

Bovine Milk Allergens: A Comprehensive Review

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Abstract: Cow milk allergy is one of the most common food allergies in early childhood and often persists through adult life, forcing an individual to a complete elimination diet. Milk proteins are present in uncounted food products, such as cheese, yogurt, or bakery item, exposing allergic persons to a constant threat. Many efforts have been made to overcome this global problem and to improve the life quality of allergic individuals. First, proper and reliable food labeling is fundamental for consumers, but the verification of its compliance is also needed, which should rely on accurate and sensitive analytical methods to detect milk allergens in processed foods. At the same time, strategies to reduce milk allergenicity, such as immunotherapy or the use of food processing techniques to modify allergen structure, have to be extensively studied. Recent research findings on the applicability of food processing, such as heat treatment, fermentation, or high pressure, have revealed great potential in reducing milk allergenicity. In this review, significant research advances on cow milk allergy are explored, focusing on prevalence, diagnosis, and therapy. Molecular characterization of cow milk allergens and cross-reactivity with other nonbovine milk species are described, as well as the effects of processing, food matrix, and digestibility on milk allergenicity. Additionally, analytical methods for the detection of milk allergens in food are described, from immunoassays and mass spectrometry methods for protein analysis to real-time polymerase chain reaction for DNA analysis.

Keywords: allergen, cross-reactivity, detection, food processing, milk proteins

Introduction

The use of milk from domesticated mammalian animals in the human diet has a very long tradition. Today, cattle, buffaloes, sheep, goats, and camels are used in various parts of the world for the production of milk and milk products for human consumption (Goff 2016). Accordingly, about 82.4% and 13.6% of the world's fresh milk comes from cows and buffaloes, respectively, while the remaining 4.0% is produced by goats, sheep, and camels. In the last 3 decades, the world's milk production has grown by more than 63%, increasing from 500 million tons in 1983 to 819.3 million tons in 2016 (FAO 2017). Asia contributed with almost 41% to global milk production in 2016, with India as its leading producer (19.6%). Both American and European continents assure almost 50% of the world's milk production, with the United States (11.8%) and Germany (4.1%) occupying the top positions, respectively. In terms of total trade of milk (share of production), only 8.7% is destined for import/export (FAO 2017). Per capita milk consumption is rather high (>150 kg/capita/y) in most developed countries (like the U.S., Finland, the Netherlands, the U.K., Sweden), while in developing ones (such as Angola, Haiti, Guinea, Mozambique, Malawi, Liberia), the intake of milk and milk products is often less than 30 kg/capita/y. The average consumption of milk and dairy products in Europe is more than 218

kg/capita/y, corresponding to 8% to 9% of dietary energy, 19% of dietary protein and 11% to 14% of dietary fat intake (FAOSTAT 2017).

As a source of vitamins and minerals (calcium, vitamin A, and vitamin B6), which are needed for the growth and development of young children, and beneficial for human bones, hair, skin, and teeth, milk is among the 1st foods to be introduced into an infant's diet (Do and others 2016). However, it is also one of the 1st and most common causes of food allergy in early childhood (Hochwallner and others 2014). Food allergy is defined as an adverse reaction mediated by the immune system and caused by the intake of some kind of food that occurs reproducibly in allergic individuals (Boyce and others 2010). Almost 90% of worldwide reported food allergies are caused by 8 groups of food products, in which milk is included (CODEX STAN 1-1985). Milk allergy is typically mediated by immunoglobulin E (IgE), inducing adverse reactions to proteins present in milk that might occur following their ingestion by sensitized/allergic individuals. Different food products might be responsible for this type of allergy since milk proteins are used as processing aids, with the possibility of being present at trace amounts in a large number of food products (Monaci and others 2006). Moreover, infant formulas, milk powders, cheeses, and yogurts are among the food products that use milk as an ingredient for their production (Fox 2001). Another important fact is that milk of all ruminant species (such as, cow, sheep, and goat) contains homologous proteins, which share the same structural, functional, and biological properties and, thus, contribute to the cross-reactivity phenomenon in allergic individuals (Monaci and others 2006).

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Sometimes milk allergy is confused with milk intolerance, which is much more common and produces clinical symptoms very similar to those of milk allergy, but not so dangerous. Contrarily to milk allergy, milk intolerance is a nonimmunological reaction to a certain milk component, causing disorders in digestion, absorption, or metabolism. A common example is the malabsorption of lactose due to an intestinal lactase deficiency, thus being classified as a metabolic disease.

In true milk allergy, typical IgE-associated symptoms appear immediately or within 2 h after the intake of milk. It can affect the skin (atopic dermatitis or eczema, angioedema, or urticaria), the respiratory system (rhinitis, asthma exacerbation, wheezing, pulmonary infiltrates, or acute rhinoconjunctivitis), and the gastrointestinal tract (vomiting, recurrent diarrhea, abdominal pain, excessive colic, or esophageal reflux). In some cases, the allergic reaction may also involve 1 or more target organs/systems, leading to a complex and systemic anaphylactic response, and often resulting in death (El-Agamy 2007; Hochwallner and others 2014; Martorell-Aragónés and others 2015). However, delayed adverse immunological manifestations may also occur, normally after 2 h of milk ingestion. In this case, non-IgE mediated mechanisms are typically involved, including a wide range of clinical presentations, such as mild rectal bleeding in milk protein induced proctocolitis or severe vomiting in food protein induced enterocolitis syndrome (Venter and others 2017). Accordingly, mild to moderate clinical symptoms in milk allergic individuals are commonly attributed to non-IgE mediated mechanism, while the severe adverse immunological responses are often IgE-mediated (Venter and others 2017). Milk allergy is the third most common food allergy that triggers anaphylactic reactions, just after peanut and tree nuts, accounting for 10% to 19% of all food-induced anaphylactic cases (Kattan and others 2011).

Currently, there is no treatment for milk allergy. Once diagnosed, the prevention of an allergic reaction relies mostly on the total avoidance of the offending food. Therefore, to guarantee consumer protection and ensure life quality to sensitized individuals, a correct and truthful food labeling system has become imperative (Costa and others 2012; Rencova and others 2013; Gomaa and Boye 2015b). Legal measures have been established and adopted by the majority of countries in the world to protect the life of those individuals (Gendel 2012; Taylor and Baumert 2015). In 1985, the *Codex Alimentarius* Commission issued, for the 1st time, a recommendation for the mandatory labeling of prepackaged food susceptible of containing potentially allergenic ingredients. Following this recommendation, 8 allergenic foods (milk, tree nuts, peanuts, gluten-containing cereals, soybean, fish, eggs, and crustaceans) and sulfites were proposed as priority for labeling systems (CODEX STAN 1-1985). Within the European Union (EU), Directive 2003/89 /EC added sesame, celery, and mustard to the previous items (CODEX STAN 1-1985), therefore totaling 12 product groups. Since then, the EU has established legislation extending the priority list to 14 groups (with the addition of mollusks and lupine) that are required to be emphasized over the rest of the ingredients enumerated in processed foods, regardless of their quantity (Directive 2007/68/EC; Regulation (EU) No 1169/2011).

Nevertheless, the total avoidance of milk consumption can cause a nutritional deficiency and may influence the growth of infants and children. As an attempt to overcome this problem, the development and optimization of new strategies of milk processing in order to destroy or modify the structure of these allergens and, therefore, reduce or eliminate their

allergenicity have been widely investigated (Bu and others 2013).

This review intends to provide an overview on the prevalence of milk allergy, its diagnosis, and therapy, with focus on the molecular characterization of milk allergens and cross-reactivity phenomena between milks of different species in allergic patients. Additionally, it describes the available methods for the detection of milk allergens in foods containing milk and milk proteins, and the effect of processing in the reduction of their allergenicity.

Prevalence, Diagnosis, and Therapy

It has been reported that nowadays 0.6% to 3% of children below the age of 6 y, 0.3% of older children and teens, and less than 0.5% of adults suffer from cow milk allergy, the most common type of milk allergy. Interestingly, the majority of milk allergic infants outgrow their allergy becoming able to consume milk and its products, although 15% of the affected children remain allergic throughout adulthood. One study reports that 45% to 50% of children outgrow milk allergy at 1 y of age, 60% to 75% at the age of 2 y, and 85% to 90% at 3 y, but the mechanisms underlying the development of this natural tolerance are not yet fully understood (Fiocchi and others 2010; Bu and others 2013). The development of natural tolerance seems to be attributed to the decline of IgE due to avoidance of milk ingestion at early stages of life or to the presence of IgE against mainly conformational epitopes (enabling the consumption of milk and milk products), rather than against sequential epitopes (Hochwallner and others 2014).

The diagnosis of IgE-mediated milk allergy is described in detail in a recent article from the World Allergy Organization (WAO) (Fiocchi and others 2010). The diagnosis begins with observation of clinical manifestations and medical history, followed by *in vitro* and *in vivo* diagnostic tests and oral provocation tests (oral food challenge [OFC] and double-blind placebo-controlled food challenge [DBPCFC]). The *in vitro* diagnostic tests include the measurement of milk allergen-specific IgE in blood serum (ImmunoCAP, Phadia AB, Uppsala, Sweden). The skin prick test (SPT) is applied *in vivo* using a commercial milk fraction or milk protein fractions that is pricked into the epidermis of a patient, resulting in the appearance of a wheal greater than the control if the patient has IgE against milk allergens. The oral provocation tests, such as DBPCFC or OFC are considered the “gold standard” methods for the correct diagnosis of food allergies. They consist in the oral administration, on different days, of placebo and progressively increasing quantities of milk until the appearance of observable (positive result) or subjective clinical symptoms upon 2nd administration of the same amount of the offending food. If the oral provocation test is considered negative (absence of clinical symptoms), the patient is advised to gradually reintroduce milk in the daily diet following a specific scheme.

The current effective treatment for milk allergy is the adoption of an elimination diet (Mousallem and Burks 2012). However, accidental exposure to milk proteins is recurrent, principally because these allergens are present in a great number of processed foods, such as meat products, fish products, desserts, bakery products, among others. In this case, medical treatment includes oral antihistamine for mild cutaneous or digestive reactions and an epinephrine autoinjector for systemic or respiratory reactions (Hochwallner and others 2014; Martorell-Aragónés and others 2015). More recently, some strategies have been developed and applied to induce desensitization or even tolerance, to different allergens. Immunotherapy has been advanced as a promising treatment approach, aiming at achieving a permanent state of tolerance

in sensitized individuals (Mousallem and Burks 2012). Based on different routes of administration (subcutaneous, epicutaneous, sublingual, and oral), the patient is exposed daily to increasing doses of the offending food, to induce immunomodulation and a desensitization state. Recent studies using immunotherapy have been proposed for milk allergy with promising results. Oral immunotherapy (OIT) shows a success rate that varied from 37% to 70% (Longo and others 2008; Skripak and others 2008; Narisety and others 2009; Brożek and others 2012).

Molecular Characterization of Milk Allergens

In recent years, the great increase of allergen identifications and the knowledge about their sequences have permitted the establishment of databases providing molecular, biochemical, and clinical data of allergens. The official list of allergens issued by the World Health Organization/Intl. Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee and the ALLERGOME databases are 2 of the numerous accessible sources (ALLERGEN 2017; ALLERGOME 2017). The milk allergens included in the official list (WHO/IUIS) were all identified as belonging to bovine milk (*Bos domesticus*).

Cow milk contains about 3 g of protein per 100 mL and includes at least 25 different proteins, all of which may act as antigens (Martorell-Aragónés and others 2015). Cow milk proteins are classified into 2 main categories that can be separated based on their solubility at pH 4.6 and 20 °C (Fox 2001). The group of proteins that precipitate are caseins (α_{S1} -casein, α_{S2} -casein, β -casein, and κ -casein) and the group that remain soluble are known as serum or whey proteins (β -lactoglobulin [β -LG], α -lactalbumin [ALA], bovine lactoferrin, bovine serum albumin [BSA], and bovine immunoglobulins), corresponding to 80% and 20%, respectively. Caseins, β -LG, and ALA are considered the major allergens; however, lactoferrin (LF), BSA, and immunoglobulins (Ig), which are present at lower quantities, have been shown to be of great importance in inducing milk allergies (Fox 2001; Hochwallner and others 2014). A summary on the known cow milk allergens, their biological functions, and accession numbers is provided in Table 1.

Caseins (Bos d 8)

Caseins are the major protein fraction of cow milk, amounting to about 80% of the total milk proteins, with sizes ranging from 19 to 25.2 kDa (Restani and others 2009; Hochwallner and others 2014). According to the WHO/IUIS official list of allergens, caseins are classified with the general designation of Bos d 8. However, in spite of this common name (Bos d 8), individual components of caseins have received different identifying names (ALLERGEN 2017). Caseins are encoded by different genes located in the same chromosome, being subdivided in distinct families. The most important are: α_{S1} - (Bos d 9), α_{S2} - (Bos d 10), β - (Bos d 11), and κ - (Bos d 12) caseins, representing 40%, 12.5%, 35%, and 12.5% of the casein fraction in milk, respectively (Wal 2002; Demeulemester and others 2006). They belong to a large family of secretory calcium-binding phosphoproteins (Smyth and others 2004), having a loose tertiary, highly hydrated structure with a phosphate group that binds strongly to polyvalent cations such as calcium, causing charge neutralization and precipitation of α_{S1} -, α_{S2} -, and β -caseins at >6 mM of Ca^{2+} and 30 °C. However, in the case of κ -casein, as it contains a small concentration of phosphate group, calcium binds weakly and is not precipitated by them (Fox 2001). In milk, where calcium is present at high concentrations, this fact leads to the formation of quaternary structures, named

Table 1—Identification of cow's milk allergens according to their biochemical classification, biological function, and respective accession numbers.

Fraction	Protein superfamily	Biochemical classification	Allergen	MW (kDa)	Biological function	Nucleotide (NCBI)	Protein (NCBI)	Protein (UniProt)
Caseins	Caseins	α_{S1} -Casein	Bos d 8	20 to 30	Caseins (individual components Bos d 9 to Bos d 12)	—	—	—
		α_{S2} -Casein	Bos d 9	23.6 (199 aa)	Calcium-binding protein. Major allergen.	NM_181029	NP_851372	P02662
		β -Casein	Bos d 10	25.2 (207 aa)	Calcium-binding protein. Major allergen.	NM_174528	NP_776953	P02663
		κ -Casein	Bos d 11	24 (209 aa)	Calcium-binding protein. Major allergen.	XM_005902037	XP_005902099	P02666
			Bos d 12	19 (169 aa)	Stabilization and coagulation of milk. Major allergen.	NM_174294	NP_776719	P02668
Whey proteins	Lysozyme Lipocalin Serum albumin Immunoglobulin Transferrin	α -Lactalbumin	Bos d 4	14.2 (123 aa)	Participates in synthesis of lactose, metal, lipid, and calcium binding. Major allergen.	M18780	AAA30615	P00711
		β -Lactoglobulin	Bos d 5	18.3 (162 aa)	Lipid binding, antioxidant activity. Major allergen.	X14712	CAA32835	P02754
		Bovine serum albumin	Bos d 6	66.3 (582 aa)	Transport, metabolism, and distribution of ligands. Defense. Major allergen.	M73993	AAA51411	P02769
		Immunoglobulins	Bos d 7	160	Defense. Minor allergen.	—	—	—
		Lactoferrin	—	80 (703 aa)	Iron-binding protein; defense. Minor allergen.	—	—	—

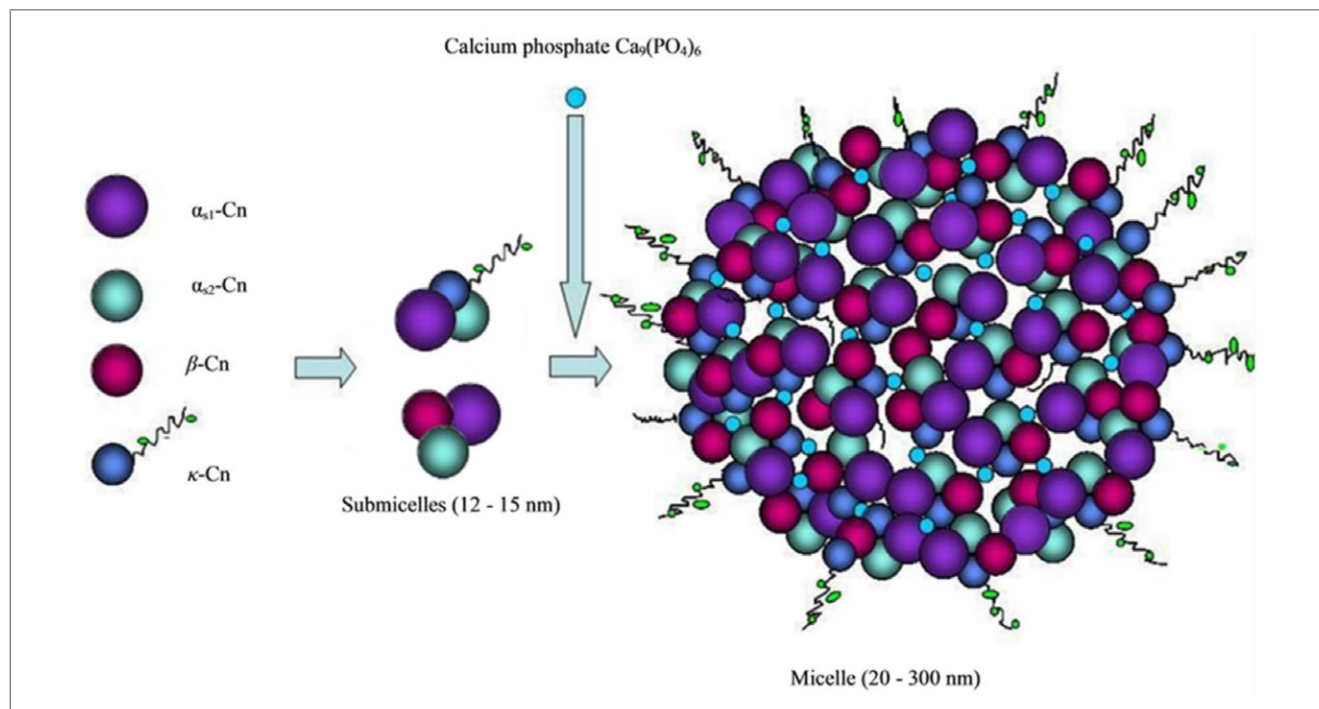


Figure 1—Structure of casein submicelles and casein micelles composed of submicelles held together by calcium phosphate. Retrieved from Rebouillat and Ortega-Requena (2015).

casein micelles, in suspension in the aqueous phase of lactosera whey. These structures are characterized by a central hydrophobic core (calcium-sensitive α_{s1} -, α_{s2} -, and β -caseins) and a peripheral hydrophilic layer (κ -casein; Figure 1) (Fox 2001; Wal 2001). The biological functions of casein micelles include the transportation and secretion of calcium and phosphate, and the digestion and absorption of nutrients by their retention in the stomach (Holt and others 2013). Caseins are considered poorly immunogenic, since their structures are noncompact and flexible. They are extensively degraded by proteolytic enzymes during digestion, susceptible to all proteinases, and exopeptidases, but not significantly affected by denaturing agents, such as urea or heat treatment. Without a tertiary structure, caseins have high surface hydrophobicity. Due to the different nature of the residues (hydrophobic, polar, and charged), they do not present a uniform distribution throughout the molecular structure, being organized in hydrophobic or hydrophilic patches. For that reason, caseins are strongly amphipathic structures, making them highly surface-active and insoluble in water (Fox 2001; Wal 2001; Hochwallner and others 2014; Goff 2016). All the caseins present genetic polymorphisms that lead to several protein variants and contribute to their high heterogeneity. These variants are characterized by point substitution of amino acids (aa), by deletions of peptide fragments of varying size or by posttranslational modifications, such as glycosylation, phosphorylation, or partial hydrolysis, which may affect their allergenic potential (Fox 2001; Wal 2001).

Bos d 9 (α_{s1} -Casein). α_{s1} -Casein is a single-chain phosphoprotein of 199 aa, with a molecular size of 23.6 kDa and characterized by a high content of proline residues. It has around 70% unordered structure, with a small fraction of secondary structure, such as α -helix and β -sheets, and a reduction in tertiary structure due to the lack of disulfide bonds. It has 2 hydrophobic regions, containing all 17 proline residues, separated by a polar region containing phosphate groups. α_{s1} -Casein possesses 7 genetic variants, charac-

terizing different cattle breeds (Wal 1998; Chatchatee and others 2001a; Goff 2016).

According to Natale and others (2004), approximately 50% of serum samples from patients with cow milk allergy react with α_{s1} -casein. However, the prevalence of sensitization to each casein fraction is not consensual, since IgE-binding capacity can be easily reduced even by a single aa substitution or increased by the unmask of hidden highly reactive epitopes (Bernard and others 1998). The identification of IgE recognition sites (IgE-binding epitopes) in the antigen is an important way to the development of new diagnostic strategies (Matsuo and others 2015). Thus, several studies have been made to identify the IgE-binding regions of α_{s1} -casein in humans. The lack of a clear tertiary structure on caseins suggests the presence of preferentially linear epitopes. Nakajima-Adachi and others (1998) identified a single immunodominant IgE-binding region at the C-terminal (residues 181 to 199), while Spuerger and others (1996) identified 3 immunodominant IgE-binding epitopes located in hydrophobic regions, where they are not accessible to antibodies, unless the casein is denatured or degraded during digestion. Chatchatee and others (2001a) identified 6 major IgE-binding regions, and they suggested that there is a difference in epitope recognition between patients with persistent and transient cow milk allergy. The region located at residues 28 to 50 recognized by Cerecedo and others (2008) was also identified by Spuerger and others (1996), Chatchatee and others (2001a), and Elsayed and others (2004). Cong and others (2012) identified 4 different regions with the recognition of the critical residue for IgE-binding.

Bos d 10 (α_{s2} -casein). α_{s2} -Casein is composed of 207 aa, with 1 disulfide bond per molecule and a molecular mass of 25.2 kDa. It presents 4 genetic variants (A, B, C, and D) with different amounts of phosphoryl groups (10 to 13), one of them (variant A) containing 11 residues of phosphoserine with a rather unstable structure to pH changes (Micinski and others 2013). The variant D has

different structural characteristics, corresponding to the deletion of a cluster with 3 phosphoserine residues, which might affect the allergenic potential of this protein (Bernard and others 2000a; Wal 2001; Busse and others 2002).

According to Natale and others (2004), the prevalence of sensitization to α_{S2} -casein in patients with cow milk allergy is 90%. Six minor and 4 major (detected in 77% of patients) sequential IgE-binding epitopes were recognized in α_{S2} -casein by Busse and others (2002), while Cerecedo and others (2008) identified only 7 regions, one of them in common with the previous study.

Bos d 11 (β -casein). β -Casein is composed of 209 aa and 5 phosphate groups, with a molecular mass of 24 kDa. It possesses 12 main genetic variants with different levels of phosphorylation. Its molecular structure is very similar to α_{S1} -casein with a globular hydrophobic domain at the C-terminal, a highly solvated and charged domain at the N-terminal, an even distribution of proline content, and without disulfide bonds. The acidic peptide sequence containing a cluster of phosphoserine residues is homologous among caseins, namely in β -casein (^{13} VESLSSEE ^{21}) and α_{S1} -casein (^{62} AESISSEE ^{70}). In α_{S2} -casein variant A, the homolog cluster of phosphoserine residues is partially repeated twice at residue positions 7 to 12 (VSSSEE) and 55 to 60 (GSSSEE). Additionally, β -casein possesses a reduced secondary structure and no rigid tertiary interactions, as in α_{S1} -casein, suggesting that their important allergenic epitopes are linear rather than conformational.

β -Casein can be cleaved by the milk plasmin (native protease) originating another family of caseins (γ_1 -, γ_2 -, and γ_3), although they are considered to be nonallergenic (Kumosinski and others 1991; Wal 1998; Chatchatee and others 2001b; Micinski and others 2013; Goff 2016). Regarding the identification of IgE-binding epitopes on β -casein, Otani and others (1987) concluded that there are at least 6 antigenic sites on the molecule and that the epitopes are sequential (Wal 1998). Chatchatee and others (2001b) have also identified 6 major and 3 minor IgE-binding epitopes, but the data about this protein are still scarce.

Bos d 12 (κ -casein). κ -Casein has a molecular mass of 19 kDa, and a primary sequence of 169 aa, with a N-terminal strongly hydrophobic and a C-terminal highly hydrophilic. This protein promotes the steric and electrostatic repulsion between micelles, thus preventing aggregation. It is also the only glycosylated casein, containing galactose, galactosamine, and sialic acid. This protein occurs as tri- or tetrasaccharides attached to threonine residues in the C-terminal region, a fact that increases its hydrophilicity. Depending on the degree of glycosylation, multiple isoforms of κ -casein can coexist in milk. There are 11 variants of κ -casein due to the differences in the number of the attached oligosaccharides (Fox 2001; Farrell and others 2004). Since it is very resistant to calcium precipitation, it contributes to the stabilization of other caseins. However, this ability is eliminated by rennet cleavage at the Phe105-Met106, leaving a hydrophobic portion, para- κ -casein, and a hydrophilic one, called κ -casein glycomacropeptide or caseinomacropeptide (CMP). The CMP has 64 aa and it is responsible for the reduction of gastric acid and serum gastrin secretion, increasing the efficiency of digestion. It also exhibits anticoagulant properties, prevents platelet agglomeration, and serotonin secretion (Micinski and others 2013; Goff 2016). The number of O-glycosylation sites in CMP can vary from 0 to 7, so both nonglycosylated and glycosylated isoforms exist in digested milk. According to Boutrou and others (2008), the glycosylated forms of CMP are less digested than the nonglycosylated ones, suggesting

that the former might be responsible for the potential immunoreactivity of CMP.

Eight major IgE-binding epitopes were detected in κ -casein by Chatchatee and others (2001b): 3 of them being recognized by 93% of patients' serum samples with cow milk allergy (^{9} IRCEKDERFFSDKIAKYI ^{26} , ^{21} KIAKYIPIQYLLSRYP SYGLNYY ^{44} , and ^{47} KPVALINNQLPYPYAKPAAVR ^{68}), and 6 epitopes by the majority of older patients. Cerecedo and others (2008) identified 2 regions (^{16} RFFSDKIAKYIPIQYVLSRY ^{35} and ^{34} RYPYGLNYYQQKPVALINN ^{53}) as dominant epitopes. Thus, the region between residues at positions 9 to 68 (at the N-terminal) may play an important role in the allergenicity of this protein. Han and others (2008) reported a total of 13 aa (at positions 17, 18, 29, 32, 35, 58, 61, 72, 97, 105, 118, 146, and 160) as critical residues for IgE-binding to linear epitopes of κ -casein. The substitution of the native residues by others resulted in overall loss/decrease of IgE-binding by pooled sera and each individual patient's serum for each epitope (Han and others 2008).

Whey proteins

The whey proteins represent 20% of cow milk protein. The main allergenic components are the globular proteins Bos d 5 (β -LG) and Bos d 4 (ALA), representing 50% and 25% of the whey protein fraction, respectively, followed by minor constituents, such as Bos d 6 (BSA), Bos d 7 (Ig), and LF (Monaci and others 2006). Contrarily to the caseins, the whey proteins possess high levels of secondary, tertiary, and, in the case of β -LG, quaternary structures. They are not phosphorylated and contain intramolecular disulfide bonds, which stabilize their structure (Fox 2001). The 3-dimensional (3D) structure seems to play an important role in maintaining the conformational epitopes, therefore contributing to the allergenic potential of the protein (Monaci and others 2006).

Bos d 4 (ALA). Bos d 4 is a monomeric globular calcium-binding metalloprotein that belongs to the family of glycosyl hydrolase (lysozyme c superfamily) and it has been identified as a major allergen in cow milk. Its primary structure is built of 123 aa, with a molecular mass of 142 kDa, and reported as having 3 genetic variants. This protein is a regulatory component of the enzymatic system of β -galactosyl transferase, responsible for the synthesis of lactose by the formation of the lactose synthetase complex. It is also known to interact with lipid membranes (stearic and palmitic acids), and it binds metals such as cobalt, magnesium, and zinc. It possesses 4 disulfide bridges and a high-affinity binding site for calcium, which stabilizes its secondary structure. Furthermore, ALA shows high thermal stability and refolding capacity. It has a highly ordered secondary structure and a compact, spherical tertiary structure; it has an "elbow-Ca $^{2+}$ -binding loop" with 2 structural domains: a large α -helical domain at the N-terminal and a short β -sheet domain at the C-terminal, flanking the calcium-binding loop (Demeulemester and others 2006; Micinski and others 2013; Hochwallner and others 2014; Goff 2016). Depending on the study population, the prevalence of ALA-specific IgE in milk allergic patients ranges from 27.6% to 62.8% (Matsuo and others 2015). The peptide ^{5} KCEVFRELKDLKGY ^{18} , which corresponds to a homologous sequence in β -LG (Bos d 5) at positions 124 to 135, is considered the major antigenic site, with a large capacity for binding with specific IgE from human sera (Adams and others 1991). Maynard and others (1997) showed IgE-binding to native ALA and to large peptides, suggesting the importance of conformational epitopes in the development of milk allergy. However, they also showed that protein denaturation might expose some linear epitopes. Hochwallner and others (2010) identified 6

IgE-reactive peptides, 3 located at the surface of the protein and one of them corresponding to the major antigenic site mentioned above. Jarvinen and others (2001) identified the same region and 3 additional conformational IgE epitopes. Hopp and Woods (1982) recognized another highly antigenic region accounting for 20% to 25% of whole ALA antigenicity at the peptide that includes the loop ⁶⁰WCKNDQDPHSSNICNISCDKF⁸⁰. More recently, Li and others (2016) identified 6 linear IgE-binding epitopes, corresponding the ones of the previous studies of Maynard and others (1997) and Jarvinen and others (2001).

Bos d 5 (β -LG). Bos d 5 is a retinol-binding protein that belongs to the lipocalin superfamily, and it is classified as a major allergen in milk. It binds a wide variety of molecules like cholesterol, vitamin D2, saturated and unsaturated fatty acids, Cu²⁺ and Fe²⁺ ions, hydrophobic ligands such as retinol, and it possesses antioxidant activity. It is a 36-kDa dimer with 2 main isoforms that differ by only 2 point mutations on residues 64 and 118. Each subunit consists of 162 aa that possesses 1 free cysteine and 2 disulfide bonds responsible for the dimerization of the molecule. This protein has a well-characterized tertiary structure common in the lipocalin superfamily, with a globular shape built up by an 8-stranded antiparallel β -barrel with a 3-turn α -helix on the outer surface and a ninth β -strand flanking the 1st strand. This structure is responsible for its main physicochemical properties and for its sulfhydryl-disulfide interactions with κ -casein during heat treatments above 75 °C. β -LG has a relative resistance to acid hydrolysis, as well as to protease activities. These features enable preserving some structural integrity after digestion, allowing its absorption through the intestinal mucosa and further presentation to the immunocompetent cells, having high allergenic potential (Wal 1998; Fox 2001; Jarvinen and others 2001; Wal 2001; Demeulemester and others 2006; Micinski and others 2013; Hochwallner and others 2014).

Food allergies associated with this protein are estimated in 80% of the population (Micinski and others 2013). For this reason, several studies focusing on the identification of IgE-binding regions have been performed. Many authors described the peptide ¹²¹CLVRTPEVDDEAL¹³⁴, located at the protein surface, as a major allergenic site (Adams and others 1991; Williams and others 1998). Jarvinen and others (2001) identified 7 IgE-binding epitopes, but only 3 were considered immunodominant, one of them corresponding to the same region (residues 121 to 134). Moreover, they stated that the presence of IgE to multiple linear allergenic epitopes might be an indicator of persistent allergy. This fact was also evidenced by Picariello and others (2010, 2013), since they suggested the same region (residues 125 to 135) with the most important allergenic potential among the β -LG determinants due to its high resistance to proteolysis. Cerecedo and others (2008) identified 3 epitopes recognized by more than 75% of sera from allergic patients with the region ⁵⁸LQKWENDECAQKKIIAEKTK⁷⁷, being significantly associated with milk protein-reactive patients. Cong and others (2012) determined 4 IgE-binding epitopes, namely ¹⁷LIVTQTMKGLDIQKV³¹, ⁷²ILLQKWENGECQKK⁸⁶, ⁹²TKIPAVFKIDALNEN¹⁰⁶, and ¹⁵²FDKALKALPMHIRLS¹⁶⁶, and 2 IgG-binding epitopes (²²TMKGLDIQKVAGTWY³⁶ and ¹²⁷AEPEQSLACQCLVRT¹⁴¹). Accordingly, the authors identified different critical residues for IgE- and IgG-binding, located at epitopes ¹⁷LIVTQTMKGLDIQKV³¹ (Thr20, Met23, and Asp27) and ²²TMKGLDIQKVAGTWY³⁶ (Leu26 and Val31), respectively. Ball and others (1994), Heinzmann and others (1999), and Selo and others (1999) also identified some major and minor linear epitopes, some of them not exposed at the surface

of the molecule and, therefore, well protected against enzymatic attacks, which suggests their relative minor importance in terms of immunoreactivity.

The recognition of specific proteins/peptides that behave as potent allergens can be considered a step forward in component-resolved diagnosis as new and highly efficient diagnostic tools (microarrays). These microarrays can determine different epitope-binding patterns, thus allowing differentiating the clinical phenotypes of milk allergy. Ultimately, Bos d 5 is considered a major allergen with multiple IgE-binding linear epitopes, highlighting the importance of its specific peptides as molecular markers for the diagnosis of persistent milk allergy (Ahrens and others 2012; Jarvinen and Sicherer 2012; Hochwallner and others 2014).

Bos d 6 (BSA). Bos d 6, although present in milk at low quantities, reacted with IgE from the sera of 50% of milk allergic patients, which rendered its classification as a major allergen. It has 582 aa and a molecular weight of 66.3 kDa, with a stable tertiary structure. Its main biological role is related to the transport, metabolism, distribution of several substances (fatty acids, ions, hormones, drugs), and protection from free radicals. It contributes to regulate the colloidal osmotic pressure of blood and it imparts free radical protection. This protein is organized in 3 homologous domains and consists of 9 loops connected by 17 disulfide bonds, many of which are protected in the core of the protein, therefore not easily accessible. The disulfide bonds play an important role in maintaining the native antigenic determinants of this molecule, mainly because of the great stability of its tertiary structure, even under denaturing conditions (Farrell and others 2004; Restani and others 2004; Hochwallner and others 2014). It has been shown that the fragment, comprising residues at positions 524 to 598, is an epitopic area for human species, from which the region of ⁵²⁴AFDEKLFTFHADICTLPDT⁵⁴² corresponds to the most critical sequence (Beretta and others 2001). Tanabe and others (2002) also identified a few epitopes from BSA to be involved in beef allergy. However, the epitopes reported by various studies were not always the same (Atassi and others 1976; Peters and others 1977; Beretta and others 2001).

Bos d 7 (Ig). Bos d 7 accounts for about 3% of total milk protein and 6% of whey proteins. It possesses a conformational structure very similar to those of human origin, occurring as polymers or protomers of a basic "Y shaped" unit composed of 4 polypeptide chains linked by inter- and intramolecular disulfide bonds. The monomers are composed of heavy (H) and light (L) chains, each of them with variable (V-) and constant (C-) domains. The V-domains of H- and L-chains converge to form the antigen-binding site, while the C-domains characterize the isotype of the Ig in cow milk: IgG, IgA, or IgM. The potential allergenicity of bovine Ig is still under study and their IgE-binding epitopes have not yet been identified. However, IgG was proposed as milk allergen due to the observation that IgE from milk allergic patients specifically binds to bovine IgG (Lefranc-Millot and others 1996; Farrell and others 2004; Natale and others 2004; D'Urbano and others 2010). Approximately 10% of patients with cow milk allergy are IgE-positive to cow IgG; therefore, this protein is considered as a minor allergen in milk (Matsuo and others 2015).

Lactoferrin. LF is an iron-binding glycoprotein that belongs to the transferrin family and it is found at levels <1% in the milk of most species. It consists of a single polypeptide chain folded into 2 globular lobes, each of them having high-affinity iron-binding sites, connected by a 3-turn helix. The molecular weight of this protein varies, depending on the extent of its glycosylation. Besides its function as a scavenger of free radicals and as an antioxidant, its

main role is to defend the organism against infections and inflammations owing to its ability to sequester iron from the environment and, thereby, removing this essential nutrient for bacterial growth (Ward and Conneely 2004). In addition, it is involved in detoxification processes and it has an antineoplastic effect by inhibiting the attachment of tumor growth factors (Micinski and others 2013). Some studies declare that some milk-allergic individuals possess LF-specific IgE, although the relevance of the allergenicity of this protein is still under discussion because these patients also present IgE against one of the major milk allergens. Until today, no data about the identification of IgE-binding epitopes have been reported (Adel-Patient and others 2005; D'Urbano and others 2010).

Cross-Reactivity of Milk Allergens

Although the milk proteins officially recognized as food allergens are of bovine origin, there are many other dairy animals whose milk is used for human consumption and, therefore, liable to initiate an allergic reaction in susceptible individuals by the ingestion of homologous proteins. Milk and milk proteins from buffalo, sheep, goat, pig, camel, mare, donkey, reindeer, and yak can be used to produce dairy products or be added to cow milk. Therefore, homologous milk proteins of different species can lead to cross-reactivity phenomena in sensitized/allergic individuals (Restani and others 2009).

Different studies reveal that the vast majority of patients with cow milk allergy have high cross-reactivity to milk from sheep, buffalo, and goat, which might be explained by their great similarity in protein composition, although presenting a different distribution. Contrarily, very few cow milk allergic individuals present cross-reactivity to donkey, mare, and camel milk, whose structures are more similar to human milk. Mare milk presents a reduction in the casein fraction, while camel milk shows a high proportion of β -casein and the lack of β -LG, as in human milk (Jarvinen and Chatchatee 2009; Restani and others 2009; Hinz and others 2012). Restani and others (1999) tested sera from cow milk allergic patients with milk proteins from mammalian species. Accordingly, the authors showed a strong IgE-reactivity of sera with the majority of milk proteins from sheep, goat, and buffalo, while no IgE-binding was observed when testing camel milk, which might be explained by the phylogenetic differences between cow and camel. Using animal monoclonal antibodies specific for cow milk proteins, Restani and others (2002) confirmed the previous results, but also observed weak immunoreactivity with mare and donkey milk. These results were also obtained by Katz and others (2008) when performing SPTs in patients with a clinical history consistent with IgE-mediated cow milk protein allergy. The authors added deer as a cross-reactive species and pig as a less reactive one, suggesting the existence of a "kosher epitope" responsible for this common allergenicity (Katz and others 2008). Suutari and others (2006) demonstrated that β -LG of reindeer milk has weak cross-reactivity with bovine β -LG, in spite of being a ruminant species, probably due to the lack of homolog bovine epitopes in the protein or a weak bond with those that are recognized.

Since goat milk contains a lower quantity of α -caseins, it has been suggested as a substitute of cow milk for allergic patients, though the results about its cross-reactivity are still controversial. Some reports suggest that cow milk allergic children can tolerate goat and sheep milk because of the weak IgE-binding to caseins (Restani and others 2009). However, the majority of studies demonstrate high cross-reactivity between cow and goat proteins (Bellioni-Businco and others 1999; Besler and others 2002; Pina

and others 2003). On the other hand, there are individuals, normally older children that present relevant, or even severe, allergic reactions to goat and sheep milk, without clinical manifestation toward cow milk. In those cases, the IgE binds the caseins (α_{S1} -, α_{S2} -, and β -caseins) with high specificity and efficiency, but not to the whey proteins, despite their pronounced sequence homology (Ah-Leung and others 2006). Several studies report the fact that patients with cow milk allergy have cross-reactivity with sheep and goat milk, but not the reverse (Wüthrich and Johansson 1995; Calvani and others 1998; Umpierrez and others 1999; Muñoz-Martín and others 2004; Viñas and others 2014). Nonetheless, the reason for this phenomenon is still unclear.

Donkey and mare milk has been revealed to be less allergenic, with a very weak IgE cross-reactivity. Some authors suggest the utilization of donkey milk in children with severe cow milk allergy, confirming that 80% of children tolerated donkey milk better than goat milk, and that it is more effective in ameliorating atopic dermatitis (Businco and others 2000; Alessandri and Mari 2007; Monti and others 2007; Vita and others 2007).

The casein proteins are present in milk of different ruminant species with high sequence homologies, varying from 80% to more than 90%, sharing the same structural, functional, and biologic properties. For example, α_{S1} -, α_{S2} -, and β -caseins from cow, goat, and sheep share 87% to 98% of sequence identity, with an IgE-sensitization to sheep and goat casein ranging from 93% to 98% in children with cow milk allergy. Moreover, it was demonstrated that human and bovine β -caseins also share approximately 50% of sequence homology. These regions correspond to clusters of phosphorylated seryl residues conserved in bovine caseins, as well as in the caseins of other species, probably playing an important role in cross-reactivity among the milk of different species (Spuergerin and others 1997; Restani and others 1999; Bernard and others 2000b). Bernard and others (1998) demonstrated that 99% of the patients' sera ($n = 58$) reacted to more than 1 casein, and 88% presented IgE against each of the 4 bovine caseins. This finding suggests the presence of common or closely related IgE epitopes, possibly associated with phosphorylation sites, described as being immunoreactive and resistant to digestive degradation, or with the polysensitization to different casein components after disruption of the casein micelles during the digestive process (Bernard and others 1998; Wal 2004). In another study, Bernard and others (2000b) showed cross-reactivity between human and bovine β -caseins, though with a lower affinity of IgE to human β -caseins. The similarity of human and bovine caseins was also demonstrated in the study of Han and others (2008), which shows 2 potential cross-reactive sites of IgE-binding epitopes between human and bovine κ -casein. Additionally, bovine and human ALA sequences share 74% of sequence homology (Wal 2001). The distribution of proteins in human milk is rather different from that of bovine milk, but more similar to donkey and mare milk because they have a minor content in caseins. Besides, β -LG is absent from human milk, in opposition to other mammalian milks. Still, sequence homology between human and bovine milk is rather high, leading to cross-allergic reactions in some patients (Tsabouri and others 2014).

Until now, camel milk seems to be the most appropriate substitute for cow milk, mainly because of the high proportion of β -casein, low proportion of α -casein, deficiency in ALA, and similarity of the Ig (Kumar and others 2016). Camel milk shows the lowest level of similarity (about 60%) with cow milk proteins (Jarvinen and Chatchatee 2009; Restani and others 2009; Tsabouri and others 2014). Many efforts have been made to

study the reliability of using camel milk in allergic patients with interesting and promising results (Shabo and others 2005; Ehlayel and others 2011; Boughellout and others 2016).

Cross-reactivity in sensitized patients can occur, not only between milk proteins of different species, but also with proteins present in other tissues, as in meat or epithelia of different mammals. BSA is very similar to human serum albumin and owing to its widespread availability, it has numerous applications in medical formulations, namely as a component of several vaccines. Most of the individuals with persistent milk allergy are known to be reactive to serum albumins of different mammalian meats, which increases their risk of developing clinical symptoms, such as rhinoconjunctivitis or asthma, due to animal epithelia (Chruszcz and others 2013). Therefore, BSA is a common example of cross-reactivity phenomenon and it is involved in the cosensitization to milk and beef with a prevalence of 13% to 20% among cow milk allergic patients (Martelli and others 2002). Vicente-Serrano and others (2007) showed that the sera from patients allergic to cow milk with IgE-binding to BSA also recognized the native serum albumin in different meats (beef, lamb, deer, and pork) and epithelia (dog, cat, and cow). However, none of them reacted with heated meats, suggesting the implication of heat denaturation in the reduction of serum albumin allergenicity. The authors also stated that albumins are involved as a panallergen (allergens responsible for wide IgE cross-reactivity between related and unrelated allergenic sources) in mammals.

Soy formulas are common substitutes to cow milk allergic individuals. Nonetheless, it has been demonstrated that some patients are intolerant to such products, suggesting the cross-reactivity between soy and cow milk proteins. It was reported that the α -subunit of beta-conglycinin (Gly m 5.0101) from the vicilin-like protein family, the G4 subunit of glycinin (Gly m 6.0401) from legumin-like proteins, and, more recently, the cysteine protease P34 (Gly m Bd30K) and the globulin P28 (Gly m Bd28K) are involved in cross-reactivity phenomena with bovine caseins (Rozenfeld and others 2002; Katz and others 2008; Smaldini and others 2012; Curciarello and others 2014; Candreva and others 2015; Candreva and others 2016).

Due to the great allergenicity of milk proteins from other species, databases such as ALLERGOME include some of these proteins in their allergen list (nonofficial). Table 2 summarizes the allergens involved in cross-reactivity to milk proteins (official and nonofficial), with the respective names from the databases ALLERGOME and WHO/IUIS.

Effect of Processing, Food Matrix, and Digestibility on Milk Allergenicity

A wide range of food products can be manufactured from milk as raw material. On a global scale, 36% of cow milk is used for cheese production, 30% for butter products, 13% for the fabrication of cream products, 11% is consumed as drinking milk, and 3% is used for powders products. Condensed, evaporated, and fermented milks are also consumed, but at smaller amounts; casein and whey proteins are used as ingredients in several products, including cheeses, bakery products, and glues. Milk from other species, such as sheep and goat, is also used predominantly for the manufacture of fermented milks and cheeses (Fox 2001; Goff 2016; Eurostat 2017; FAOSTAT 2017). Thus, milk and milk proteins can be present in several food matrices, being submitted to different types of processing, until they become available to consumers as final products. Food processes applied to

Table 2–Milk proteins of other species associated with cross-reactivity phenomena and respective allergen names.

Allergens	Buffalo (<i>Bubalus bubalis</i>)	Goat (<i>Capra aegagrus hircus</i>)	Sheep (<i>Ovis aries</i>)	Reindeer (<i>Rangifer tarandus</i>)	Mare (<i>Equus caballus</i>)	Donkey (<i>Equus asinus</i>)	Mule (<i>Equus mulus</i>)	Camel (<i>Camelus dromedarius</i>)	Pig (<i>Sus scrofa domestica</i>)
α S1-Casein	Bub b 9	Cap h 9	Ovi a 9	Not found	Equ c 9	Not found	Not found	Cam d 9	Sus s 9
α S2-Casein	Bub b 10	Cap h 10	Ovi a 10	Not found	Equ c 10	Not found	Not found	Cam d 10	Sus s 10
β -Casein	Bub b 11	Cap h 11	Ovi a 11	Not found	Equ c 11	Not found	Not found	Cam d 11	Sus s 11
κ -Casein	Bub b 12	Cap h 12	Ovi a 12	Not found	Equ c 12	Not found	Not found	Cam d 12	Sus s 12
α -Lactalbumin	Bub a 4	Cap h 4	Ovi a 4	Not found	Equ c ALA	Not found	Not found	Cam d 4	Sus s 4
β -Lactoglobulin	Bub a 5	Cap h 5	Ovi a 5	Ran t 5	Equ c BLG	Equ as BLG	Equ mu BLG	Absent	Sus s 5
Serum albumin	Not found	Cap h 6	Ovi a 6 (also present in meat, and urine)	Not found	Equ c 3 ^a (also present in meat, and skin)	Equ as 6	Not found	Not found	Sus s 1 ^a (also present in meat, and urine)

^a Present in WHO/IUIS Official List of Allergens.

milk and milk products can include pasteurization or ultra-high temperature (UHT) treatment to eliminate pathogens from liquid milk, fermentation to produce yogurts, and evaporation and spray-drying to obtain concentrates and milk powders for infant formulas, respectively (Verhoeckx and others 2015).

Food processing can induce different modifications in the structure of proteins, including aggregation, unfolding, and glycation, and also the occurrence of Maillard reaction products. All these alterations may affect the IgE-binding capacity and, consequently, increase or reduce the allergenicity of proteins (Rahaman and others 2016). The latter is normally attributed to the destruction of conformational epitopes or to the occurrence of chemical reactions in the food matrix between proteins, fat, and sugars, limiting the availability of the protein to the immune system. On the other hand, the formation of neoepitopes and the effect of food matrix that decrease protein digestibility (and consequently the preservation of the existing epitopes) might potentially increase protein allergenicity (Nowak-Węgrzyn and Fiocchi 2009). The high content of proteins in a food matrix seems to enhance the stability against simulated gastrointestinal degradation and create a competitive environment for enzyme cleavage, thereby delaying gastrointestinal proteolysis of food allergens (Schulten and others 2011).

Many efforts have been made to study the influence of milk processing technologies on the reduction of allergenicity, to find new and effective processes to be applied to milk products and, therefore, control milk allergy. Table 3 and 4 summarize several recent studies about the modification of milk allergens and allergenicity upon conventional and novel food processing technologies.

Conventional food processing

Heat treatment. Heat treatment is an important step in the manufacturing of most dairy products with the use of techniques such as pasteurization, sterilization, and UHT processing. By nature, caseins are considered as intrinsically disordered proteins, possessing very little secondary and tertiary structures (such as the case of β - and κ -caseins), but still able to perform their function. Consequently, they are very stable to heat treatments, showing only a partial reduction or no change in their allergenicity (Bhat and others 2016). Bloom and others (2014) demonstrated the presence of caseins after 60 min at 95 °C, not affecting substantially their immunoreactivity. Although casein allergenicity can be influenced by the period, temperature, and presence of other foods (for example, wheat) during the heat process, all serum sample taken from milk-allergic subjects remained IgE-reactive to caseins, even after extensive thermal treatment. Similarly, Morisawa and others (2009) showed that α -caseins submitted to thermal treatment did not affect the amount of histamine released from basophils, but a combination of heat treatment with enzymatic digestion led to a decrease of histamine release, confirming the relation of α -casein specific-IgE with linear epitopes.

In opposition, whey proteins are thermolabile, with changes on their allergenicity (Verhoeckx and others 2015). β -LG shows an increased antigenicity and allergenicity, when subjected to temperatures ranging from 50 to 90 °C due to the exposure of hidden allergenic epitopes after an unfolding of the native structure of the protein. Above 90 °C, the allergenicity of β -LG seemed to decrease because of sulphhydryl–disulfide exchange, which enhances conformational changes with the subsequent destruction or mask of conformational epitopes on the surface of the molecule (Bu and others 2009b). In addition to disulfide-mediated aggregation at those temperatures (90 to 120 °C), Maillard reactions might

cause the loss of linear epitopes that lead to a general reduction in the allergenicity of β -LG (Kleber and Hinrichs 2007; Bu and others 2009b; Bloom and others 2014; Xu and others 2016). Figure 2 presents a schematic representation of the effect of heat treatment at different temperatures in the β -LG structure. The combination of heat treatment with pepsin digestion also showed a reduction in the allergenicity of β -LG (Sletten and others 2008; Morisawa and others 2009). ALA is more heat-stable than β -LG, but at high temperatures it presents a greater decrease in antigenicity because its conformational epitopes are thought to be more IgE-reactive, while the most relevant epitopes in β -LG are linear (Jarvinen and others 2001; Bu and others 2009b). In addition, heating of β -LG or ALA results in the formation of intermolecular disulfide bonds and subsequent binding to other food proteins. Therefore, these mechanisms of aggregation make IgE-epitopes less accessible and, consequently, less allergenic (Nowak-Węgrzyn and Fiocchi 2009; Bloom and others 2014). This matrix effect has led some authors to suggest a diet with baked milk, since in their study about 70% of tested children were able to ingest a muffin containing baked milk without any immediate clinical symptoms (Nowak-Węgrzyn and others 2008). After sequential food challenges with baked cheese and unheated milk in a test population of children that previously tolerated extensively heated (baked) milk products, Kim and others (2011) revealed that 28% and 60% of them were able to tolerate baked milk/baked cheese and unheated milk, respectively. Sopo and others (2016) evaluated the effect of wheat matrix on baked milk tolerance in children with IgE-mediated cow milk allergy. They demonstrated that 81% of children tolerated baked cow milk in a wheat matrix (ciambellone), 56% tolerated liquid baked cow milk, 78% Parmigiano Reggiano (a typical Italian cheese), and 82% partially hydrolyzed formula, revealing that matrix effect was relevant only in half of the cases. The tolerance to Parmigiano Reggiano was also studied by Alessandri and others (2012) in patients with suspected cow milk allergy, reporting that 56% of children tolerated the Italian cheese after 36 mo of its maturation. These data were correlated with the extent of cheese maturation, in which the milk proteins, especially caseins, are gradually and constantly broken by the proteolytic enzymes of lactic acid bacteria and milk rennet, resulting in a decrease of allergenicity during gut digestion.

Techniques such as sterilization cause the denaturation of 75% of whey proteins and promote Maillard reactions, which occur between free aa and aldehyde/ketone groups of sugars present in milk or in other food matrices, and are known to change conformational structures and to affect protein allergenicity (Thomas and others 2007; Verhoeckx and others 2015). The effect of conjugating allergenic proteins with reducing sugars through Maillard reactions has been widely studied as a possible solution to reduce milk allergenicity. Even in proteins highly resistant to proteolysis, it has been reported that there is an increase in *in vitro* digestibility and a reduction in their immunoreactivity (Kobayashi and others 2001; Corzo-Martínez and others 2010; Wu and others 2013). Glucose (Bu and others 2009a, 2010a), chitosan (Aoki and others 2006), nystose, fructofuranosyl nystose, and fructooligosaccharides (Zhong and others 2013, 2015), oligoisomaltose and maltose (Aalberse 2007; Li and others 2011, 2013), and carboxymethyl dextran (Kobayashi and others 2001) are some sugars with a reported effect on the reduction of β -LG and ALA antigenicity and allergenicity. Maillard reactions between lactose (disaccharide) and the accessible amino groups of lysine residue in whey proteins might also occur, leading to the formation of Amadori products (Liu and others 2016). As reported for other sugars, heating at 130 °C for

Table 3—Recent studies about the effect of traditional processing techniques on milk allergens.

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	References
β -LG	Heat treatment	80 to 90 °C—increased allergenicity; above 100 °C—decreased allergenicity	–	Purified protein, skim milk and sweet whey	Ehn and others (2004); Kleber and Hinrichs (2007)
Caseins, β -LG	Heat treatment hydrolysis	Caseins—decreased allergenicity after hydrolysis; β -LG—little effect on allergenicity	κ -Caseins—unaffected without heat treatment; caseins—proteolytic degradation with heat treatment; β -LG—increased digestibility by pepsin after heat denaturation, matrix fat content did not affect digestibility	Whole, semiskimmed, skimmed and UHT. Caseins—IgE epitopes more stable to digestion with UHT process; β -LG—high reactivity to IgE in both UHT and semiskimmed milks;	Sletten and others (2008)
ALA, β -LG	Heat treatment	50 to 90 °C—increased antigenicity (higher in β -LG); 90 to 120 °C—decreased antigenicity (higher in ALA)	–	Whey protein isolates	Bu and others (2009b)
α -Caseins, β -LG	Heat treatment hydrolysis	Decreased allergenicity with the combination of heat treatment and pepsin digestion	Pepsin digested all untreated and heat treated α -caseins in 30 min; pepsin digested β -LG only after heat treatment	Purified proteins	Morisawa and others (2009)
β -LG	Heat treatment	–	Native β -LG resistant to simulated gastric digestion; 90 °C—increased digestibility	Purified protein	Peram and others (2013)
Caseins, β -LG, ALA	Heat treatment	Caseins—unaffected; β -LG/ALA—decreased allergenicity at 90 to 100 °C after 20 min	–	Fresh skim milk. Caseins—unaffected by matrix; β -LG/ALA—decreased allergenicity with a wheat matrix	Bloom and others (2014)
α -Caseins, β -caseins, β -LG, ALA	Heat treatment	Caseins—unaltered; β -LG—increased allergenicity at 65 to 85 °C during 25 min; decreased allergenicity at 85 to 90 °C during 25 min; ALA—decreased allergenicity	–	Milk protein concentrates	Xu and others (2016)
β -LG	Glycation reaction, (acidic oligosaccharides, carboxymethyl dextran, chitosan)	Decreased allergenicity	–	Purified protein (genotype AA)	Kobayashi and others (2001); Hattori and others (2004); Aoki and others (2006)
β -LG	Heat treatment, glycation reaction	Decreased allergenicity	–	Purified protein (variant A)	Taheri-Kafrani and others (2009)
β -LG, ALA	Glycation reaction (glucose)	Decreased antigenicity: β -LG—51.88 °C, 75.7 h, and 2.59 WR; ALA—52.8 °C, 78 h, and 5.96 WR	–	Whey protein isolates	Bu and others (2009a, 2010a)
β -LG	Glycation (galactose, tagatose, dextran)	Glycation (gal/tag) at 40 °C, 24 h—unaffected allergenicity; glycation (gal/tag) at 50 °C, 48 h—decreased allergenicity; glycation with dextran—unaffected allergenicity	Glycation (gal/tag) at 40 °C, 24 h—unaffected digestibility; glycation (gal/tag) at 50 °C, 48 h—increased digestibility with an aggregation inhibitor (Pyridoxamine); glycation with dextran—unaffected digestibility	Mixture of variants A and B	Corzo-Martínez and others (2010)
β -LG, ALA	Glycation reaction (oligosomaltose, maltose)	Decreased antigenicity: β -LG (oligosomaltose)—68.48 °C, 29 h, and 4.7 weight ratio (WR); β -LG (maltose)—60.8 °C, 54.3 h, and 1.04 WR; ALA (maltose)—61.6 °C, 57.6 h, and 1.1 WR	β -LG/ALA—More susceptible to simulated gastric digestion after glycation with oligosomaltose; reduced antigenicity after glycation with oligosomaltose and digestion	Purified proteins	Li and others (2011, 2013)
β -LG	Glycation (fructooligosaccharides, galactooligosaccharides and isomaltoligosaccharides)	Decreased allergenicity	–	Purified protein	Wu and others (2013)

(Continued)

Table 3—Continued.

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	References
β -LG	Glycation reaction (mPEG; nystose and fructofuranosyl fructooligosaccharides)	Decreased antigenicity	–	Purified protein	Zhong and others (2013, 2015, 2016)
ALA β -LG	Fermentation (<i>L. helveticus</i> and <i>S. thermophilus</i>)	Lower antigenicity at 6 h of fermentation with combined strains and 0.5 d of cold storage	–	Skim milk	Bu and others (2010b)
β -LG	Fermentation (<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CRL 656)	Higher decrease of allergenicity induced by proteases of <i>L. delbrueckii</i> than with heat treatment	–	Purified protein and whey protein isolates. Greater hydrolysis of β -LG in WPI	Pescuma and others (2011)
α_{51} -Caseins, β -caseins	Fermentation (<i>L. helveticus</i> A75)	Decreased allergenicity induced by proteases of <i>L. helveticus</i>	–	Reconstituted skim milk	Ahmadova and others (2013)
ALA, β -LG, α -caseins, β -caseins	Fermentation (<i>L. casei</i>)	Decreased allergenicity of all proteins; cold storage after fermentation did not affect allergenicity significantly; heat treatment before fermentation: reduced allergenicity α -LA and β -LG	–	Reconstituted skim milk	Shi and others (2014)
ALA, β -LG, α -caseins, β -caseins	Fermentation (<i>L. rhamnosus</i> GG)	Decreased allergenicity of all proteins; lower antigenicity at 12 h of fermentation and 0.5 d of cold storage; heat treatment before fermentation: reduced allergenicity α -LA and β -LG	–	Reconstituted skim milk	Yao and others (2014)
ALA, β -LG, α -caseins, β -caseins, κ -caseins, BSA, LF	Fermentation (<i>L. casei</i> LcY, <i>S. thermophilus</i> MK10, <i>B. animalis</i> B130)	Decreased allergenicity: different rates for each allergen, bacteria, or stage of simulated gastric digestion	Simulated gastric digestion with fermentation: decreased allergenicity	Mare's milk	Fotschki and others (2015)
ALA, β -LG, α -caseins, β -caseins, κ -caseins, BSA, LF	Fermentation (<i>L. casei</i> LcY)	Fermentation with or without simulated digestion: decreased allergenicity (higher with digestion)	Simulated gastric digestion with and without fermentation: decreased allergenicity	Buttermilk	Wróblewska and others (2016)
Whey proteins	Enzymatic hydrolysis	Decreased allergenicity (proteins degradation, inhibition of mast cell degranulation, reduction of ear swelling, reduction of T-cell proliferation)	Hydrolysis has a time-dependent effect on allergenicity	Whey protein concentrates	Knipping and others (2012)
Whey proteins	Enzymatic hydrolysis (trypsin)	Decreased allergenicity (reduced spleen lymphocyte proliferation, low levels of specific IgE and plasma histamine, increased secretion of IFN- γ)	–	Whey protein concentrates	Duan and others (2014)
Caseins, β -LG, ALA	Enzymatic hydrolysis	Decreased allergenicity (decrease IgE recognition, basophil activation, and T cell response)	In most patients, increasing time of hydrolysis decrease allergenicity and immunogenicity	Whey protein hydrolysates	Meulenbroek and others (2014)
β -LG, ALA, α -caseins, β -caseins	Enzymatic hydrolysis (proteases of <i>E. faecalis</i>)	–	α -caseins and β -caseins: complete hydrolysis; β -LG and ALA: partial hydrolysis	Skim milk and sodium caseinate	Biscola and others (2016)
β -Caseins	<i>In vitro</i> protein digestion	Increased immunoreactivity after gastric digestion, higher with commercial porcine pepsin; decreased immunoreactivity after duodenal digestion particularly with human enzymes	Faster degradation of β -caseins with human digestive enzymes than with commercial enzymes; similar hydrolysates after gastric digestion; less numerous and shorter peptides after gastroduodenal digestion with human fluids	Purified protein	Benedé and others (2014)
Caseins, β -LG	<i>In vitro</i> protein digestion	Simulated gastric digestion (0 to 60 min): decreased allergenicity; simulated intestinal digestion (0 to 60 min): unaltered immunoreactivity	Different rates of hydrolysis in simulated digestions for each allergen, caseins, and β -LG	Pasteurized nonfat dry milk	Do and others (2016)

WR, weight ratio of sugar:protein isolates.

Table 4—Recent studies about the effect of novel food processing techniques on milk allergens.

Allergen	Treatment	Allergenicity/ antigenicity	Digestibility	Matrix	References
ALA β -LG	High pressure (HP)	HP treatment alone—unaffected allergenicity; HP in combination with pepsin and trypsin—decreased antigenicity of ALA and β -LG	β -LG—hydrolyzed by chymotrypsin and trypsin with and without HP; hydrolyzed by pepsin only with HP; ALA—hydrolyzed by pepsin and trypsin with and without HP, not hydrolyzed by chymotrypsin	Skimmed bovine milk	Peñas and others (2006)
β -LG	High pressure Heat treatment	Skim milk and sweet whey—increased antigenicity with increasing pressure and time but decreased with increasing temperature; whey protein isolate—increased antigenicity with increasing temperature and pressure	—	Skim milk and sweet whey—antigenicity more susceptible to heat treatment	Kleber and others (2007)
β -LG	High pressure	—	400 mPa, 10 min—slight increased digestibility; 600 to 800 mPa—complete proteolysis by pepsin in 1 min	Spray-dried powder (85% pure)	Zeece and others (2008)
β -LG	High pressure	>200 mPa—decreased antigenicity; proteolysis at atmospheric pressure—unaffected allergenicity; 400 mPa and proteolysis—decreased allergenicity	Atmospheric pressure—unaffected digestibility; above 400 mPa—increased digestibility by pepsin and chymotrypsin	Purified protein	Chicon and others (2009)
β -LG	High hydrostatic (HHP) pressure Proteolysis	Chymotrypsin and pepsin in combination with HHP—decreased allergenicity (absence of anaphylaxis, mast cell activation and basophils)	—	Purified protein	López-Expósito and others (2012)
β -LG	Dynamic high-pressure microfluidization (DHPM)	DHPM in combination with heat treatment—increased antigenicity; DHPM in combination with tryptic hydrolysis—decreased antigenicity	Improved digestibility by trypsin at increased pressure	Purified protein	Zhong and others (2011, 2014)
Caseins, ALA, β -LG	Controlled pressure drop (DIC)	Caseins—increased allergenicity; whey proteins—decreased allergenicity at 0.4 and 0.6 mPa	—	Whey protein isolates Skimmed milk	Boughellout and others (2015)
α -Caseins, β -LG	Irradiation (γ -radiation)	Decreased allergenicity (doses up to 10 kGy)	—	Purified proteins	Lee and others (2001)
α -Caseins, β -caseins ALA	Irradiation (γ -radiation)	Decreased allergenicity	—	Purified protein	Byun and others (2002)
	Irradiation (γ -radiation)	Decreased total ratio of proteins (10 kGy)	—	Cow's milk and Queso Blanco: similar results in both matrix	Ham and others (2009)
	Irradiation (γ -radiation)	Decreased allergenicity (low levels of ALA-specific IgE, inhibition of mast cells and basophils activity, reduced levels of plasma histamine and reduced anaphylaxis in mice)	—	Purified protein	Meng and others (2016a, 2016b)
α -Caseins	High pressure, UV-C, far-IR radiation	HPP—decreased allergenicity, high after simulated gastric and intestinal digestion; UV-C and FIR—decreased allergenicity	Increased digestibility after HPP treatment	Purified protein	Hu and others (2016)
α -Caseins β -LG, ALA	UV-C, high intensity ultrasound, NAPT	UV-C—decreased allergenicity; high-intensity ultrasound and NAPT—unaffected allergenicity	—	Purified proteins	Tammineedi and others (2013)
Whey proteins	Microwave irradiation Enzymatic treatments	Decreased allergenicity with the combination of microwave (200 W) and hydrolysis with pronase, papain and alcalase	Increased hydrolysis by pronase, papain, alcalase and chymotrypsin after microwave treatment	Whey protein concentrates	Izquierdo and others (2008)
β -LG, whey proteins	Microwave irradiation Enzymatic treatments	Decreased allergenicity with the combination of microwave (200W) and peptic hydrolysis	Increased β -LG and whey proteins digestion with microwave treatment	Whey protein isolates and purified β -LG	El Mecherfi and others (2015)
β -LG	Genetic modification (Ala86Gln)	Decreased IgE binding (mutated β -LG 9 times less recognized than native and recombinant wild type)	—	Purified protein (variant A)	Kazem-Farzandi and others (2015)
β -LG	Genetic modification (Lys69Asn)	Decreased IgE binding (mutated β -LG 9 times less recognized than wild type)	—	Purified protein (variant A)	Taheri-Kafrani and others (2015)

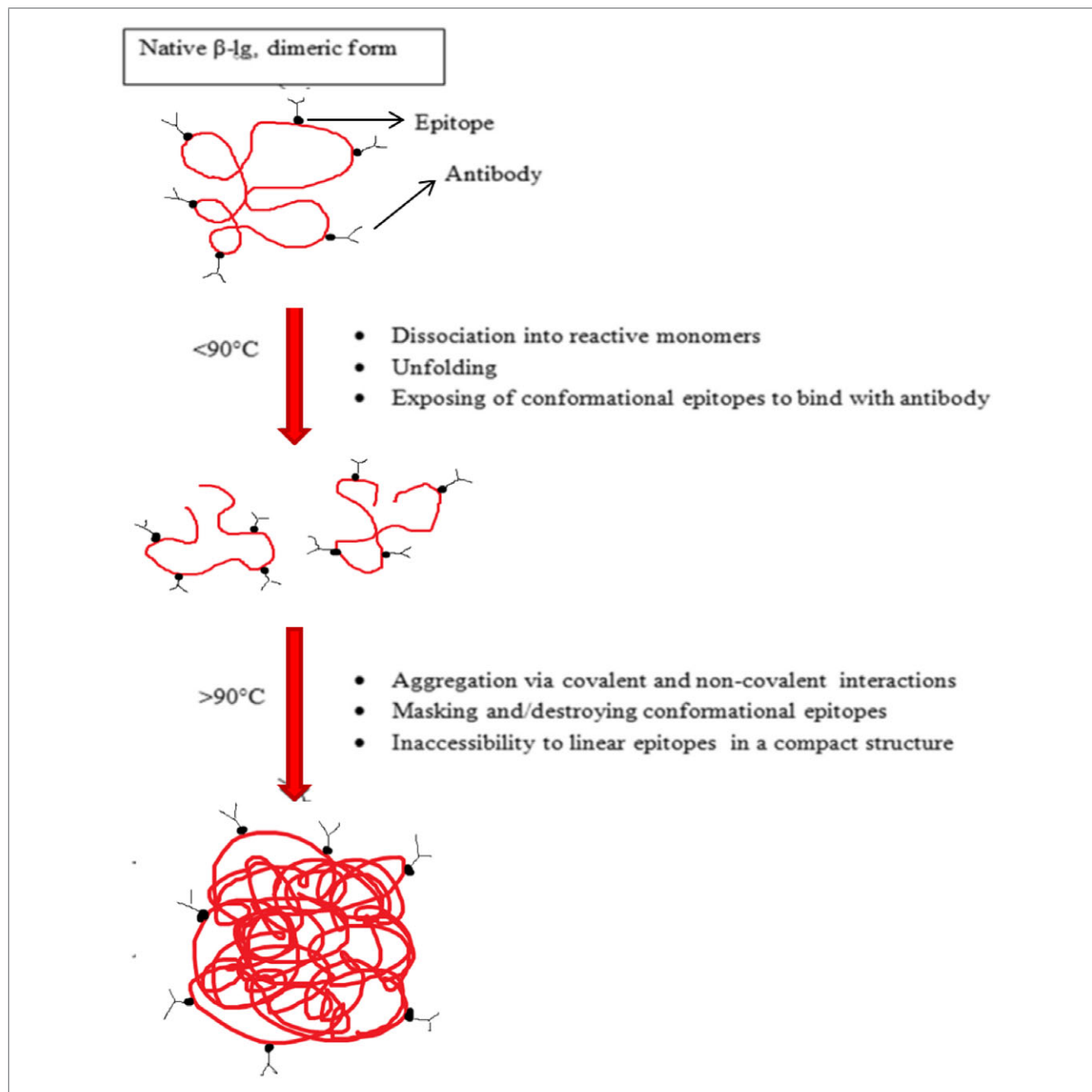


Figure 2—Schematic representation of the alterations in β -LG epitopes to bind with antibody at different level of heat treatment. Reprinted from Rahaman and others (2016) with permission from Elsevier Ltd.

20 min lead to the formation of Maillard products from lactose and whey proteins (mainly ALA and β -LG), with a decrease in the IgG-binding capacity of at least 67% compared to unheated samples. Since both ALA and β -LG present several residues of lysine located at the IgG-binding regions, the blockage of lysine residues during the Maillard product formation probably induced conformation alterations in their epitopes, thus affecting the IgG-binding capacity of whey proteins (Liu and others 2016). On the other hand, proteins that undergo lactosylation process are more resistant to proteolysis, which might contribute to the formation of immunoreactive species and, thus increase the allergenicity of whey proteins (ALA and β -LG) (Milkovska-Stamenova and Hoff-

mann 2016), although the heat treatment seems to reduce their immunoreactivity (Taheri-Kafrani and others 2009; Liu and others 2016).

Masking native/conformational epitopes is a possible explanation (Taheri-Kafrani and others 2009), but new epitopes can also emerge after conjugation with some substances due to the exposure of hydrophobic regions (Bu and others 2013). In addition, conditions such as pH, temperature, duration of exposure, weight ratio of sugar/protein, and previous digestion assays need to be well established to induce the maximum effect on antigenicity and allergenicity (Corzo-Martínez and others 2010; Li and others 2011, 2013).

Unfortunately, there are no reports about the effects of other heat treatments, such as UHT, vacuum evaporation, or spray-drying on allergenicity, despite the fact that some recent studies describe their effect on functional properties of milk proteins (Schuck and others 2013; Verhoeckx and others 2015).

Fermentation and enzymatic hydrolysis. Fermentation by lactic acid bacteria is a process commonly used to produce different types of milk products, such as yogurt and ripened-cream butters. These bacteria possess a complex proteolytic system that includes peptidases, proteinases, and transport systems, all essential for their growth in milk and dairy products. During fermentation, these enzymes hydrolyze milk proteins into peptides and aa, which greatly increase the possibility of cleaving relevant epitopes and, consequently, decrease their antigenicity and allergenicity (Shi and others 2014). Wróblewska and others (2016) reported a significant reduction in the immunoreactivity of ALA, β -LG, α -casein, β -casein, κ -casein, BSA, and LF after buttermilk fermentation by *Lactobacillus casei*, which was even higher after simulated digestion. Despite the 21% reduction of α -casein immunoreactivity, this protein was still the most reactive. This was due to the higher concentration of anticasein-specific serum IgE compared to anti-ALA and anti- β -LG, because the patients were mostly sensitized to caseins (98%) and less to β -LG (69%) or ALA (51%). The change on allergenicity was also explained by the lactic acid bacteria species, the fermentation, and the storage conditions. Fotschki and others (2015) tested 3 different strains of bacteria and verified that *L. casei* LcY caused the highest decrease in the immunoreactivity of mare milk after fermentation, while *Streptococcus thermophilus* MK10 caused the lowest effect. Bu and others (2010b) also concluded that the combination of *Lactobacillus helveticus* and *S. thermophilus* induced a decrease in β -LG and ALA antigenicity. Fermentation with a proper cold storage also seems to have an interesting effect since the activity of microorganisms is then higher, producing more proteases that contribute to a reduction of protein antigenicity (Bu and others 2010b; Yao and others 2014). Several authors have studied the effect of simulated gastric digestion with saliva, pepsin, and pancreatin/bile salts after fermentation with lactic acid bacteria. The results showed a synergistic effect on the reduction of immunoreactivity with different rates at each stage of digestion for each tested allergen (Fotschki and others 2015; Wróblewska and others 2016). Wróblewska and others (2016) showed the fragmentation of ALA dimeric structure, the hydrolysis of BSA and β -LG after the pepsin step, and the complete degradation of caseins by porcine pancreatin/bile extract.

A matrix effect seems to be involved in the reduction of allergenicity. In the study of Pescuma and others (2011), fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 showed a greater hydrolysis percentage of β -LG in whey protein concentrates than in free protein, possibly due to the codenaturation of ALA with β -LG, increasing their aggregation, which led to complete exposure of peptic cleavage sites. An interesting approach carried out by Phromraksa and others (2008) was the identification of different proteolytic bacteria from a Thai traditional fermented food with reducing allergenic potentials. The concentrated crude enzyme of *Bacillus subtilis* reduced β -LG allergenicity, making it suitable for use in the production of hypoallergenic milk food products. The use of proteolytic bacteria has received much attention for their application in the design of new hypoallergenic dairy products. Biscola and others (2016) isolated a new proteolytic strain of *Enterococcus faecalis* from raw bovine milk, whose proteases demonstrated strong activity against α - and β -caseins at

optimal conditions of 42 °C and pH 6.5, in both skim milk and sodium caseinate.

Enzymatic hydrolysis has been used in the development of a variety of protein hydrolysate-based infant formulas to feed infants with cow milk allergy, proving to be an effective method to change the immunoreactivity of allergens (Duan and others 2014). Prioult and others (2004, 2005) studied the effect of hydrolysis with *Lactobacillus paracasei* and *Bifidobacterium lactis* enzymes on the allergenicity of acidic peptides from bovine β -LG. Their results indicated that the IgE-binding capacity was reduced by the hydrolysis of β -LG peptides, by repressing the lymphocyte stimulation. Moreover, these peptide fragments significantly up-regulated interferon (IFN)- γ and interleukin (IL)-10 production and downregulated IL-4 secretion by murine splenocytes. A decreased allergenicity, confirmed by protein degradation, inhibition of mast cell degranulation, reduction of ear swelling, and reduction of T-cell proliferation was also observed in whey protein concentrates after hydrolysis with a time-dependent effect (Knipping and others 2012). Meulenbroek and others (2014) stated that in some patients this time effect is not evident, indicating that the degree of hydrolysis is not decisive, but the presence and stability of IgE and T-cell epitopes in the hydrolysates are recognized by individual patients. As stated previously, heat treatment seems to increase the effect of enzymatic hydrolysis due to the possible exposure of cleavage sites as a result of thermal denaturation and, subsequently, enhancing the susceptibility of protein to undergo proteolysis. The molecular weight of peptides obtained after hydrolysis has also different effects on allergenicity, though there is a disagreement about the optimal molecular weight to be used depending on the chosen hydrolysis process. The specificity of enzyme, the sensitivity of the patients against the antigen, and the optimization of hydrolysis conditions may alter the final effect on allergenicity (Bu and others 2013). For example, the enzymatic digestion of β -LG may generate new antigenic substances, suggesting the existence of numerous epitopes scattered in hydrophobic regions of the molecules that became bioavailable after enzymatic digestion (Selo and others 1999). Thus, the development of hypoallergenic formulas for cow milk patients requires a careful evaluation of all these parameters.

Digestibility. During the gastrointestinal digestion, the majority of proteins are extensively cleaved throughout the digestive tract by gastrointestinal enzymes and the peptidases of the intestinal brush border to small peptides and aa (Sanchón and others 2018). However, some larger peptides are known to survive to the harsh conditions of the digestion process, being absorbed by the intestinal mucosa and further presented to the immune system. When a significant portion of the protein (large peptide) resists to digestion, it is more likely to be presented to the inductive mucosal immune system, thus increasing its potential for sensitization (Bøgh and Madsen 2016). Upon a reexposure to the allergenic peptide, which retained the proper size and conformation to be recognized by the immunocompetent cells, it increases the probability for eliciting an allergic response. The most common route of exposure to food allergens is via the gastrointestinal tract or the skin, which may occur at different pre- and postnatal stages. In the specific case of cow milk allergy, the gastrointestinal tract is the principal route of sensitization in children, normally during their 1st year of age, although the exposure by inhalation (especially in patients with asthma) to milk proteins might also be relevant as primary sensitizer (Leonardi and others 2014; Tran and others 2017).

As already stated, some authors combined the evaluation of the effect processing with the simulation of *in vitro* human

gastrointestinal digestion to identify large stable fragments that come massively in contact with the mucosa and the immunocompetent cells. Some allergens are resistant to gastric and intestinal luminal digestion, easily reaching the intestinal mucosa where absorption can occur and trigger an immune response. However, alterations in allergen conformation caused by processing may affect the ability of food allergens to reach the jejunal mucosa and reduce their allergenicity. In addition, the intestinal digestion may potentiate the effect of processing on allergens and reduce even more their allergenicity (Do and others 2016). Heat treatment (Peram and others 2013), glycation (Li and others 2011, 2013), and fermentation (Fotschki and others 2015; Wróblewska and others 2016), when combined with simulated gastric digestion, presented an increased digestibility of milk proteins and, consequently, a reduction in their allergenicity. Studies focusing on the evaluation of different digestibility models are also very relevant for improved results (Mandalari and others 2009; Benedé and others 2014; Do and others 2016; Sanchón and others 2018). The study of Benedé and others (2014) evaluated the effect of commercial enzymes in comparison with human fluids during gastric and duodenal digestion. They found a faster degradation of β -caseins with the production of less numerous and shorter peptides and a decreased immunoreactivity after duodenal digestion, particularly with human digestive enzymes. An increased immunoreactivity of β -caseins after gastric digestion, higher with commercial porcine pepsin, was also observed, suggesting the unmasking of some IgE epitopes following hydrolysis. Sanchón and others (2018) compared the peptides obtained from *in vitro* digestion process with the ones collected *in vivo* from human jejunum. The authors verified that the common resistant regions of milk proteins were similar *in vitro* and *in vivo* digestion processes, revealing that the *in vitro* process might present a good approximation to the physiological gastrointestinal digestion of milk proteins. Damodaran and Li (2017) evaluated a 2-step enzymatic approach to reduce the immunoreactivity of whey protein isolate and casein. The method consisted of a partial hydrolysis using different proteases (chymotrypsin, trypsin, or thermolysin) followed by repolymerization with microbial transglutaminase. After partial hydrolysis with chymotrypsin, trypsin, and thermolysin, whey protein hydrolysates preserved about 80%, 30%, and 20% of the original immunoreactivity, which decreased to 45%, 35%, and 5% upon repolymerization, respectively. Accordingly, the results suggested the possibility of producing hypoallergenic milk products. In another study performed by Quintieri and others (2017), whey protein concentrate digested with pepsin followed by ultrafiltration markedly reduced the antigenicity of whey hydrolysates, suggesting this method as a potential tool for the production of hypoallergenic infant food formulas.

Novel food processing technologies

High-pressure processing. High-pressure processing (HPP) is a novel technology able to inactivate microorganisms and enzymes in food, maintaining its original flavor and nutritional value, with the use of ultra-high pressures above 100 mPa at room temperature (Huang and others 2014). It is known that high pressures alter the conformational state of milk proteins, leading to enhanced flexibilities, unfolding, and aggregation. This causes the exposure of epitopes buried in the native molecule and increases allergenicity of whey proteins, but it also enhances susceptibility to the action of key digestive proteases with an eventual decrease of allergenicity. Moreover, aggregation of casein monomers reveals new determinants absent in monomeric forms, shown by the high IgE-reactivity of some patients only against these aggregates, but

not against their individual components (Kleber and others 2007; Zhong and others 2011; López-Expósito and others 2012). The effect of high pressure in whey proteins is time dependent, being influenced by different milk matrix and temperature levels. Kleber and others (2007) observed that skim milk and sweet whey (by-product of rennet-coagulated cheese) presented an augmented antigenicity with increasing pressure and time, but decreased antigenicity with increasing temperature, while in whey protein isolates the antigenicity was enhanced in all tested conditions of pressure and temperature. The diversity among the allergenicity rates detected in distinct types of milk and other products highlights the complexity of food ingredients and the importance of conducting studies on the effect of HPP in different food matrices (Huang and others 2014). High-pressure treatment is still unable to completely eliminate the allergenicity directly, but the combination with other strategies may result in possible solutions, such as HPP with enzymatic hydrolysis. López-Expósito and others (2012) demonstrated that β -LG hydrolysates (obtained with chymotrypsin and pepsin digestion) lost their allergenicity as revealed by the absence of anaphylactic reactions, mast cell activation, and a decrease in body temperature. A novel strategy named instant controlled pressure drop (DIC) that combines the effect of pressure and high temperature in a short time, followed by an instant pressure drop to vacuum was also tested. An augmented allergenicity of caseins and a reduction in whey protein immunoreactivity was caused by the dissociation of the casein micelles or aggregation of casein monomers and by changes in tertiary and secondary molecular structures of whey proteins, respectively (Boughellout and others 2015). The development of hypoallergenic milk formulas after HPP, combined with heat treatment or enzymatic hydrolysis, can reduce milk allergenicity and maintain sensory quality and nutritional value.

Food irradiation. Food irradiation uses ionizing radiation such as X-rays, high-energy electron beams (β -particles), or γ -rays for food sterilization, thereby improving the safety and shelf-stability without compromising nutritional or sensory quality, when applying the appropriate dose. Irradiation creates changes in the ability of IgE-allergen binding by the induction of structural denaturation, fragmentation, and/or aggregation of proteins, and, at least, the destruction of IgE epitopes (Ham and others 2009; Oduke and others 2016). Using γ -irradiation, Lee and others (2001) successfully reduced the allergenicity of β -LG by 7-fold. Meng and others (2016a) also proved the low potential *in vivo* allergenicity of irradiated ALA by the decrease in ALA-specific IgE levels, the inhibition of mast cells, and basophil activation, a significant decrease of histamine levels and a reduction of anaphylactic reactions in mice.

Ultraviolet and infrared radiation. Similar effects on the changes in conformational epitope structures of milk allergens were observed after the applications of ultraviolet (UV) and infrared (IR) radiations (Anugu 2009; Tammineedi and others 2013; Hu and others 2016). UV radiation has been used as a bactericidal agent since the year 1928. More recently, it has been used by the food industry as a sanitizing and disinfecting agent. Similarly, IR radiation can inactivate microorganisms by damaging intracellular components, such as DNA, RNA, and ribosomes in the cell, and modify the protein structure in food. According to Hu and others (2016), the allergenicity of α -caseins decreased after 15 min of the UV-C treatment and 5 min of far-IR treatment, with the 1st treatment being the most efficient as confirmed by the simulated digestion tests. Tammineedi and others (2013) also showed a reduction in the allergenicity of α -caseins and whey proteins

in 25% and 27.7%, respectively, after 15 min of the UV-C treatment. However, it seems that these alterations are not enough for the production of hypoallergenic formulas. Microwave radiation combined with enzymatic hydrolysis could be an alternative to reduce the antigenicity of milk proteins, based on few studies that reported interesting results (Izquierdo and others 2008; El Mecherfi and others 2015).

Other technologies. Ultrasound has gained much attention because it seems to have an effect on the allergenicity of shrimp and soy allergens (Li and others 2006). This new technology alters the conformation and reactivity of allergens by the implosion of sonication bubbles formed during the process, causing localized high pressure and temperature (Tammineedi and Choudhary 2014). However, ultrasound seems to have no effect in reducing the allergenicity of milk proteins, as stated by Stanic-Vucinic and others (2012), and Tammineedi and Choudhary (2014).

The application of nonthermal atmospheric plasma also revealed having no effect on α -casein and whey protein allergenicity (Tammineedi and others 2013), despite the recent developments on the reduction of shrimp and wheat allergenicity (Nooji 2001; Shriver 2001). As demonstrated, it destroys mainly the 3D structure and the conformational epitopes of proteins, without the selective damage of allergenic IgE epitopes.

Genetic modification of key residues in binding sites of the allergens, without interrupting the global structure would be a possible solution for allergen-specific immunotherapy. Until now, 2 mutations on major epitopes of β -LG, namely Ala86Gln (Kazem-Farzandi and others 2015) and Lys69Asn (Taheri-Kafrani and others 2015), have been studied. The results indicated that both mutated proteins are recognized with 9-fold less potency by IgE in cow milk allergic patients than the native or recombinant β -LG. Mutations were responsible for the disappearance of important epitopes and, consequently, for the reduced IgE binding to mutated β -LG.

Many efforts have been made to study the effect of processing on the allergenicity of milk proteins, with promising results showing the applicability of these technologies on the production of hypoallergenic formulas. Moreover, it seems that all the nutritional and sensory characteristics are maintained, in opposition to some conventional processes, such as heat treatment and enzymatic hydrolysis. The applicability of other novel processing technologies, such as pulsed UV light, pulsed electric field, and ohmic treatment, not yet evaluated in milk allergenicity, could be interesting approaches in the near future (Johnson and others 2010; Tammineedi and Choudhary 2014; Verhoeckx and others 2015; Cappato and others 2017).

Analytical Methods for the Detection of Milk Allergens in Processed Foods

The increased awareness about the public health implications of food allergies has resulted in the need of developing analytical methodologies to control the presence of hidden allergenic ingredients in processed foods, allowing the enforcement of labeling regulations (Taylor and others 2014). Allergic consumers are fully dependent and supposedly protected by the label information of processed foods. However, accidental exposure to hidden allergens in foods owing to mislabeling or cross-contaminations during food processing constitutes a real risk for these individuals (Costa and others 2014, 2015). Milk proteins are often applied as technological aids, and, thus, they are present in several types of foods as an ingredient. However, owing to the common practice of using

shared production lines to manufacture different food formulations, accidental cross-contaminations are very likely to occur. To increase the well-being and safety of sensitized individuals, food products for human consumption must declare all potentially allergenic ingredients irrespective of their amount. Therefore, proper and highly sensitive analytical methodologies represent essential assets to aid the industrial management of allergenic foods and, subsequently, to facilitate allergen control/monitoring by regulatory authorities.

The choice of the best method for allergen analysis depends on specific criteria, such as target analyte (proteins or DNA), basis of detection (chemical or biological), cost per run/analysis, setup, cross-reactivity phenomena, need for expertise knowledge, and possibility for multitarget detection (Johnson and others 2011). Moreover, appropriate sensitivity and specificity to trace minute amounts in complex food matrices are important requirements (Costa and others 2012). The ideal limit of detection (LOD) for allergens in food products has been considered in the range of 1 to 100 mg/kg (Poms and others 2004), although these values of reference are currently being revised. Morisset and others (2003) established a threshold of clinical reactivity to milk of 30 mg/kg for milk proteins, to guarantee 95% safety for patients who are allergic to milk, based on the consumption of 100 g of product. More recently, using appropriate statistical dose-distribution models, the reference dose for milk was defined as 0.1 mg of protein, considering the eliciting dose that protects 99% of the milk-allergic population (ED_{01}). According to the conversion factors available from the U.S. Dept. of Agriculture (USDA Food Composition Database), this reference dose represents 3.03 mg of liquid milk per kg of food and 0.28 mg of nondry fat milk per kg of food (Taylor and others 2014). Presently, there are several technical possibilities for the detection of milk allergens in foodstuffs, and recent developments are summarized in Table 5.

Protein-based methods

The classical protein-based methods are still the most commonly used for the detection of allergens in foods. They are based on allergen-antibody interactions and available in different formats, such as lateral flow devices (LFD), dipstick tests, enzyme-linked immunosorbent assay (ELISA), and immunoblotting. Currently, leading-edge technologies have reached particular attention for allergen analysis, namely immunosensors and mass spectrometry (MS) platforms (Costa and others 2017).

Immunoassays. Antibodies play an important role in most of the allergen detection methods due to their specific binding to respective antigens, which create very sensitive and specific systems. Most immunoassays for the detection of milk allergens are based on ELISA, which can provide quantitative results through the comparison of optical or fluorescent signals of the unknown samples with standard curves (Costa and others 2017). Different formats are available, namely direct ELISA, indirect ELISA, competitive ELISA, although the sandwich ELISA is the most commonly used. Presently, owing to the increased demand for rapid and reliable tests for the detection of specific allergens in food, various commercial ELISA kits have entered the market (Table 6). They are able to detect specific proteins, such as caseins and β -LG, or total milk proteins with reported LOD values ranging from 0.015 to 2 mg/L, though the type of food matrix and the effect of processing can affect these values. Heating can result in the formation of insoluble protein aggregates, which may be undetectable by ELISA. In addition, the interaction with compounds of the food matrix and differences in antibody recognition of heat-denatured proteins can

Table 5—Methods for the detection of cow's milk allergens in different food products.

Method	Matrix	Target allergen	Limit of detection	Analytical range	References
IC-ELISA and S-ELISA	Model processed foods (sausage, bread, and pâté); commercial samples labeled with milk ingredients	β -LG	0.05 mg/kg (S-ELISA) 0.5 mg/kg (IC-ELISA)	5 to 100 μ g/L (S-ELISA) 15 to 500 μ g/L (IC-ELISA)	de Luis and others (2009)
ELISA, commercial kits	Flours and bread	Casein	Not reported	Not reported	Heick and others (2011a)
S-ELISA, Commercial kit	Meat-based products	β -LG	0.2 mg/kg	0.5 to 13.5 mg/kg	Decastelli and others (2012)
ELISA, commercial kits	Cookie dough and cookies	Caseins, β -LG	Not reported	Not reported	Khuda and others (2012b)
ELISA, commercial kits	Chocolate	Caseins, β -LG	Not reported	Not reported	Khuda and others (2012a)
I-ELISA and IS-ELISA	Red and white wines	Caseins	0.1 and 0.2 mg/L (white and red wine, I-ELISA), 0.01 and 0.1 mg/L (white and red wines, S-ELISA)	10 to 1000 and 10 to 3000 μ g/L (white and red wines, indirect ELISA) 1000 and 10 to 10000 μ g/L (white and red wines, sandwich ELISA)	Deckwart and others (2014)
ELISA, commercial kits	Desserts	Caseins, β -LG, Milk proteins	3 mg/kg	0 to 30 mg/kg	Johnson and others (2014)
ELISA, commercial kits	Cookie dough, cookies	Caseins	0.04 mg/kg	0.2 to 6 mg/kg	Török and others (2014)
ELISA, Commercial kits	Dry mixes and processed cookies	Caseins	10 mg/kg	2.5 to 15 mg/kg	Gomaa and Boye (2015b)
ELISA, commercial kit	Working surfaces	β -LG	0.2 μ g	Not reported	Galan-Malo and others (2017)
Optical immunobiosensor (SPR)	Bovine milk, colostrum, and infant formulas	LF	19.9 μ g/mL	0 to 1000 ng/mL	Indyk and Filonzi (2005)
Optical immunosensor (resonance enhanced absorption)	Processed milk matrices	β -LG	Not reported	100 to 100 ng/mL	Hohensinner and others (2007)
SPR immunosensor	Bovine milk	Caseins	0.01 mg/L	0.1 to 10 mg/L	Hiep and others (2007)
SPR immunosensor	Milk	ALA, β -LG, BSA, LF	Not reported	0 to 1000 ng/mL	Billakanti and others (2010)
Optical immunobiosensor (SPR)	Milk-based products	ALA	0.12 mg/mL	10 to 1000 ng/mL	Indyk (2009)
Optical immunochip biosensor (resonance enhanced absorption)	Milk- and whey protein-based food samples	β -LG	1 ng/mL	Not reported	Maier and others (2009)
Antibody-microarrayed chip using iSPR	Cookies, chocolates	κ -Casein	0.2 mg/kg (cookies)	0.1 to 10 mg/L	Raz and others (2010)
Electrochemical immunosensor	Cheese	Caseins	0.05 ng/mL	0.1 to 10 ng/mL	Cao and others (2011)
Electrochemical immunosensor	Cake, cheese snacks, and biscuits	β -LG	0.85 pg/mL	0.001 to 100 ng/mL	Eissa and others (2012)
Amperometric	Cow and human milk (raw, UHT and pasteurized)	β -LG	0.8 ng/mL	2.8 to 100 ng/mL	Ruiz-Valdepeñas and others (2015)
Magnetoimmunobiosensor	Cow and human milk (raw, UHT and pasteurized)	ALA	11.0 pg/mL	37.0 to 5000 pg/mL	Ruiz-Valdepeñas and others (2016)

(Continued)

Table 5–Continued.

Method	Matrix	Target allergen	Limit of detection	Analytical range	References
LC-MS	Fruit juices	ALA (2 peptides), β -LG (4 peptides)	1 μ g/mL	Not reported	Monaci and van Hengel (2008)
LC-ESI-Q-TOF-MS	Cookies	α ₅₁ -casein, α ₅₂ -casein, BSA, β -casein	100 mg/kg	10 to 1000 mg/kg	Monaci and others (2010b)
LC-ESI-MS/MS	Wine	α ₅₁ -casein (3 peptides) α ₅₂ -casein (2 peptides) β -casein (3 peptides) κ -casein (1 peptide)	50 μ g/mL	10 to 100 g/hL	Monaci and others (2010a)
LC-MS/MS	Bread	α ₅₁ -casein (2 peptides) α ₅₂ -casein (2 peptides)	5 mg/kg	10 to 500 mg/kg	Heick and others (2011b)
LC-MS/MS	Bread, flours	α ₅₁ -casein (2 peptides)	5 mg/kg	10 to 500 mg/kg	Heick and others (2011a)
LC/HCD-MS	Wine, cookies	β -casein (1 peptide)	1.6 mg/kg	1.6 to 22 mg/kg (Cookies) 1.6 to 6 mg/kg (Wines)	Monaci and others (2011)
MALDI-TOF/MS and MS/MS analysis	Wheat and glucose, matrix, chocolate, cookies	α ₅₁ -casein (3 peptide)	Not reported	Not reported	Cucu and others (2012)
LC-MS/MS	Chocolates, milk-based products	ALA (3 peptides), β -LG (6 peptides)	1 ng/mL		Ansari and others (2011)
LC-ESI-3D-IT-MS	White wine	ALA (1 peptide) β -LG (2 peptides) α ₅₁ -casein (2 peptides) β -casein (2 peptides)	0.09 to 0.23 mg/kg 0.6 fmol	10 to 500 mg/L 0 to 100 mg/kg	Losito and others (2013) Newsome and Scholl (2012)
Protein standard absolute Quantification (PSAQ) using UPLC-ESI-MS	Cookies, biscuits	β -casein (1 peptide) Labeled α ₅₁ -casein			
UPLC-TQ-MS/MS	Pasta, dough	β -Casein	0.5 mg/kg	1 to 100 μ g/mL	Chen and others (2015)
LC-ESI-MS/MS	Red wine	α ₅₁ -Casein (1 peptide) β -casein (1 peptide)	0.5 mg/L (α ₅₁ -casein) 0.01 mg/L (β -casein)	1 to 100 mg/L 0.03 to 50 mg/L	Mattarozzi and others (2014)
Micro-HPLC-ESI-MS/MS	Cookies	α ₅₁ -Casein (2 peptides)	0.1 mg/kg	0.5 to 20 mg/L	Monaci and others (2014)
Orbitrap monostage MS compared with hybrid linear ion trap MS	Wine	α ₅₁ -Casein (6 peptides) α ₅₂ -casein (1 peptide) β -casein (2 peptide) κ -casein (1 peptide)	0.2 to 2 μ g/mL	0.2 to 2 μ g/mL	Pilolli and others (2014)
LC-MS	Dry mixes and processed cookies	Caseins (5 peptides)	10 mg/kg	10 to 1000 mg/kg	Gomaa and Boye (2015b)
HPLC-MS/MS	Meat products	Caseins (2 peptides) Whey proteins (2 peptides)	1 mg/kg	0 to 139 mg/kg 0 to 4000 mg/kg	Jira and Schwägele (2015)
LC-ESI-MS/MS	Bakery products	α ₅₁ -casein (1 peptide)	0.11 mg/kg	1 to 150 mg/kg	Lamberti and others (2016)
UHPLC-MS/MS	Tomato sauce, cookies, chocolate, ice-cream	Caseins (4 peptides), whey proteins (3 peptides)	0.5 mg/kg (caseins), 5 mg/kg (whey)	0 to 5 mg/kg	Planque and others (2016)
LC-MRM/MS	Cookies, biscuits, peanut jam, waffles, patisseries, pork pie, wheat meal	β -LG (2 peptides), ALA (2 peptides), α ₅₁ -casein (1 peptide)	0.2 mg/L (β -LG), 0.39 mg/L (ALA), 0.2 mg/L (α ₅₁ -casein)	0.48 to 31.25 mg/L (β -LG) 0.97 to 31.25 mg/L (ALA) 0.48 to 31.25 mg/L (α ₅₁ -casein)	Ji and others (2017)
Tetraplex real-time PCR	Bakery products	Mitochondrial DNA (tRNA-Lys)	0.64 μ g DNA/L	0.64 to 20 μ g DNA/L	Köppel and others (2010)
Hexaplex Real-time PCR	Sausages, cookies, chocolates, sandwiches, parfaits	Mitochondrial DNA (tRNA-Lys)	2 μ g DNA/L	2 to 20 μ g DNA/L	Köppel and others (2012)
Real-time PCR with TaqMan probe	Candies, biscuits, chocolate, drinks	ALA	0.025 mg/L	0.00025 to 25 mg/L	Xiao and others (2016)

CE-ELISA, competitive ELISA; I-ELISA, indirect-ELISA; S-ELISA, Sandwich ELISA; LC, liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; UHP, ultra-high pressure; MRM, multiple reaction monitoring; IT, ion trap.

Table 6—Commercially available LFD and ELISA kits for milk allergen detection/quantification in foods.

Commercial kit (brand)	Matrix	Type of assay (cat nr)	Analytical range	LOD (mg/kg)	Performance time
Veratox [®] for Total Milk (Neogen [®] , Lansing, Mich., U.S.A.)	Products noncontaining milk (juice, wine, sauces)	Sandwich quantitative ELISA (8470)	2.5 to 25 mg/kg	1	30 min
RIDASCREEN [®] Fast Milk (r-Biopharm AG, Darmstadt, Germany)	Bovine milk in ovine or caprine milk and cheese	Competitive quantitative ELISA (R4652)	2.5 to 67.5 mg/kg	0.7	30 min
ELISA Systems Casein (ELISA Systems TM , Queensland, Australia)	Nondairy products	Sandwich semiquantitative ELISA (ESCAPRD-48)	1.0 to 10 mg/kg	0.5	45 min
Veratox [®] for Casein Allergen (Neogen [®])	Products noncontaining milk (juice, wine, sauces)	Sandwich quantitative ELISA (8460)	2.5 to 15 mg/kg	1	30 min
RIDASCREEN [®] Casein (r-Biopharm AG, Darmstadt, Germany)	Ice cream, wine, chocolate, beverages, infant formula, bakery goods, sausages, cake, bread	Competitive quantitative ELISA (R4652)	0.5 to 13.5 mg/kg	0.12	30 min
ELISA Systems BLG (ELISA Systems TM , Queensland, Australia)	Nondairy products	Sandwich semiquantitative ELISA (ESMRDLG-48)	0.1 to 1 mg/kg	0.05	45 min
BioKits BLG Assay Kit (Neogen [®])	Food products	Sandwich quantitative ELISA (902061Y)	2.5 to 40 mg/kg	2	120 min
RIDASCREEN [®] Fast β -LG (r-Biopharm AG, Darmstadt, Germany)	Whey protein, whey protein containing products	Competitive quantitative ELISA (R4901)	0.5 to 13.5 mg/kg	0.19	30 min
AgraQuant [®] β -LG (Romer Labs Div., Getzersdorf, Austria)	Food products	ELISA (COKAL1048)	0.01 to 0.4 mg/kg	0.015	NR
AgraStrip [®] Total Milk (Romer Labs Div., Getzersdorf, Austria)	Food products	LFD (COKAL2410AS)	1 to 10000 mg/kg of milk protein; 3 to 30000 mg/kg of skimmed milk powder	1	NR
Reveal [®] 3-D for Total Milk Allergen (Neogen [®])	Liquid products (juices, sorbets)	LFD (8479)	5 to 1000 mg/kg	5 to 10	5 min
Reveal [®] for Total Milk (Neogen [®])	Liquid products (juices, sorbets)	LFD (8478)	NR	5	5 min
Bovine Total Milk Rapid Test (Elution Technologies, Colchester, Vt., U.S.A.)	NR	LFD (MILK-1004)	1 to 1000 mg/kg	1 to 2	10 min
AgraStrip [®] Caseins (Romer Labs Div., Getzersdorf, Austria)	Food products	LFD (COKAL1210AS)	1 to 10000 mg/kg	1	NR
Casein Lateral Flow IIR (MioBS, Yokohama, Japan)	Processed and unprocessed food	LFD	NR	5	25 min
Casein Lateral Flow Kit (Crystal Chem, Downers Grove, Ill., U.S.A.)	Raw and processed food	LFD (M2202)	NR	5	15 min
AgraStrip [®] β -LG (Romer Labs Div., Getzersdorf, Austria)	Food products	LFD (COKAL1010AS)	0.5 to 100 mg/kg of milk protein, 1.5 to 3000 mg/kg of skimmed milk powder	0.5	NR (excluding extraction)

LOD, limit of detection; NR, not referred.

affect the detection and, particularly, the quantitative determination of allergenic proteins in food products. Therefore, a careful choice of the proper kit is always needed (Downs and Taylor 2010). Table 6 presents a set of commercially available ELISA kits for the detection of milk allergens. Table 5 gathers not only the information regarding the application of commercial kits to assess the detection/quantification of milk in foods, but also the in-house developed ELISA. Deckwart and others (2014) have developed 2 systems (indirect and indirect sandwich ELISA) for the detection of caseins in white and red wines, achieving a LOD between 10 and 200 $\mu\text{g/L}$. The reported sensitivities were in accordance with the threshold of 0.25 mg/L requested by the OIV (Organisation Intl. de la Vigne et du Vin, Paris, France), regarding the presence of fining agents (milk caseins, ovalbumin) in wines. Their work also demonstrated the influence of a complex food matrix on the performance of this type of assay. The development of 2 ELISA formats (indirect competitive and sandwich) was performed by de Luis and others (2009) for the detection of β -LG in processed foods. The competitive and sandwich systems, with LOD of 0.5 and 0.05 mg/kg, respectively, were able to detect undeclared milk ingredients in 14% of the tested commercial samples. The sandwich format proved to be more specific and sensitive because of being less affected by the matrix than the indirect competitive one.

LFD are other rapid and specific immunochemical tests for allergen detection. The principle of the method is the same as that of ELISA, but it allows a simpler and faster performance with qualitative or semiquantitative results that can be interpreted visually. The lack of quantitative information and the susceptibility of these devices in providing false-negative results are the major drawbacks associated with their use. However, they are largely applied in the food industry to monitor the cleaning of food processing equipment and food product contamination (Courtney and others 2016). There are several LFD kits in the market that detect milk allergens in food products in a few minutes and on-site, with LOD down to 0.5 mg/L, as demonstrated in Table 6.

Biosensors are considered emerging tools for allergen detection, since they are fast, repeatable, and highly sensitive approaches with great potential for full automation. In brief, biosensors are based on the direct recognition of a biological interaction between a receptor (antibody or probes) and a target molecule (protein or DNA) by means of a transducer that produces a measurable signal (Schubert-Ullrich and others 2009; Prado and others 2016; Costa and others 2017). Several studies have been performed using SPR immunosensors to detect milk allergens in different food matrices, reaching sensitivities of 1 ng/L to 0.12 mg/mL (Indyk and Filonzi 2005; Hiep and others 2007; Indyk 2009; Billakanti and others 2010; Raz and others 2010). Electrochemical immunosensors are also used for milk protein detection (Cao and others 2011; Eissa and others 2012; Ruiz-Valdepeñas and others 2015; Ruiz-Valdepeñas and others 2016). Eissa and others (2012) developed an immunosensor able to detect down to 0.85 pg/mL of β -LG in food products, which is the lowest LOD reported for this protein by electrochemical immunosensors (Table 5). Nonetheless, owing to the fact that these systems are based on the biological interaction between an antibody and the respective allergen/marker protein, a careful interpretation is always needed to avoid false positive or false negative results (Johnson and others 2011; Costa and others 2012; Khuda and others 2012b; Gomaa and Boye 2015a, 2015b).

In general, the immunoassays are able to detect major allergenic milk proteins, such as ALA and β -LG, in a wide diversity of food matrices. However, information on their development is still lacking to determine the presence of hydrolyzed milk proteins

in foods, as they often retain their immunoreactivity. So far, few studies have been carried out to evaluate the immunoreactivity of pure whey and casein hydrolysates (Pessato and others 2016; Damodaran and Li 2017), but with no application to the detection of peptides from hydrolyzed milk proteins in different food matrices.

MS platforms. MS has played an important role in proteomic research and has proved to be a powerful analytical technique for both protein and peptide analysis, encompassing the identification, characterization, and determination of food allergens (Monaci and Visconti 2009). MS platforms offer several advantages, such as high rapidity, accuracy, sensitivity, specificity, and reproducibility (Picariello and others 2011). Its sensitivity is comparable to ELISA and quantitative polymerase chain reaction (PCR), allowing a multitarget detection in a single run with high specificity. Additionally, the problems related to cross-reactivity often linked to immunoassays are eliminated because the detection of the target peptide/protein does not require interaction with a biological receptor (antibody), enabling a direct and unequivocal identification of the target analytes. The proteomic analysis of a sample commonly consists of one/several separation steps at protein and/or peptide level (gel electrophoresis, liquid chromatography [LC]), followed by MS analysis. There are 2 main approaches for allergen detection, quantification, and characterization: the bottom-up approach, where proteins are digested with enzymes, such as trypsin, prior to MS analysis; and the top-down approach, where the whole proteins are fragmented directly inside the mass spectrometer, avoiding the variable step of protein digestion. The bottom-up approach is the most commonly used due to the current limited performance of top-down-based instruments (Prado and others 2016).

There is an increasing number of reports regarding the detection of milk allergens in foodstuffs by MS technologies. The majority of these works use multitarget approaches, enabling the discrimination of different milk allergens (caseins, ALA, and β -LG) in different food matrices, such as wines, cookies, infant formulas, and bakery products, with sensitivities ranging from 0.01 to 5 mg/kg (Table 5). Very recently, Ji and others (2017) developed a LC-tandem MS (LC-MS/MS) method for the confirmation and quantification of 3 milk allergens (ALA, β -LG, and α _{S1}-casein) in different food products, namely cookies, biscuits, waffles, patisseries, yolk pie, among others, with a reported LOD of 0.2 mg/kg for β -LG and α _{S1}-casein, and 0.39 mg/kg for ALA. Similar sensitivities were obtained by Losito and others (2013) whose method, based on LC-electrospray ionization-ion trap-MS (LC-ESI-IT-MS), was able to detect caseinate at trace levels in different Italian white wines, with LOD ranging from 0.09 to 0.29 mg/L, depending on the wine.

Like in immunochemical methods, food matrix also affects the sensitivity of MS methods. Accordingly, the reported LOD values are higher for the analysis of allergens in complex food matrices such as chocolates. The effect of food processing on the target allergens should also be accounted for because their structure is known to be differently affected by distinct types of processing, requiring the identification of marker peptides in both raw and processed matrices. Food processing alters the extractability and solubility of allergenic or other marker proteins, which can compromise the good performance of the MS-based method. However, the advantages of high accuracy, specificity, and multitarget analysis, make the MS-platforms more widely used for allergen analysis than the classical immunochemical assays (Prado and others 2016).

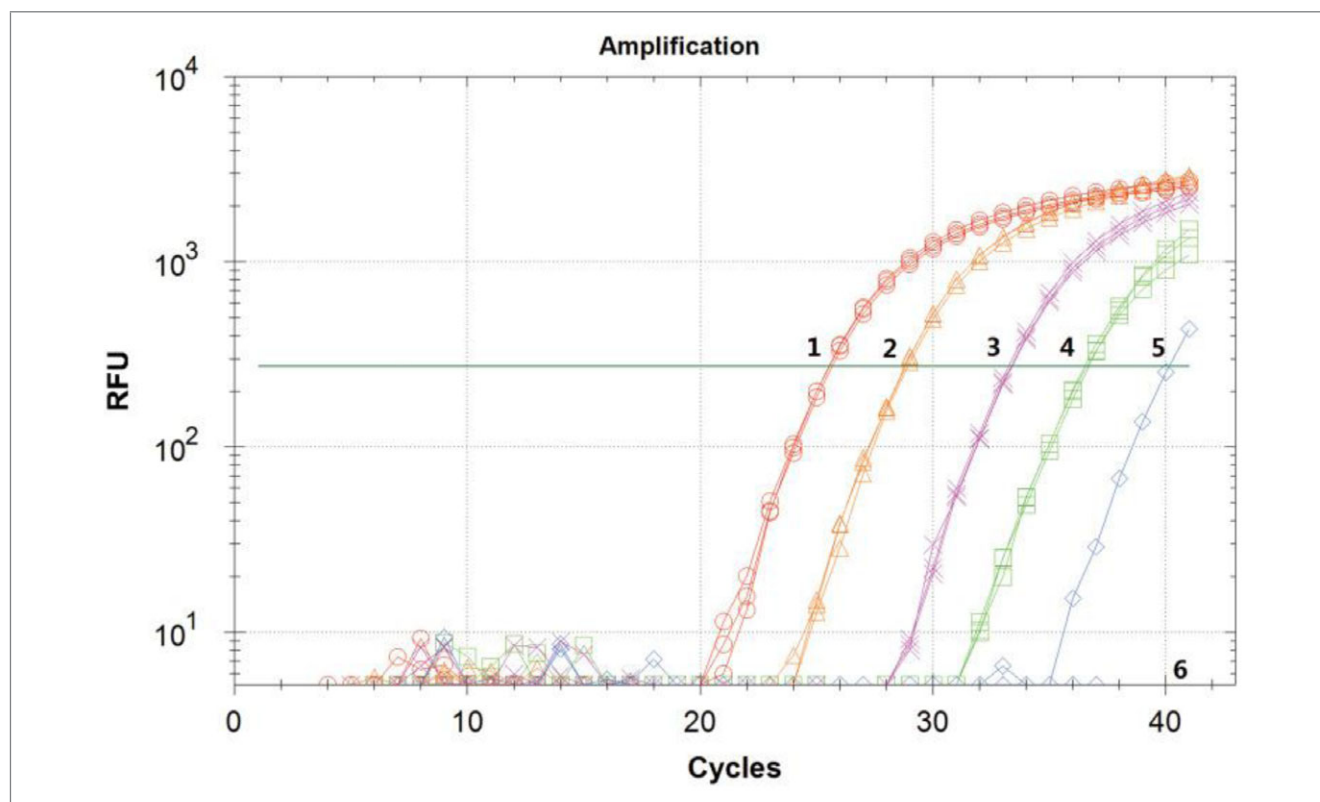


Figure 3—Amplification of an ALA gene fragment from 10-fold serial dilutions of cow's milk DNA. Legend: 1 to 6: 50 ng (circle), 5 ng (triangle), 0.5 ng (cross), 0.05 ng (square), 0.005 ng (diamond), and 0 (straight line) ng of DNA, respectively. Reprinted from Xiao and others (2016) with permission from Elsevier Ltd.

DNA-based methods

Recently, DNA-based methods for allergen detection have been received with increasing interest due to their high specificity, sensitivity, independence from possible biological effects associated with antibody production, and high thermal stability of DNA molecules, particularly relevant to analyze processed foodstuffs. Therefore, DNA-based methods have proved to be excellent alternatives to protein-based methods, especially when analyzing highly processed foods (Costa and others 2017). DNA targets might be genes that encode allergenic proteins or other specific sequences, therefore they are considered as indirect markers of the presence of an allergenic ingredient. Most of the published works using DNA-based methods consist of the amplification of the initial target DNA sequences by PCR with the use of specific primers, responsible for conferring a high specificity level to the assays (Mafra and others 2008; Prado and others 2016). The main approaches used for allergen detection are end point PCR, multiplex PCR, real-time PCR, and PCR-ELISA, but recently new promising advances have gained much interest, such as real-time PCR coupled to high-resolution melting (HRM) analysis, single-tube nested real-time PCR, DNA arrays, and genosensors. Most reports apply DNA-based methods for the authentication of milk products, such as cheeses (Dalmasso and others 2011), and for the identification of different species in milk products (Bottero and others 2003; Mafra and others 2004; Lopez-Calleja and others 2007; Zhang and others 2007; De and others 2011). In contrast, few studies describe the detection of milk allergens in foodstuffs, with real-time PCR being the main technique used for this purpose. Real-time PCR methods have the advantages of providing quantitative results with adequate setup cost, reasonable running

time, and moderate requirements for specialized equipment and personnel. Köppel and others (2010, 2012) developed 2 tetraplex and 2 hexaplex real-time PCR systems with TaqMan probes for the simultaneous detection of several allergens in food, including milk allergens (Table 5). The methods exhibited good specificity with a sensitivity down to 0.64 $\mu\text{g/mL}$ of bovine DNA. A real-time PCR method with a TaqMan minor groove binder probe for the specific detection of ALA gene in food was developed by Xiao and others (2016). The method showed a sensitivity of 0.05 ng of bovine DNA (Figure 3) and it was applied to 42 commercial samples in order to verify the compliance with the label for the presence of milk as an ingredient.

Final Remarks

The concern with milk allergy has increased over the last few years, mainly because most of the affected individuals are infants below the age of 3. Currently, there is no treatment for food allergies and, consequently, the sensitized individuals have to avoid milk products and all foodstuffs containing milk derivatives. In the case of accidental exposure, different pharmaceuticals (H1- and H2-antihistamines, beta-2 agonists, or glucocorticosteroids) can be used to relieve the clinical symptoms associated with adverse immunological responses, although epinephrine is commonly used to treat very severe and life-threatening allergic reactions (anaphylaxis).

Recent advances have been made in the development of effective strategies to treat milk allergy and induce tolerance in allergic patients. OIT seems to be a promising approach, with a success rate varying from 37% to 70%. Another approach is focused on the reduction of milk allergenicity by the use of

new food processing technologies. Processing induces changes to milk proteins that can largely affect their susceptibility to gastrointestinal digestion, absorption kinetics, and, consequently, their immunoreactivity. Therefore, the allergenic potential of milk proteins may be diminished by selecting appropriate parameters during processing. In spite of reported reductions in allergenicity with some types of processing, no method is completely effective. Due to differences in the degree of allergenic reactions and in the tolerance among different patients, it is important to conduct more *in vitro* and *in vivo* studies to test different conditions and combinations of milk processing methods.

To improve consumer protection and to ensure life quality of sensitized individuals, several regulations and directives have been established, which state the obligation of labeling the potentially allergenic ingredients/foods, including milk. Thus, the development of methodologies to detect and quantify allergens is imperative to allow the enforcement of the labeling regulations and control the presence of hidden allergenic ingredients. ELISA have provided sensitive and specific methods for the detection of milk proteins in food products, although with the limitation of being seriously affected by food processing that could lead to false negative results. Immunosensors have also been applied, resulting in fast, repeatable, and potentially fully automated analysis. MS platforms, such as LC-MS/MS, have shown their efficacy in the detection of allergenic milk proteins and peptides, which can be used as confirmatory tools for the identification of multiple allergens. DNA-based methods, despite consisting of indirect detection approaches, are considered efficient alternatives, being less prone to be affected by food processing. The capability of methods to detect allergens in food products at trace levels depends on many factors, including the food matrix, the extraction method, the food processing operation, and the form in which the allergen is present. Despite these limitations, the currently available methods for the detection of milk allergens are playing a crucial role in the provision of information to allergic consumers, which is essential for an elimination diet required to protect their health.

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Author Contributions

Caterina Villa wrote the manuscript with critical input and corrections by Joana Costa, Maria Beatriz P.P. Oliveira, and Isabel Mafra. Isabel Mafra did the final editing. All authors contributed to locating and to interpreting the literature sources.

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