mode diameter 6.6-6.7 µm, specific surface area 10-12.3 m^2/g fat) after 60 and 90 min of gastric digestion (Fig 1C). These aggregates dissolved by SDS addition and not by EDTA addition (data no shown). On the opposite, pasteurized HM samples did not show any modification of the particle size during gastric digestion (Fig 1B - G60 and G90).

6.2.3 Proteolysis kinetics

6.2.3.1 NH_2 release after digestion of HM

The proportion of NH_2 released after gastric and intestinal digestion was not significantly different between raw and pasteurized milks. A mean NH_2 release of 1.7 ± 0.5% was determined in the gastric phase for the three types of milk. Intestinal digestion led to an increase in NH_2 release from 16.1 ± 1% to 46 ± 0.6% at the end of the digestion (Fig 4).

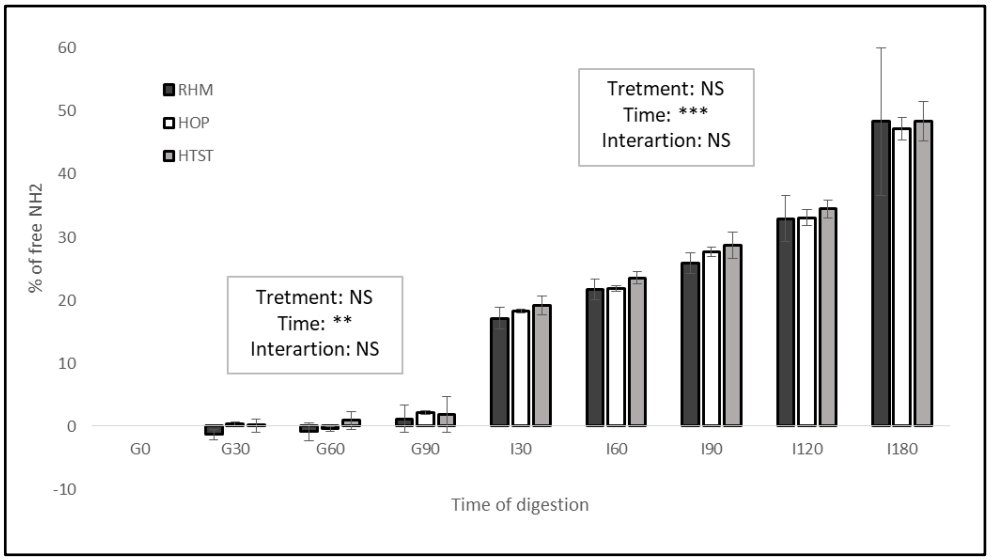


Fig 4. NH_2 release by OPA test of aw (RHM) and pasteurized (HoP and HTST) HM samples during gastric (G) and intestinal (I) digestion. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS).

6.2.3.2 Gastric and intestinal proteolysis

The electrophoretic protein profiles of gastric (A) and intestinal (B) digests in reducing (A_R,B_R) and non-reducing (A_{NR}, B_{NR}) conditions are reported in Figure 5. The identification of the protein in the bands is detailed in Table 2. Figure 6 reports the relative abundance of bands showing significant variation in one or more conditions, of the gels under reducing (Fig 6A) and not reducing (Fig 6B) conditions.

Gastric proteolysis: the majority of significant variations occurred between raw and pasteurized samples. The only significant variation between the two different pasteurization methods in band abundance during gastric digestion was found, in non-reducing conditions, for bands 4-5-6nr, that resulted to be slightly more digested in HoP sample. The band contained mainly immunoglobulin components, and Lactoferrin. Regarding the major proteins, only 21-24% of native Lactoferrin was detected in HTST and HoP after 90 minutes of gastric digestion, while in RHM Lactoferrin remained almost intact. The high molecular weight aggregate (bands 1-2-3nr), containing Lactoferrin and other proteins, was also almost unaffected by gastric digestion in all samples. The other major proteins (such as β-casein, serum albumin and α-lactalbumin) were digested in similar ways in all the samples. Differences in the digestion of minor bands were found between raw and pasteurized samples in reducing conditions for several high molecular weight bands (Macrophage mannose receptor 1 – band R2; Xanthine dehydrogenase – band R3; BSSL + Lactotransferrin – band R4) . These bands showed faster gastric digestion kinetics in pasteurized milk than in RHM, as already seen for Lactoferrin.

Intestinal proteolysis: under non-reducing condition, the high molecular weight aggregates (bands 1-2-3nr) were digested to almost undetectable levels during the intestinal phase in all samples, despite the initial double abundance in undigested samples. The slight, although significantly, lower relative abundance in HoP treated digests of band 4-5-6nr, mainly containing immunoglobulin components and Lactoferrin, was confirmed. The band abundance was largely unaffected by intestinal digestion. BSSL band (7-8-9nr) was significantly higher in RHM than in pasteurized samples at the beginning of the intestinal phase, but it was rapidly digested to almost undetectable levels in the intestine. In reducing conditions, at the beginning of the intestinal digestion, pasteurization increased the resistance to digestion of α-lactalbumin with respect to RHM. Nevertheless, the extent of the difference was small, and, after 180 min, virtually no intact α-lactalbumin is detected in any sample. As already seen in the gastric phase, Lactoferrin (detected in several bands at native molecular weight and as fragments) is more rapidly digested in the pasteurized samples with respect to RHM, and remains largely unaffected by digestion when HM is not pasteurized.

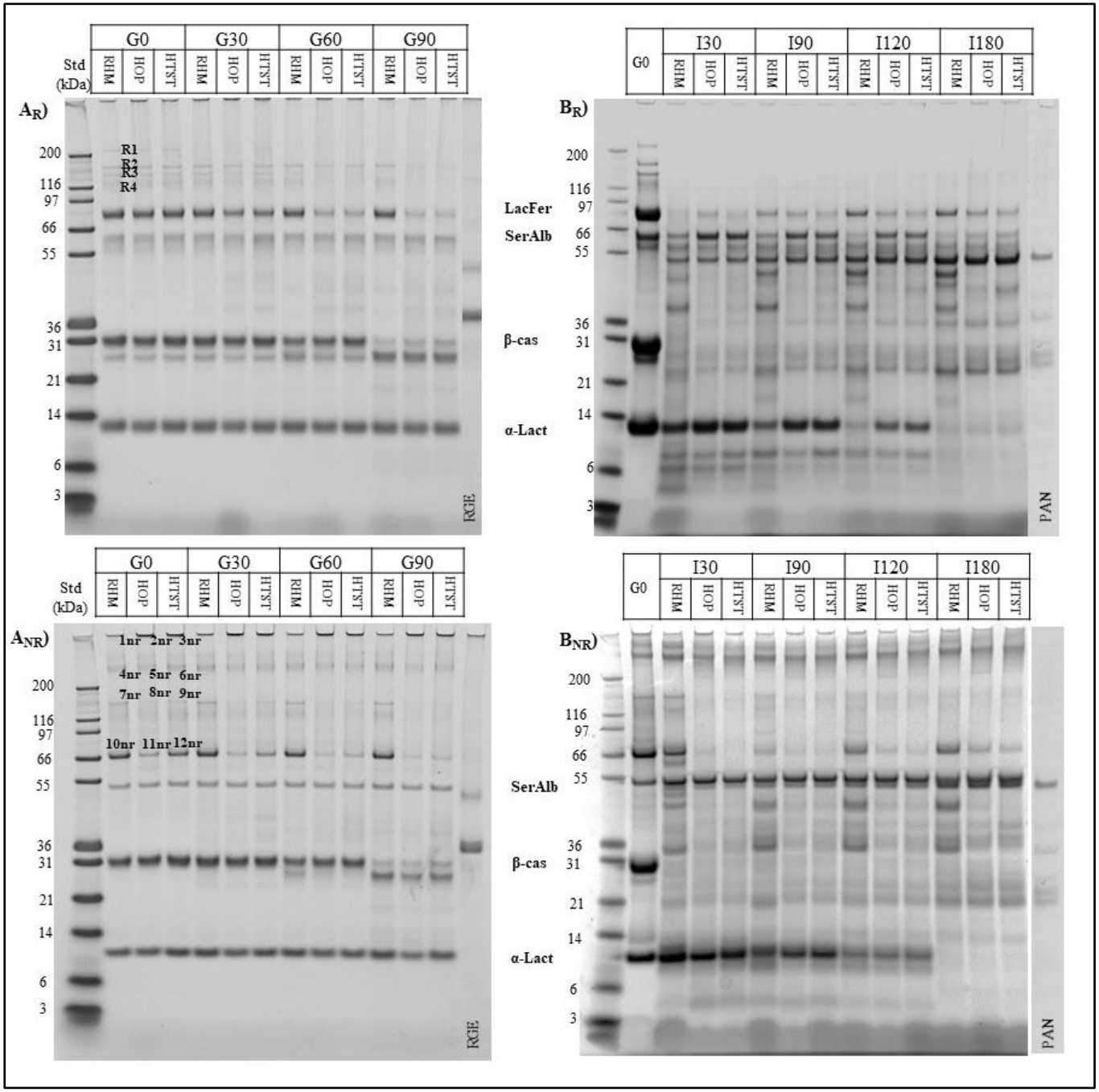


Fig 5. Protein profiles of raw (RHM) and pasteurized (HoP and HTST) HM samples in reducing (A_R and B_R) and non-reducing (A_{NR} and B_{NR}) conditions, during gastric (A) and intestinal (B) phases. Samples were loaded in the gels taking into account the dilution of the gastric and intestinal juices at different sampling times.

G0: undigested HM; G30, G60 and G90: gastric digestion at 30, 60 and 90 minutes; I30, I90, I120 and I180: intestinal digestion at 30, 90, 120 and 180 minutes.

Pan: pancreatin; RGE: Rabbit Gastric Extract.

Lactoferrin (LacFer) and α-lactalbumin (α-Lact) were identified by antibody recognition. Serum albumin (SerAlb) and β-casein (β-cas) were identified in previous papers. Differently coded protein bands were identified by LC-MS/MS (Table 2).

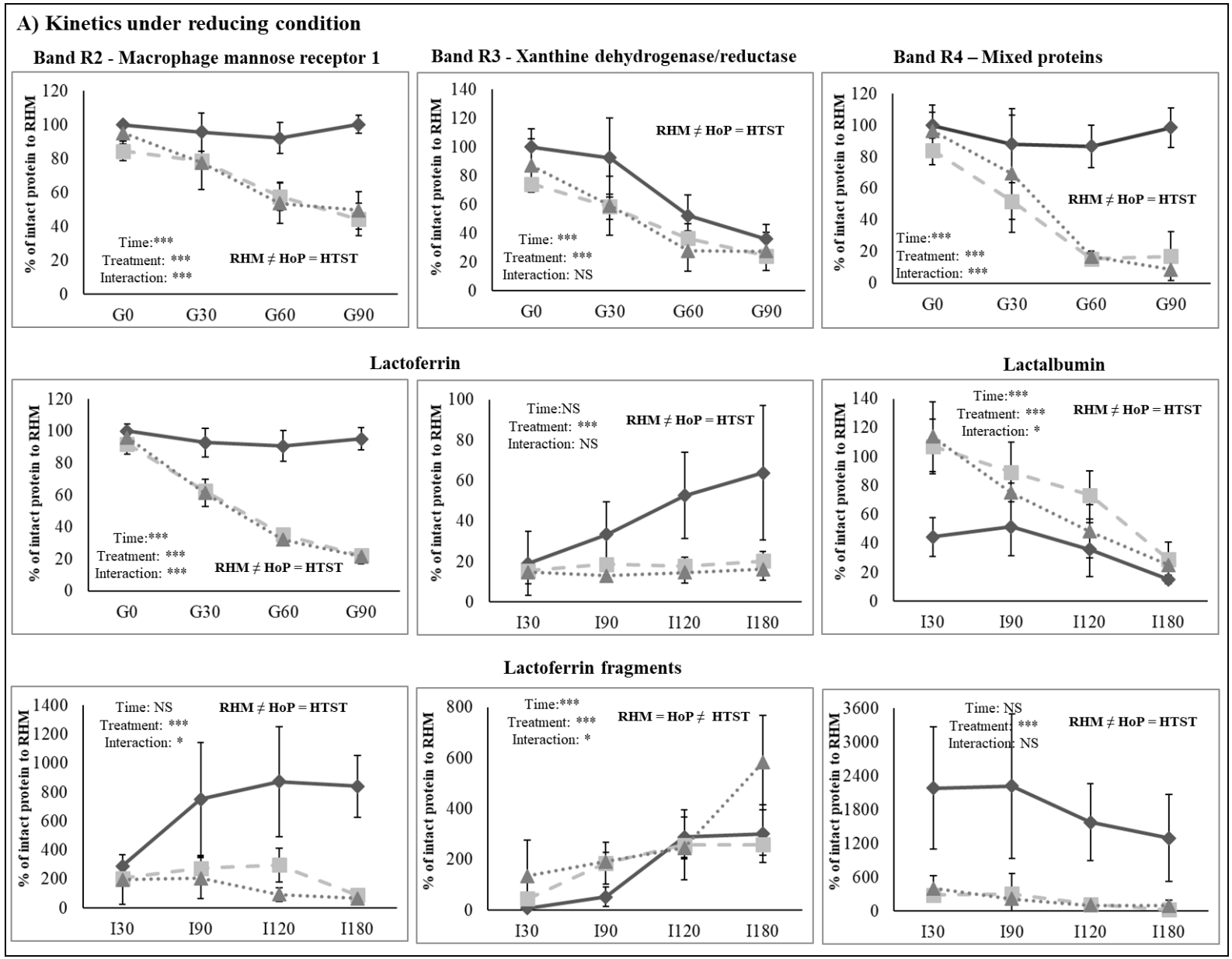


Fig 6A. Percentage of intact band of raw (RHM) and pasteurized (HoP and HTST) HM samples (as relative abundance to undigested raw HM band) during gastric and intestinal digestion, as obtained by densitometric analysis of the gels under **reducing conditions.**
$p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS).

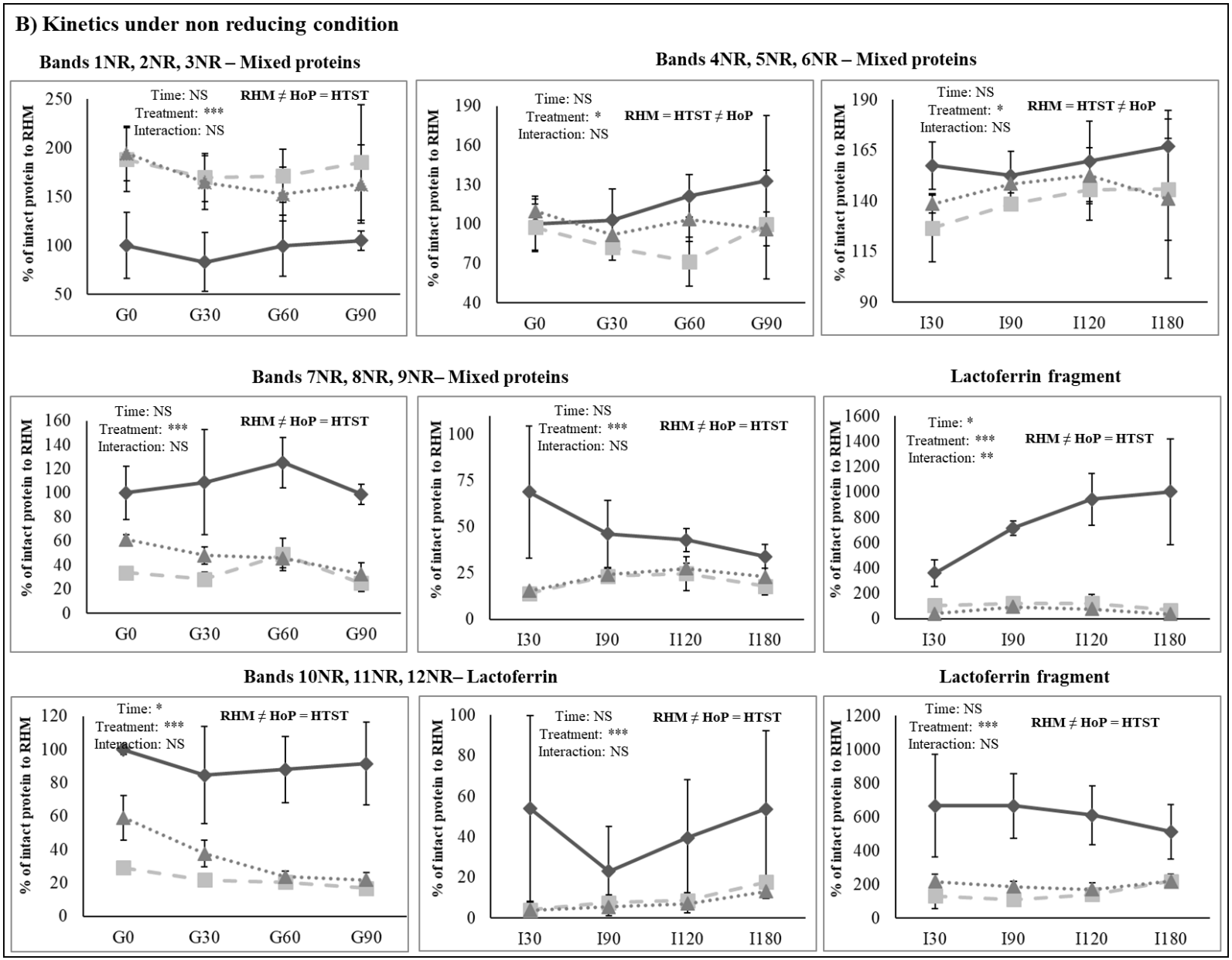


Fig 6B. Percentage of intact band of raw (RHM) and pasteurized (HoP and HTST) HM samples (as relative abundance to undigested raw HM band) during gastric and intestinal digestion, as obtained by densitometric analysis of the gels under **not reducing conditions.** $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS).

6.2.3.3 Intestinal amino acid liberation

The free AA profile of HM samples during intestinal digestion was also determined and quantified (Fig 7). Although a significant difference in the amount of non-essential AAs (Fig 7D) was observed between RHM, HoP and HTST, the release of total (Fig 7A), essential (7B) and semi-essential AAs (7C) was always similar between RHM and HTST, and significantly higher than in HoP.

6.2.4 Lipolysis kinetics

Figure 8 reports the TAGs disappearance during gastric and intestinal digestion. TAG percentage decreased to 72.5 ± 6.7% for RHM, 71.3 ± 4.2% for HoP and 71.9 ± 1.6% for HTST after 30 min of gastric digestion, and remained quite stable afterwards. TAGs hydrolysis was highly increased after 30 min of intestinal digestion, and remaining TAGs reached at end digestion 32.2 ± 1.9% for RHM, 34.3 ± 6.3% for HoP and 29.3 ± 0.8% for HTST of the initial quantity. The lipolysis kinetics was not significantly different between the treatments.

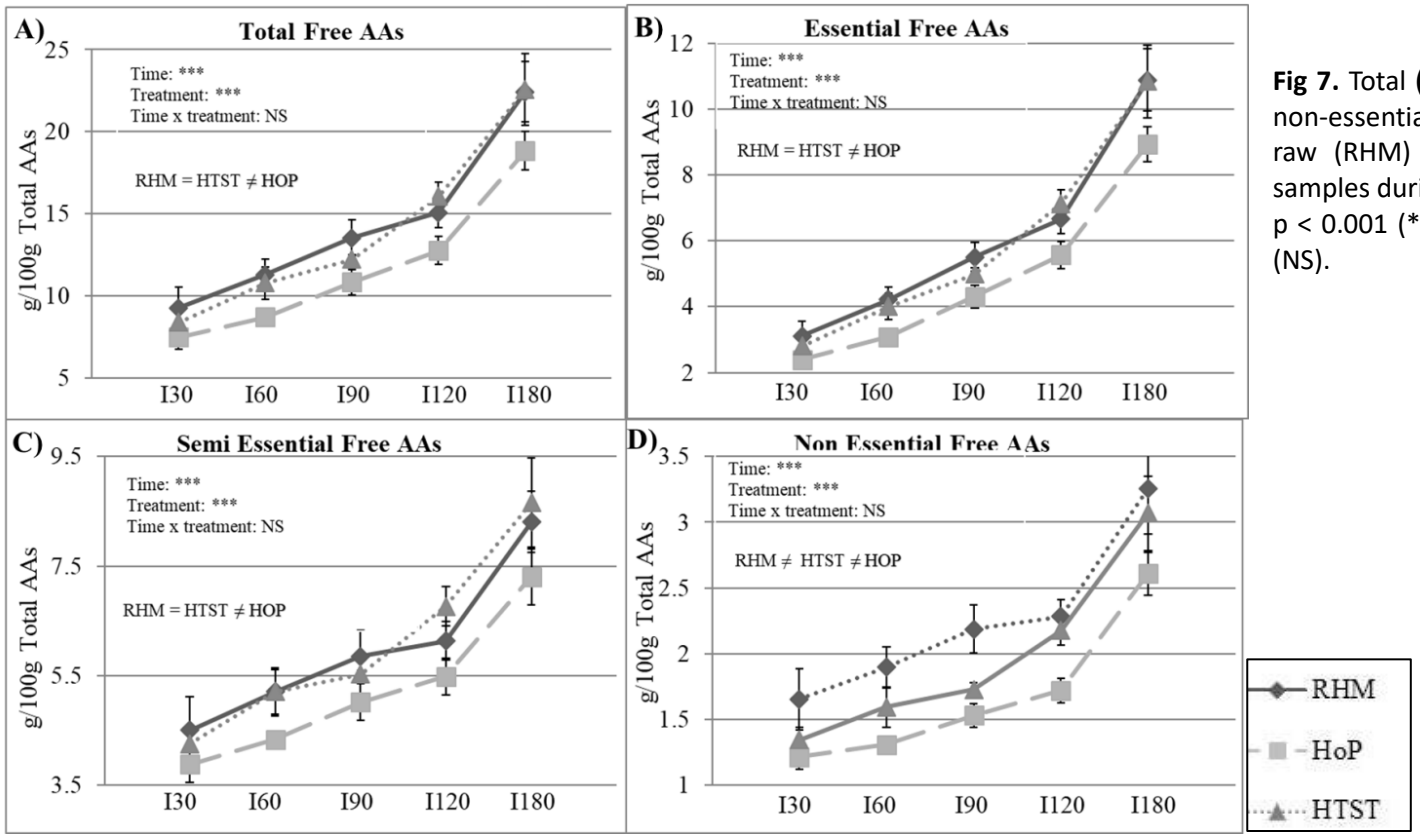


Fig 7. Total **(A)**, essential **(B)**, semi-essential **(C)** and non-essential **(D)** free Amino acids (AAs) release of raw (RHM) and pasteurized (HoP and HTST) HM samples during intestinal digestion.
$p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS).

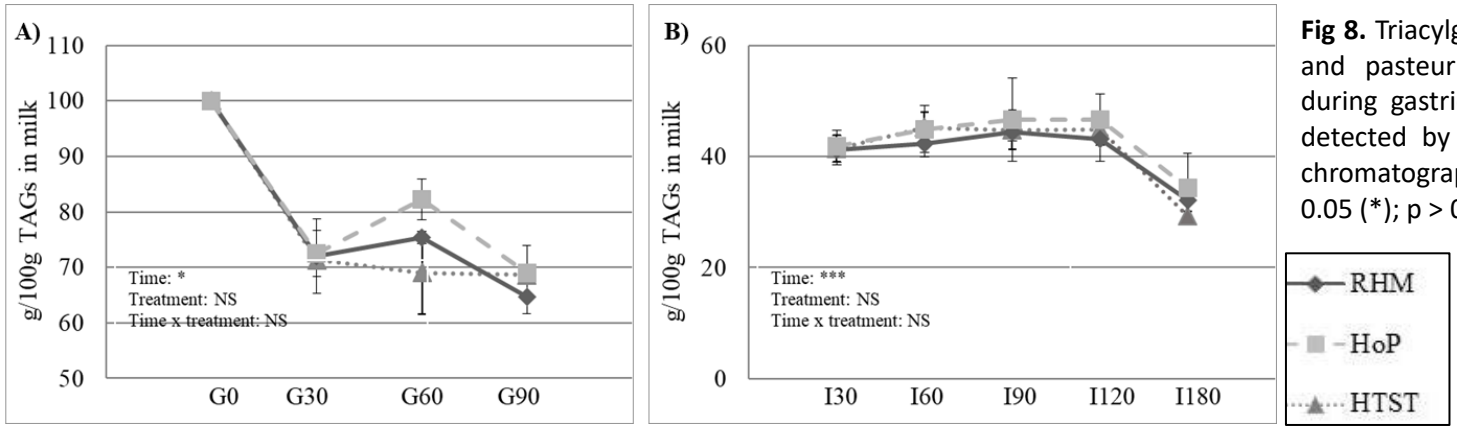


Fig 8. Triacylglycerol (TAG) hydrolysis of raw (RHM) and pasteurized (HoP and HTST) HM samples during gastric **(A)** and intestinal **(B)** digestion, as detected by densitometric analysis of thin layer chromatography. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS).

6.3 Discussion

The present study evaluated, for the first time, whether different thermal pasteurization techniques may differently affect HM digestive kinetics in a preterm newborn model of dynamic digestion. To date, several reports have reported that HTST pasteurization allows a better preservation of mother's milk, in term of both nutritional and biological value in comparison to standard Holder technique. In particular, HTST treated HM was shown to have higher secretory IgAs (sIgAs) content, and higher lysozyme and BSSL activities (Giribaldi *et al*., 2016) in comparison to Holder pasteurized HM. More recently, it was demonstrated that HTST pasteurization led to improved preservation of raw HM antiviral activity (Donalisio *et al*., 2018). Nevertheless, data about possible implications of different pasteurization methods on the profile of digestion, if any, were lacking. In the present experiment, we demonstrated that standard Holder pasteurization and the continuous-flow HTST share the majority of digestive patterns at both lipid and protein level. Nevertheless, some minor significant differences occur, thus indicating that further characterization, especially at peptidomic level, may reveal interesting differential features in the specific digestive patterns of the proteins.

Both pasteurization processes affected HM protein profiles. In particular, Lactoferrin content was notably reduced by pasteurization, however to a much lower extent in HTST than in HoP samples. This is in agreement with what previously described and summarized in the review by Peila *et al*. (2017). These results were largely expected from previous observations (Baro *et al*., 2011; Giribaldi *et al*., 2016). The different trends of soluble proteins in reducing and non-reducing conditions in response to the two pasteurization techniques was noteworthy, since the bands showing differential behavior in the two conditions were composed by the protein fractions that are probably responsible for the formation of high molecular weight aggregates. Besides Lactoferrin, these include BSSL, Macrophage mannose receptor and Xanthine dehydrogenase. This phenomena, which is reversed by addition of DTT, involves the formation of disulfide-bonds between these fractions (Baro *et al*., 2011; Giribaldi *et al*., 2016). Interesting, measuring Lactoferrin in pasteurized HM samples by ELISA tests led to under-estimation of its concentration (data not shown), indicating that the structural modification due to thermal processing is involved in epitope misrecognition by the antibody.

The observation of the milk macrostructure by confocal microscopy revealed that protein aggregates were located in the soluble phase, as well as at the interface of the milk fat globule membrane, and that this type of aggregation seemed to be more visible in the HoP than in HTST milk, although its extent was not quantified. The protein adhesion phenomena to fat globule observed in our study were already observed using confocal microscopy (de Oliveira, Bourlieu, *et al*., 2016; de Oliveira, Deglaire, *et al*., 2016) and a proteomic approach (Ma *et al*., 2019; Ye, Singh, Taylor, &

Anema, 2004) on RHM and HoP. To gain deeper insight on differences between the two pasteurization methods, we performed here, for the first time, the analysis of the HM protein fraction associated to the fat globule membrane in non-reducing condition. MFGPs have an important physiological role, as they may contribute to the protection against bacteria and viruses in the newborn gastrointestinal tract. Different clinical trials also indicate that the MFGPs have potential beneficial effects on the newborn immune system and on their cognitive functions due to the bioactive proteins attached to the fat globules (Demmelmair, Prell, Timby, & Lönnerdal, 2017). Protein profile of MFGPs was highly impacted by the thermal processing, with a lower alteration of the native MFGPs profile in the HTST milk with respect to HoP. Even for the fat globule associated fraction, a clear involvement of Lactoferrin, xanthine dehydrogenase and bile salt-activated lipase in the formation of aggregates was appreciated. Fatty acid-binding protein and α-lactalbumin were detected in MFGPs fraction only after pasteurization, indicating a protein transfer from the soluble phase to the fat globule membrane after pasteurization, as already described (Ma, Zhang, Wu, & Zhou, 2019). Protein modification after pasteurization seemed to affect also Lysozyme and β-casein.

A strong particle destabilization was detected in RHM from 60 minutes of gastric digestion with the formation of larger aggregates, while both pasteurized milks didn't show similar particle size shifts. The emulsion destabilization observed in this study for RHM was in agreement with the previous studies, conducted both *in vitro* (de Oliveira, Bourlieu, *et al*., 2016 and de Oliveira, Deglaire, *et al*., 2016) and *in vivo* (de Oliveira *et al*., 2017). Confocal microscopy of gastric samples showed the formation of big aggregates only in raw sample as described by de Oliveira, Bourlieu, et al., 2016 and de Oliveira, Deglaire, et al., 2016. These aggregates were based on clusters of milk fat globules around which protein aggregates built up. These aggregates presented lower density than milk aqueous phase and these lipoprotein clusters were prone to cream in the gastric compartment. Pasteurization selectively affected the gastric proteolysis, since the susceptibility to proteolysis depends on the specific structure of the protein. This difference in protein accessibility due to structural modifications induced by pasteurization could modify the aggregate grade of the proteins with the milk fat globules during gastric digestion. The lack of formation of large gastric aggregates in HoP treated HM was previously shown to not interfere with gastric emptying (de Oliveira *et al*., 2017), a factor previously addressed as indicator of the feeding intolerance development and in necrotizing entherocolititis for preterm newborns (Li *et al*., 2014).

Despite significant differences in the protein pattern of the starting undigested HM, the proteolysis profiles originating from the two pasteurization methods were similar, and differences with respect to RHM were observed. The only significant difference between the two methods was related to the higher resistance to digestion in RHM and HTST of one

band containing immunoglobulin components with respect to HoP, although its extent was limited. Electrophoretic patterns showed that all immunoglobulin containing bands were poorly affected by digestion.

All the pasteurization procedures have a major impact on Lactoferrin structure, as already seen in undigested milk, resulting in increased digestibility, with respect to RHM. These observations are in agreement with previous *in vivo* data, showing that intact Lactoferrin and IgAs could be detected in the stool of breastfed infants (Lönnerdal, 2016). IgAs have a leading role in the correct development of newborn immune system, and their higher resistance following HTST with respect to HoP, although slight, may contribute to maintaining immune homeostasis in the particularly fragile preterm newborns. Lactoferrin facilitates the first step of the iron absorption process, and stimulate epithelial cell proliferation and differentiation (Lönnerdal, 2016). Its faster hydrolysis and higher aggregation following all pasteurization techniques may thus be detrimental for the overall protective effect on the pretem newborns, although its main siderophore activity could be preserved when aggregated, and warrants further functional investigations. On the other hand, Lactoferrin is a recognized source of bioactive peptides with important antimicrobial role in the gastrointestinal tract (Wada & Lönnerdal, 2014), and its increased digestibility following pasteurization may, therefore, be beneficial. Further peptidomic analysis, currently underway, may shed light on this peculiar aspect.

In our study we didn't find any significant difference in the initial free AA content between the samples, as well as in the content of essential and semi essential AAs. Despite the minor differences in the kinetics of intestinal digestion of the different protein fraction between HM samples pasteurized by HoP and by HTST, a significant difference was found in their total and essential AA content during digestion. In particular, a significantly lower amount of both was found in HoP digests, up to -20% with respect to HTST. The impact of pasteurization on the AA release during digestion was poorly investigated in preterm model. Indeed, only de Oliveira, Deglaire, *et al*. (2016) investigate the AA release during digestion on HoP pasteurized HM. The higher released of both total and essential AAs in the intestinal tract found in RHM and HTST samples could be a positive factor for the physiological state of the preterm infant, since they represent an important source of energy, fuel lymphocyte differentiation, contribute to the intestinal cell integrity and to protection against oxidative stress (Valentine *et al*., 2010). Since these differential release of AAs between HoP and HTST does not reflect protein degradation pattern, further peptidomic investigation may contribute to a further understanding of these discrepancies.

No impact of the pasteurization on lipid hydrolysis kinetics was found in the present study, although we observed a high level of pre-lipolysis in undigested milk, probably related to the long storage in HMB before thermal treatments. Results confirmed previous observations on preterm milk lipolysis by de Oliveira, Bourlieu, *et al*. (2016). Since lipids are an

important energy source for the newborns, as well as being involved in the regulation of immune system, and converted to metabolites that regulate inflammatory processes and organogenesis, the low impact of pasteurization on their physiological release is thus important. Clinical evidence suggests that alterations in FA intake in preterm infants may have implications on the risk of chronic lung disease, necrotizing enterocolitis, retinopathy and neurodevelopment (Robinson & Martin, 2017). Within this framework, the decreased activity of BSSL following both pasteurizations seemed to have little or no impact on the total degradation of TAGs, its function being balanced by endogenous intestinal lipases.

The use of an *in vitro* dynamic model of digestion allows to unravel the differential impact of HTST pasteurization on the digestive kinetics of proteins and lipids in donated HM, thus providing an alternative to costly and controversial *in vivo* tests, especially when dealing with the most vulnerable infants, such as preterm. The specific design of the HTST equipment, tailored for use with donor human milk, was confirmed to be less damaging than standard HoP on specific bioactive proteins, mainly involved in immune responses. The present study has proven, for the first time to our knowledge, that HTST may also provide a better retention of some of these bioactive proteins throughout gastrointestinal digestion, although the extent of this retention was limited. Nevertheless, further peptidomic analysis is expected to provide insight into specific features that differently affect the two pasteurization methods, and that seems to lead to a total and essential free aminoacid release more similar between HTST and raw human milk.

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CHAPTER 7

CONCLUSIONS

The aim of this thesis was to investigate the changes in food allergenicity and digestibility induced upon processing

In the first part of this work, the effect of roasting was evaluated on the allergenicity of hazelnut OB associated proteins in a cohort of hazelnut allergic pediatric patients. In our study, the majority of patients immune-recognized oleosins in both raw and processed hazelnuts. Moreover, pediatric patients with hazelnut allergic symptoms and negative diagnostic tests to major allergens recognized oleosins exclusively. The results demonstrated the crucial role of oleosins in the routine hospital diagnostic tests and, particularly, in patients for which it was not possible to make a clear correlation between symptoms and IgE molecular recognition. In addition, a new allergenic oleosin was discovered. The sequence of this new oleosin was accepted by the WHO/IUIS allergen nomenclature sub-committee with the official name of Cor a 15. In our experiment, the new allergenic oleosin Cor a 15 was the most frequently recognized hazelnut oleosin by the patients' IgEs, in both raw and roasted hazelnut.

The results obtained in the second part of this work allowed to highlight that the modification of hazelnut proteins induced by roasting modified the level of IgEs recognition by pediatric allergic patients. We evaluated the effect of roasting on hazelnut allergenicity, using different roasting technologies and conditions. At low temperature, roasting did not affect the hazelnut allergenicity and a similar immunoreactivity pattern between hot air and infrared roasting was found. For both technologies, roasting at high temperature resulted in lower hazelnut immune-reactivity, in comparison to low temperature roasting and to raw samples. Oleosin allergenicity showed a strong resistance to high temperature roasting, for both techniques tested in this study.

In the third part of this thesis, the effect of processing was evaluated for animal allergens. Proteins of edible insects were subjected to two different processing (boiling and frying) and insects cross-allergenicity was evaluated in a cohort of house dust mite (HDM), shrimps and primary mealworm allergic patients. Results allowed to observe for the first time that the cockroach allergen-like protein was involved in primary respiratory and food allergy in mealworm. In general, the effect of processing did not eliminate the allergenicity of edible insects and only partially reduced their allergenicity potential. Moreover, HDM, shrimp and mealworm allergic patients should consume insects with caution, since different proteins are involved in insect's cross-sensitization.

In the last chapter, the effect of thermal processing on food protein digestibility was evaluated using a simulated preterm newborn *in vitro* dynamic digestion system. With this system, the impact of two pasteurization methods (Holder vs HTST) on human milk digestibility was investigated. The lactoferrin content, the profile of proteins associated to the membrane of fatty acids globules, and the milk macrostructure were highly impacted by pasteurization, to a much lower extent following HTST than HoP pasteurization. Human milk digestibility resulted to be differentially affected by the two pasteurization methods, as far as the essential amino acid release and resistance to digestion of

immunoglobulins were concerned. In particular, HTST pasteurization resulted to allow a digestion profile closer to that of raw milk, in comparison to Holder pasteurization.

Despite the food matrices selected for the present study were quite different, the processing resulted in similar effects, as far as the two following aspects are concerned:

- Processing modified the allergenicity in both water-soluble and not soluble protein fractions of hazelnut and edible insects. For both food matrices, allergenicity resulted to be either increased or decreased by heat processing, depending on the allergen type and processing conditions. In both cases, processing did not eliminate the food allergenic power.

- The lipid component of foods was affected by processing and, consequently, the associated lipid proteins. Microscopy images of hazelnuts and human milk showed a relevant effect of processing on the fat structure. In the first case, the hazelnut OBs decreased in size and their integrity was lost after roasting. In human milk, the human MFG and whey proteins appeared aggregated after pasteurization. The structural changes induced upon processing in both hazelnut OB and human MFG affected the associated proteins. In both cases, the protein profile of processed material was very different when compared to the raw ones. In addition to the native proteins associated to fat/oil membranes, water soluble allergens (Cor a 9 and Cor a 11) were found in the hazelnut OBs associated protein extract after roasting and, Fatty acid-binding protein and α-lactalbumin were detected in the human MFG associated protein fraction after pasteurization. The protein adhesion to the fat component after heat processing is probably due to the obtaining a different folding of the molecule exposing its hydrophobic parts and, as consequence, allowing the adhesion of the protein to the fat membrane.

The results obtained in this work allowed to obtain novel evidences on the effect of processing in food protein allergenicity and gastro-intestinal digestion.

Further studies should be performed in order to better understand the clinical relevance of allergenic oleosin Cor a 15 in a wider cohort of hazelnut allergic pediatric patients using *in vivo* and *in vitro* tests. Moreover, it would be of interest to evaluate the clinical relevance and the effect of thermal processing on Cor a 15 allergenicity, in a cohort of hazelnut allergic adult patients.

In addition, in order to better characterize the allergenicity potential of insects as food source, HDM and shrimp allergic volunteers should be *in vivo* challenged in a controlled double blind placebo clinical trial.

Last, the kinetic of human milk digestion should be further investigated, in order to obtain evidences on the effect of pasteurization on peptide release during the digestive process, with a focus on the release of bioactive peptides.

List of Abbreviations

1DE: one dimentional electrophoresis
2DE: two dimentional electrophoresis
AA: amino acid
BAT: basophil activation test
BSSL: bile salt-dependent lipase
CBB: coomassie brilliant blue
DDA: data-dependent acquisition
DTT: DL-dithiothreitol
ESI-Q-TOF: electrospray ionization quadrupole time-of-flight
HDM: house dust mite
HM: human milk
HoP: holder
HTST: hight temperature short time
IAA: iodoacetamide
IgE: Immunoglobulin E
LC-MS: liquid chromatography- mass spectrometry
LDS-PAGE: lithium dodecyl sulfate- polyacrylamide gel electrophoresis
MALDI-TOF: matrix-assisted laser desorption/ionization- time-of-flight
MGF: milk fat globule membrane
MGFP: milk fat globule membrane proteins
OBs: oil bodies
OPA: o-Phthalaldehyde
PAS: Periodic Acid Schiff
PbP: prick-by-Prick
RAST: radioallergosorbent test
SPT: skin prick test
TAG: triacylglycerol
TEM: transmission electron microscopy

List of Publications and Congress Contributions

Publications

Research articles

Nebbia S, Lamberti C, Giorgis V, Giuffrida MG, Manfredi M, Marengo E, Pessione E, Schiavone A, Boita M, Brussino L, Cavallarin L, Rolla G (2019). *The Cockroach Allergen-like protein is involved in primary respiratory and food allergy to yellow mealworm (Tenebrio molitor)*. Clinical & Experimental Allergy, 49(10), 1379-1382. https://doi.org/10.1111/cea.13461

Giorgis V, Rolla G, Raie A, Geuna M, Boita M, Lamberti C, Nebbia S, Giribaldi M, Giuffrida MG, Brussino L, Corradi F, Bacco B, Cassarino Gallo S, Nicola S, Cavallarin L. (2018). *A case of work-related donkey milk allergy.* Journal of Investigational Allergology & Clinical Immunology, 28(3):197-199. https://doi.org/10.18176/jiaci.0237

Manuscripts under evaluation

Lamberti C, Nebbia S, Balestrini R, Marengo E, Manfredi M, Cirrincione S, Giuffrida MG, Cavallarin L, Abbà S, Acquadro A. (2019). *Identification of caleosin in hazelnut*. Submitted to Plant Science.

Nebbia S, Lamberti C, Cirrincione S, Acquadro A, Marinoni A, Manfredi M, Marengo E, Calzedda S, Abbà S, Monti G, Cavallarin L, Giuffrida MG. (2019). *Oleosin Cor a 15 is a novel allergen in hazelnut allergic Italian children*. Submitted to Allergy.

Manuscripts in preparation

Nebbia S, Lamberti C, Giorgis V, Cirrincione S., Giuffrida MG, Rolla G, Cavallarin L. *Influence of processing on the allergic cross-reactivity of five edible insects*.

Nebbia S, Giribaldi M, Cavallarin L, Coscia A, Briard-Bion V, Ossemond J, Henry G, Ménard O, Dupont D, Deglaire A. *Impact of HTST pasteurization of human milk on preterm newborn in vitro digestion: gastrointestinal disintegration, lipolysis and proteolysis.*

Nebbia S, Giribaldi M, Cavallarin L, Coscia A, Briard-Bion V, Ossemond J, Henry G, Ménard O, Dupont D, Deglaire A. *Impact of human milk HTST*

pasteurization on the kinetics of peptide release during in vitro dynamic digestion at the preterm newborn stage.

Lamberti C, Nebbia S, Balestrini R, Marengo E, Manfredi M, Cirrincione S, Giuffrida MG, Cavallarin L. *Effect of hot-air and infrared-roasting on hazelnut allergenicity*.

Extended abstract

Nebbia S, Lamberti C, Bua A, Manfredi M, Marengo E, Monti G, Cavallarin L. Giuffrida MG. (2018). *Characterization of allergy to hazelnut oleosins in pediatric patiets in Italy*. Wiley online library, Allergy.

Congress Contributions

Oral presentation

Nebbia S. (2018). Extraction and 2D electrophoresis set up protocols of proteins associated to tree nuts oil bodies. “Proteomix International”, 7-8 September, Padova, Italy.

Oral poster session

Nebbia S, Lamberti C, Bua A, Manfredi M, Marengo E, Monti G, Cavallarin L. Giuffrida MG. (2018). Characterization of allergy to hazelnut oleosins in pediatric patients in Italy. "EAACI congress", 26-30 May, Munich, Germany.

Posters

Lamberti C, Nebbia S, Cirrincione S, Antoniazzi S, Giribaldi M, Manfredi M, Marengo E, Giuffrida MG, Cavallarin L. (2019). *Effect of different types of roasting on hazelnut immunoreactivity.* ‘‘2nd Food Chemistry Conference”, 17-19 September, Seville, Spain.

Cirrincione S, Nebbia S, Lamberti C, Bufo A, Giorgis V, Manfredi M, Marengo E, Giuffrida MG, Rolla G, Cavallarin L. (2019). *Effect of thermal processing on the immune cross-reactivity of five edible insects.* “2nd Food Chemistry Conference”, 17-19 September, Seville, Spain.

Nebbia S, Giribaldi M, Cavallarin L, Coscia A, Ménard O, Dupont D, Deglaire A. (2019). *Impact of HTST pasteurization of human milk on the kinetic of digestion of macronutrients after in vitro dynamic digestion.* "6TH International Conference of Food Digestion", 2-4 April, Granada, Spain.

Giribaldi M, Nebbia S, Cavallarin L, Sottemano S, Briard-Bion V, Jardin J, Ménard O, Dupont D, Deglaire A. (2019). *Do different techniques of human*

milk pasteurization impact the kinetics of peptide release during in vitro dynamic digestion? "6TH International Conference of Food Digestion", 2-4 April, Granada, Spain.

Nebbia S, Lamberti C, Giorgis V, Basile S, Giuffrida MG, Rolla G, Cavallarin L. (2018). *Effect of thermal processing on the proteins profiles and allergenicity of edible insects*. "EFSA science, food, society", 18-21 September, Parma, Italy.

Lamberti C, Nebbia S, Cirrincione S, Manfredi M, Marengo E, Monti G, Cavallarin L, Giuffrida MG. (2018). *Characterization by 2DE and LC-MS of hazelnut oleosins allergy in Italian pediatric patients*. "3rd INPPO World Congress", 9-12 September, Padova, Italy.

Nebbia S, Lamberti C, Manfredi M, Marengo E, Cavallarin L, Giuffrida MG. (2018). *The synergism of MALDI LIFT-TOF/TOF MS and ESI –Q-TOF MS instruments to discover new isoforms of allergens from hazelnut oleosin extract.* "XXII international mass spectra conference", 26-31 August, Firenze, Italy.

Lamberti C, Nebbia S, Giorgis V, Fornero M, Giuffrida MG, Rolla G, Cavallarin L. (2018). *A case of two allergic patients sensitized to Tenebrio molitor by occupational exposure*. "EAACI congress" 26-30 May, Munich, Germany.

Nebbia S. (2017). *Identification of 30 KDa Bombyx mori allergen by immunoblotting and MALDI- TOF/TOF mass spectrometry*. School "3rd App-ms intensive and travelling school of applied mass spectrometry to complementary techniques", 23-30 April.

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