Casein : Allergenicity and molecular Properties

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Abstract

Caseins represent about 80% of total protein in cow's milk. Individual caseins, αs1, αs2, β and κ-casein, are characterized by specific structure and function. Besides their role in cheese-making, casein subunits exhibit molecular properties and structure which confer them technological and functional properties exploited in food and pharmaceutical industries for a long time. Nutritional value and beneficial effects due the release of bioactive peptides are also widely reported. Despite their valuable properties, caseins are able to trigger even severe immuno reactions in sensitive individuals thus representing one of the most important allergenic source in the food allergens landscape. Thanks to the exploitation of high throughput technologies combined with advanced bioinformatic tools, the forefront research is directed to identify and characterize new epitopes capable of inducing an onset of allergic reaction in predisposed consumers. On the other hand, several efforts are underway to develop innovative solutions to reduce caseins allergy by preserving nutritional quality of proteins. The paper aims to give a broad overview on the main proteins constituting cow's milk, the related molecular properties as well as to provide some knowout about their functioning properties in food processing. The focus is then moved forward to allergenic properties of caseins and capability of milk proteins to trigger cow's milk allergy (CMA) in sensitive consumers. Processing strategies to mitigate casein allergenicity and CMA prevention, management and therapeutic approaches are also presented.

Keywords: caseins, molecular properties, functioning properties, food allergens, cow's milk allergy, IgE, innovative strategies, allergenicity reduction.

1. Introduction

Milk proteins represent an important source of nutritional compounds due to their high biological value and richness in essential aminoacids. Through acidification at pH 4.6 or ultracentrifugation, these proteins can be divided in two main fractions: caseins and whey proteins, which proportions in the total milk vary among the species. For instance, in human, cow, goat, camel and mare milk, casein represents the most dominant fraction, with percentages ranging from 40% and 80%. Cow milk shows the highest content of casein (80%), followed by goat (64.52%), mare (55%), camel (52%) and human (40%). Cow milk represents the most widespread source of dairy products worldwide accounting for around 82.4% of the world's fresh milk (Villa et al., 2018). As other species, casein fraction from cow milk is composed by α-S1-casein, α-S2-casein, β-casein and κ-casein representing 40%, 12.5%, 35% and 12.5% of the whole caseins, respectively (Huppertz, 2013; Huppertz et al., 2018). Different proportions of α-S1-, α-S2-, β- and κ-casein were found in other milk species (Zhao et al., 2023).

Structure and molecular properties of casein subunits are responsible for their technological and functional properties. For example, κ-casein genetic variants show different effects on cheesemaking (Schaar et al., 1985) whereas the structure of individual molecules can affect flavor binding, color, and digestibility (Foegeding, 2015). Aminoacid sequences are also responsible for biological effects due to the release of bioactive peptides (Silva and Malcata, 2005). Caseins have open and flexible conformations and contain hydrophilic and hydrophobic segments. The high hydrophobicity promotes the formation of micellar structure (size from 50 to 300 nm), all four types of caseins (94% on dry weight basis) and amorphous calcium phosphate, magnesium and citrate (6% d.w.b.) (Ranadheera et al., 2016). Other than the micellar form, sodium or calcium salts of casein (namely caseinates) are widely used ingredients in the food industry. Caseins are Generally Regarded as Safe (GRAS status) and therefore are also used as a protein ingredient in various food products to enhance their physical, functional (foaming, thickening, emulsification, texture) and nutritional properties. Caseins possess ions and small molecules binding capacity, stabilizing and emulsification activities, and gelation and water binding capacity under defined conditions (Ranadheera et al., 2016); these physical properties and behaviour, make them as good candidates to develop delivery systems in food and pharmaceutical industries (Sadiq et al., 2021).

Despite their valuable properties, caseins are able to trigger even severe immuno reactions in sensitive individuals thus representing one of the most important allergenic source in the food allergens landscape.

Several investigations demonstrated that the allergenicity of cow milk is higher than what found for goat, camel and mare, even if no report has clearly determined the reasons (Zhao et al., 2023). Cow caseins proteins are well known for their allergenic potential and according to the International Union of Immunological Societies (WHO/IUIS) they are designed as Bos d 9 (α-S1 -), Bos d 10 (α-S2), Bos d 11 (β-) and Bos d 12 (κ-), while the general code Bos d 8 is used for identifying the whole casein fraction. Likewise, to functioning and functional properties, the composition and sequence of amino acids determine the higher structure of proteins, which, in turn, entails the biological function of proteins, including allergenicity. Although conflicting data exist concerning, milk processing techniques can alter the milk fat and protein's molecular structure and serve them as allergens to the immune system of allergic individuals. For istance, homogenization might increase the

allergenicity of the milk due to disintegration of casein micelles and milk fat globules (Poulsen et al., 1987; Geiselhartet al., 2021).

Cow's milk allergy (CMA) is one of the most common food allergies in early life with an estimated prevalence in developed countries ranging from 0.5% to 3% at age 1 year (Flom and Sicherer, 2019). Allergic responses by both whey proteins and caseins in adults are rare but severe (Lam et al., 2008). Multiple symptoms were produced by milk ingestion; vomiting, diarrhea, abdominal pain, asthma, rhinitis, and atopic dermatitis were frequently presenting and challenge symptoms. Some patients had central nervous system symptoms, urticaria, or anaphylactic reactions.

The allergy is typically treated by eliminating cow's milk proteins from the diet of allergeic individuals. Formula alternatives include hydrolyzed cow's milk formula, rice-based formula, soy-based formula, and amino acid–based formula, which are all nutritionally adequate alternatives to cow's milk formula (Kipfer and Goldman, 2021). However infants with CMA can also react to alternative formula (Agostoni, et al. 2006). Despite the relevance of the quality of diet for infants on an elimination diet, evidence of dietary nutrient deficiencies, growth deficits, and low food intake are reported in patients subjected to cow's milk-free diet (Meyer, 2018; Vieira et al., 2010). Therefore, milk processing strategies to inactivate allergic proteins by preserving nutritional value of milk are a current challenge (Jaiswal and Worku, 2022).

In this context this paper details main proteins in cow milk and related molecular properties at the basis of functioning properties in food processing; caseins are then deeply discussed for their allergenicity in CMA. Milk processing strategies to mitigate casein allergenicity and CMA prevention, management and therapeutic approaches are also presented.

2. Main molecular properties of caseins

Casein (CN) subunits are classified into 4 types: α_{s1} (40% of the total casein in bovine milk), α_{s2} (10%), β (35%), and κ (15%). Molecular weight ranges from 20 to 30 kDa depending on the subunit (Huppertz, 2013; Huppertz et al., 2018).

α_{s1}-CN contains 199 amino acids and has a molecular mass of *ca*. 23.0 kDa prior to phosphorylation, which increases to ~ 23.6 kDa as a result of the phosphorylation of 8 Ser residues. Based on its primary sequence, expected isoelectric point (pI) is 4.9, but this decreases by *ca*. 0.5 pH units on phosphorylation of the 8 Ser residues. α_{s1}-CN is a moderately hydrophobic protein. Estimated percentage of α-helix and β-sheets in αs1-casein ranged from 5 to 20% and 17 to 40% (Huppertz, 2013; Huppertz et al., 2018).

α_{s2}-CN contains 207 amino acids resulting in a molar mass of *ca*. 24.3 kDa for the non-phosphorylated protein and 25.2 kDa for the variant containing 11 phosphorylated Ser residues. Non-phosphorylated protein has a pI of 8.3 but the phosphorylation reduces the pI to 4.9. Protein can be divided into five distinct regions of high charge and low hydrophobicity (residues 1–41 and 42–80), a hydrophobic region with a slight positive charge (residues 81–125), a phospho-peptide analog (residues 126–170) with high negative charge, and a region with high hydrophobicity and strong positive charge (residues 171–207). Several studies report variable percentages of α-helix (up to 54%), β-sheet (approximately 15%) of the secondary structure (Huppertz, 2013; Huppertz et al., 2018). The presence of two Cys residues favour the formation of intra- or intermolecular disulphide bonds with other α_{s2}-casein molecules.

β-CN sequence consists of 209-amino acids corresponding to molecular mass of 23.6 kDa for the non-phosphorylated form and 24.0 kDa following phosphorylation of 5 Ser residues; consequently, pI is estimated at 5.1 and 4.7 for non-phosphorylated and phosphorylated β-CN, respectively. (Huppertz, 2013; Huppertz et al., 2018). β-CN is strongly amphipathic; the *N*-terminus region (residues 1–40) contain only 2 Pro residues and have low hydrophobicity, the middle section (residues 41–135) has moderate hydrophobicity, whereas the C-terminal section, (residues 136–209) contains many of the apolar residues and is characterized by high hydrophobicity. The presence of 7%–25% α-helix structure and 15%–33% β-sheet were reported.

κ-CN is the smallest of the caseins with 169-amino, a theoretical molecular weight of 18.98 Da and a theoretical pI of 5.93. Likewise to other caseins, variable degrees of phosphorylation have also been found for κ-CN (Huppertz, 2013). Both hydrophobicity and charge are distributed unevenly throughout the protein: segment 1–20 shows predominantly hydrophilic behaviour, whereas segment 21–110 contains some strongly hydrophobic patches; segment 110–120 is strongly hydrophilic, whereas the segment 121–169 shows some hydrophilic and hydrophobic areas (Huppertz, 2013). As concern the secondary structure, several studies report that κ-CN may contain 10–20% α-helix, 20–30% β-structure and 15–25% turns (Huppertz, 2013).

3. Functioning properties and applications

The diverse protein caseins have been extensively studied due to their importance in milk, as food additives and as emulsifiers and stabilizers for glue, paint, and other materials (Audic et al., 2003; O'Donoghue and Murphy, 2023). Although the exact structure and nature of casein micelles are still under debate, these proteins lack well-defined secondary and tertiary structure due to large amount of propyl residues (Holt and Sawyer, 1993). This aspect, apparently in contrast with the known relationship between structure and function of a protein, is actually very crucial for casein functional properties basically due to their primary amino acid sequence.

Thus, caseins are considered flexible, unfolded or random-coil peptides capable of creating several intermolecular interactions (Audic et al., 2003; Holt et al., 2013). Depending on the specific product, caseins assume different meso or macro-structures with different functional properties that impact both with perception and with their digestibility, accessibility and/or allergenicity. This concept would also explain the versatility of milk caseins both in dairy products and non-food settings (Foegeding and Davis, 2011; Foegeding, 2015).

Caseins α_{s1}-CN, α_{s2}-CN and β-CN are calcium-binding phosphoproteins as they are involved in trapping of calcium phosphate up to form calcium phosphate nanoclusters. Moreover, the same proteins complexing with calcium phosphate become tighter than globular proteins (Holt et al., 1998). Thus, α_{s1}-CN, α_{s2}-CN and β-CN are sensitive to the Ca2+ ion and precipitate at the concentration present in milk (De Kruif and Holt, 2003). Conversely, κ-CN is insensitive to Ca2+ and if present in sufficient quantity it stabilizes the other caseins by preventing their precipitation (De Kruif and Holt, 2003).

The calcium-sensitive caseins are located within the micellar structure, while κ-CN is located outside with hydrophilic amino acid and glycosylated residues that protruding outwards from the micellar structure as flexible filaments surrounded by the aqueous medium (Walstra model; Walstra and Jenness, 1984). The submicelle structures, held together by phosphoserines complexing calcium

phosphate (CaP), are covered by κ-CN hydrophilic shell allowing the casein micelles to remain in solution, although dependent on the pH-sensitive balance between soluble Ca2+ and that in micelles (Huppertz et al., 2013). Thus, unlike its protein constituents, casein micelle is an orderd structure providing fluidity to casein molecules and solubilize phosphate and calcium and preventing calcium stones in the mammary gland in addition to providing nutritional compounds (Doherty et al., 2003; Holt et al., 2013).

Casein proteins play an important nutritionally role because of their high phosphate content due to which they bind significant quantities of calcium and also are rich in lysine which is an essential amino acid in humans (Fox et al., 2015).

Caseins are also able to form oligomers and polymers after treatment of oxidases (laccases, peroxidase, tyrosinase) in the absence or presence of redox mediators (i.e. ferulic acid or chlorogenic acid; Loi et al., 2020; Selinheimo et al., 2008; Li et al., 2020). Mattinen et al. (2005) reported the production of homopolymers of tyrosine-containing peptides or tyrosine alone with only trace amounts of the mediator cross-linked to the substrate; they also proved that a reactive mediator radical was produced preferentially, and that cross-linking of the substrates occurred by formation of isodityrosine bonds (C–O–C) and to a lesser extent by dityrosine bonds (C–C). The formation of crosslinking also affected digestibility, functional and allergenic properties of caseins (Stanic et al., 2010; Loi et al., 2018).

A function has been demonstrated for each of the caseins. α_{S1}-CN plays an important role in the ability of milk to transport calcium phosphate. It has also been found that one of its peptide (f(64-68)) has radical scavenging activity (Creamer et al., 1981; Kitts and Weiler, 2003). Also, β-CN plays an important role in determining the surface property of casein micelle. Some β-CN peptides f(1-18), f(105-117), f(191-193) act as a macrophage activator an immunomodulators, increasing the phagocytic activity and peroxide release; an other β-CN peptide (f(98-105)) also possesses antioxidant activity (Migliore-Samour and Jolles, 1988; Azuma et al., 1989; Rival et al., 2001).

αs1- and β-casein are also responsible for the stabilization of micelle by preventing aggregation of αs2- and k-casein, respectively (chaperonic activity). This activity has also been demonstrated towards other proteins like bovine serum albumin, whey proteins, β-lactoglobulin, carbonic anhydrase and alcohol dehydrogenase preventing their stress-induced aggregation and forming soluble, high molecular weight complexes (Bhattacharyya and Das, 1999).

Besides, following the oral ingestion of dairy products including milk, fermented milk, cheese, and yoghurt β-casomorphins are formed from β-CN. These peptides, acting as ligands to opioid receptors generating an analgesic effect (Chabance et al., 1998; Woodford, 2021; Thiruvengadam et al., 2021). In 2009 European Food Safety Authority stated that there are no health risks associated with the eptapeptide β-casomorphin-7 (BCM7; EFSA, 2009).

k-CN shows, due to its calcium solubility, play the role of casein micelle stabilization preventing their precipitation in milk. Two peptides k-CN are casoxins and casoplatelin which possess opioid antagonist and platelet aggregation, respectively. This casein is highly sensitive to proteolysis by chymosin and pepsin (Farrell et al., 2004; Delfour et al., 1965) cleaving k-CN at the Phe105-Met106 peptide bond and releasing highly soluble caseinomacropeptide (CMP; with several the carbohydrate residues such as fucose, galactose, N-acetylglucosamine, Nacetyl-galactosamine and N-acetylneuraminic acid) and insoluble para-k-casein. CMP shows, in vitro, several biological

activities, including neutralization of enterotoxin, inhibition of bacterial and viral adhesion to Caco-2 cells, promotion of bifidobacterial growth and modulation of the immune system response (Karimidastjerd and Gulsunoglu-Konuskan, 2021).

α_{s2}-casein which possesses two cysteine residues plays important role in the transport of calcium phosphate. Its anti-microbial peptide casocidin-I has the ability to inhibit growth of *E. coli* and other bacteria (Brignon et al., 1977; López-Expósito et al., 2006).

Phosphopeptides (CaseinoPhosphoPeptides, CPP) are released from casein, following enzymatic hydrolysis of the casein fractions κ-, αs1-, αs2-, and β-CN (2). They are characterized by a consensus sequence of the type SerP-X-SerP/ThrP/Glu/Asp, where X is any amino acid residue except Pro and do not contain phosphorylated tyrosine and histidine residues. A sequence common to the αs1-, αs2-, and βCN moieties is -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-. CPPs are interesting because they survive the gastrointestinal passage and are found in the stomach, duodenum and distal ileum after ingestion of milk. They act as carriers of di- or trivalent mineral cations, increasing their bioavailability for absorption in the small intestine. In fact, it has been demonstrated that CPPs, in particular the peptides f(1-25)4P of β-casein and f(59-79)5P of αs1-casein, increase calcium uptake in differentiated human tumor cells enterocytic (HT-29), in Caco2 cells (8), and in osteoblasts (9). These data have suggested the possibility of using CPPs as vectors of Ca2+ in functional foods to increase their bioavailability in the development of the organism, in bone calcification and in the prevention of osteoporosis (Clare and Swaisgood, 2000, Korhonen et al., 1998; Latham, 1999).

4. Cow's milk allergenicity (CMA)

Cow's milk allergy (CMA) is one of the most common food allergies in infants, and its prevalence has increased over recent years (Flom and Sicherer, 2019; Mousan and Kamat, 2016). CMA is defined as a reproducible adverse reaction to one or more cow's milk (CM) protein and it has a range of clinical manifestations, with variable intensity and time, which can differ from "immediate" to "delayed" reactions, reflecting the different pathogenesis (Giannetti et al., 2021). According to the definitions issued by the European Academy of Allergy and Clinical Immunology and the American National Institute of Allergy and Infectious Diseases, the CMA can be classified based on the underlying immune mechanism into three categories: immunoglobulin E (IgE) mediated (IgE-CMA), non-IgE mediated, and mixed (Boyce et al., 2011). About 60% of CMA are IgE-mediated, although estimates change according to the study population and age (Flom and Sicherer, 2019). The remaining 40% is divided into non IgE-mediated and mixed forms. In **figure 1** are schematized the main factors accounting for a IgE food allergy.

Allergens present in the serum fraction, which represent approximately 20% of total proteins, include α-lactalbumin (Bos d 4) and β-lactoglobulin (Bos d 5), which are the most abundant, and immunoglobulins (Bos d 7), serum albumin (BSA, Bos d 6), and traces of lactoferrin, lysozyme, proteose-peptone, and transferrin. The remaining 80% of milk proteins including casein fraction, was globally indicated according to the official allergens nomenclature as Bos d 8 (http://allergen.org/index.php). Later on, each casein was classified with its own allergen code: αS1-casein, which is the most important as Bos d 9, αS2-casein as Bos d 10 and β-casein as Bos d 11 and κ-casein as Bos d 12.

Most children with milk allergy are sensitized to more than one protein, with a greater variability of symptoms. Typically patients are sensitized to caseins (Bos d 8), β-lactoglobulin (Bos d 5), and α-lactalbumin (Bos d 4), which are the major milk allergens. **Table 1** and **Figure 2** provide a short description of linear epitopes located along the entire length of each casein subunit, the epitopic sequences and the overlapping regions.

4.1. CMA prevalence and diagnosis

CMA usually occurs in the first two years of life and may resolve spontaneously during childhood or adolescence (Giannetti et al., 2021). Currently, there is a significant heterogeneity in the prevalence data of CMA in the majority of papers, ranged from 1.8% to 7.5%, depending on differences in study design or methodology or differences between populations and geographic areas (Savage and Johns, 2015; Giannetti et al., 2021). Moreover, many investigations assessing prevalence relies on self-reports, with the consequent limitations linked to the subjective nature of the data (Savage and Johns, 2015).

CMA diagnosis is based on the combination of clinical history and physical examination by allergy tests such as specific IgE (sIgE) blood assay and skin prick test (SPT), and, when eligible, oral food challenge (OFC). It is important to emphasize that sensitization, i.e., raised sIgE directed against a specific antigen or positive SPT, in the absence of a supporting clinical history, is common in the general population but insufficient for a diagnosis of CMA. A precise evaluation would require a confirmatory oral food challenge (OFC) at predetermined intervals over time, however, such studies are rarely conducted due to their intrinsically reduced feasibility and ethical issues (Savage and Johns, 2015; Arasi et al., 2022).

Despite these limitations during the assessment, a large number of investigation have attempted to estimate the prevalence of CMA. The results of two main contributors to such data by meta analysis were reported in **table 2.** These meta-analyses show that prevalence estimates can be influenced by many factors such as geographic region, source population, age and participation rates, and limitations of diagnosis (Rona et al., 2007, Nwaru et al., 2014).

An important contribution to prevalence studies was the EuroPrevall birth cohort study, published in 2015 (Schoemaker et al., 2015). In this study 12,049 children from across nine European centres from different climatic and cultural regions were studied to generate the first reliable, multinational incidence data on CMA. 9336 (77.5%) were followed up until the age of 2 years. They showed that the incidence of CMA across Europe in the first two years of life was 0.74% (95% CI 0.56–0.97%), ranging from 0.00% to 1.29% across the centres, with most affected infants having IgE-associated CMA (incidence of 0.59%, 95% CI 0.43–0.80). CMA has a good prognosis with two-thirds of affected infants becoming tolerant within one year after diagnosis. This tolerance development differed according to the presence of sIgE to cow's milk: 57% of children with IgE-associated CMA tolerated cow's milk and 100% of children with non-IgE-associated CMA.

Numerous papers have analyzed the possibility of establishing a cutoff for sIgEs and SPTs for CM and its proteins that could predict whether a patient would react to an OFC. Indeed, it has been demonstrated that the greater the food sIgE levels are and the SPT wheal size is, the higher the

chances that the patient will manifest adverse reactions during an OFC (Cuomo et al., 2017). Actually, several studies showed that cutoffs can vary with age (Komata et al., 2007, Nowak-Wegrzyn et al., 2009), with the type of allergen used to perform SPTs (commercial extract vs raw milk) or because of the degree of cooking (Sampson et al., 2014).

Although OFC remains the gold standard for the diagnosis, data show that OFC results are not predictive of the severity of subsequent reactions (Pettersson, 2018) and there is no direct correlation between the eliciting threshold experienced by children during an OFC and the reaction's severity upon accidental exposure (Eigenmann et al., 2021). Serious reactions to the OFC have been described, up to a case of fatal reaction (Arasi et al., 2022). Therefore, predictors of the OFC outcomes and alternative diagnostic tests are under investigation. Among these, the basophil activation test (BAT) has been shown to provide some insights to distinguish patients who are clinically allergic from those who are tolerant albeit sensitized (Santos et al., 2014, Rubio et al., 2011) and potentially to support the risk stratification assessment for the severity of allergic reactions (Santos et al., 2020, Kawahara, 2019). However, routine applications of BAT for clinical use are not yet feasible due to the lack of standardized protocols and large clinical validation studies.

4.2. Casein allergenicity

Casein subunits (S1-casein, α-S2-casein, β-casein and κ-casein) induce different allergic responses (Jaiswal and Worku, 2022). Among these subtypes, α-CN is reported as the most allergenic protein, followed by κ-CN (Natale et al., 2004). The composition and sequence of amino acids determine the higher structure of proteins and it is responsible for the biological function as well as allergenicity of proteins (Zhao et al., 2023). To this regard the advancement in bioinformatics technology allows to resolve the primary structures of many proteins and to predict functional proteins (Zhang et al., 2017), active peptides (Auestad and Layman, 2021; Tondo et al., 2019; Gambacorta et al., 2022), and adverse effects (Wang et al., 2020a). Today, databases with allergenic proteins and their aminoacid sequence responsible for allergenic response are available and costantly updated (e.g., DNASTAR Protean, BepiPred1.0, ABCpred, IEDB, and NetMHCIIpan-4.0 server; Zhao et al., 2023; Sarkar, et al., 2023). To provide an overview of casein allergenicity, epitopes and their reactivity by each casein subunits are briefly discussed in the following sections.

4.2.1 α-S1- casein (Bos d 9)

Among casein subtypes, α-casein fraction is the most allergenic protein, followed by κ-casein (Natale et al., 2004). Indeed, it was found that approximately 50% of serum samples from patients with cow milk allergy react with α-S1-casein (Natale et al., 2004). Since the IgE-biding capacity is strongly dependent on epitope sequence, a number of studies have been tailored to identify the IgE-biding regions of α-S1-casein in humans. Grounding on the base that caseins don't have a rigid tertiarty structure but develop a random coil conformation stabilized by hydrophobic interactions, it should be supposed that caseins show preferentially linear epitope. In 1998 Nakajima-Adachi and co-workers investigated the determinants of IgE, IgG4, and T cells specific for bovine α-S1-casein from patients by using its synthetic peptides and cyanogen bromide–digested fragments. By means of ELISA for epitope mapping, an immunodominant IgE-binding region at the C-terminal (residues 181 to 199) was identified, while sites for anti-αs1-casein IgG4 were found to be located in multiple

regions of α-S1-casein (Nakajima-Adachi et al., 1998). In addition, Spuergin and others (1996), by using a screening approach based on synthetic peptides, identified 3 immunodominant B-cell epitopes of bovine α-S1-casein corresponding to amino acids 19-30, 93-98, and 141-150. Anyway the reactivity of other parts of the proteins was observed when the sera of allergic patients were singly analyzed (Spuergin et al., 1996). In 2001 Chatchatee and others identified 6 major IgE-binding regions suggesting that there is a difference in epitope recognition between patients with persistent and transient cow milk allergy (Chatchatee et al., 2001), while Cong and others in a study dated 2012, identified 4 different epitopic regions of α-S1-casein, with the recognition of the critical residue for IgE-binding (Cong et al., 2002). More recently, Ruiter and others (2006) identified four main regions (amino acid residues 43–66, 73–96, 91–114 and 127–180) in the α-S1-casein molecule immunogenic to T cells, among which the amino acid residues 133–156 spanned the immunodominant part (Ruiter et al., 2006). Interestingly, Yang and others demonstrated that the digestion and transport characteristics of milk proteins, along with the epitope peptides release, strictly depend from the dairy processing, indeed by investigating 3 commercial dairy products, (pasteurized milk, ultra-heat-treated milk and dried skim milk) they found that only 2 peptides of α-S1-casein (AA 84-90 and 125-132) of pasteurized milk and ultra-heat-treated milk persist after gastrointestinal digestion and transportation via an Ussing chamber (Yang et al., 2022).

In the Immuno Epitope Database and Analyisis Resource online platform (IEDB, https://www.iedb.org) all the B cell and T cell epitopes currently identified for a multitude of allergens are listed, and regarding α-S1-casein of cow milk (Bos taurus ID 9913), a total of 245 epitopes able to trigger allergenic reaction to humans were reported. In **figure 2** the sequential linear epitopes located along the entire length of the allergen molecules is shown, while in **table 1** the aminoacid sequence of the sequential epitopes along with additional information, such as the mapped position in the protein sequence (start and end point), the number of epitopes substring or those spanning between two adjacent regions, was displayed. Of 245 IgE-binding epitopes recognized for cow milk α-S1-casein allergen, 212 are specific for B-cell and 43 for T-cell. It was recently demonstrated that the genetic polymorphisms of caseins influence the allergenic potential of some immunodominant epitopes. Indeed, single amino acid substitutions or deletion resulted in a loss or decrease or increase in immunoreactivity in some α S1- and β-casein epitopes according to RepliTop analyis performed using allergenic patients' sera (Lisson et al., 2013). Furthermore, it was observed that goat and water buffaloes milk harbor an allergenic potential due to cross-reactivity between cow and these species proteins, although individual epitopes from goats and water buffaloes showed lower immunoreactivity compared with epitopes from cows, suggesting a reduced allergenic activity of α-S1-casein from goat (Lisson et al., 2013).

4.2.2 α-S2- casein (Bos d 10)

α-S2- casein fraction of cow milk counts for approximately 12.5% of the whole casein proteins and four different genetic variants (A, B, C and D) were identified with each of them showing different structural characteristics (Villa et al., 2018). A sequence similarity of 90.1%, 54% and 58% was observed in α-S2- casein of goat, camel and mare milk, respectively, when compared with cow milk (Zhao et al., 2023). According to Natale and co-workers, the prevalence of sensitization to α-S2-casein in patients with cow milk allergy is 90% (Natale et al., 2004) and four major and six minor

sequential IgE-binding regions were identified on this protein (Busse et al., 2002). The first major region is located in the middle of the protein at amino acids (AA) position 83-100, while the other three major regions are located in the carboxy terminal portion of the protein at AA 143-158, 157-172 and 165-188. Minor IgE-binding regions were also identified at AA 31-44, 43-56, 93-106, 105-114, 117-128, and 191-200 (Busse et al., 2002). Interstingly, the epitopic sequence ^{171}YQKFALPQYL^{180} was recognized as a marker of persistent cow milk allergy (Järvinen et al., 2002). More recently Cerecedo and others (2008) investigated the IgE- and IgG-binding areas of αs1-, αs2-, β-, and κ-caseins and b-lactoglobulin in a population of patients by mean of a peptide microarray–based immunoassay. The authors found 7 peptide sequences of α-S2-casein recognized by more than 75% of patients, six of which never reported in literature before (Cerecedo et al., 2008). Finally, in an interesting study of Yang et co-workers (2022), it was observed that, after gastrointestinal digestion of pasteurized milk and ultra-heat-treated milk and transportation of the release products in Ussing chamber, 1 peptide (AA 25–32) of α-S2-casein still survive, thus demonstration that although processed, daily products retain their allergenicity (Yang et al., 2022). By the IEDB online platform it is possible to retrieve all the epitopic sequences currently identified for α-S2-casein of different species. Regarding cow milk, 68 epitopes were reported and 10 sequential IgE binding sequences approximately covering the whole proteins were found (**figure 2).** In **table 1** all the relevant information about the mapped position of the sequential epitopes along with the epitope substring and overlapping among the different protein regions are also reported. All the sequences listed in IEDB database are recognized as linear B-cell epitopes.

4.2.3 β - casein (Bos d 11)

Among cow milk proteins, β - casein represents the second highest fraction constituting the 34.13% of the total content of caseins. A different situation could be observed for other species where the concentration of β - casein is the first highest among all proteins, such as in human (69%), goat (55%), camel (65%) and mare (79%) (Zhao et al., 2023). A total of thirteen-casein genetic variants have been identified in β - casein of cow milk, including A1, A2, B, C, D, E, F, H1, H2, I and G showing changes in aminoacid sequence that led to different level of phosphorylation (Kaminski et al., 2007; Caroli et al. 2002; Petrat-Melin et al., 2015). The genetic variants of bovine β-casein have drawn a special interest and attention to scientist and dairy consumers due to the potential relationship existing between the β - casein genotype and the health of cow's milk consumers. A1 and A2 represent the most common forms of β - casein in dairy cow breeds, whereas B variant is less common, and A3 and C alleles are rare (Giglioti et al., 2020). The difference between A1 and A2 bovine relies in an amino acid substitution at the 67th position of the protein chain, where it is possible to find an histidine in A1 milk and a proline in A2 milk (Heyman et al., 1988). During human digestion A1 bovine milk proteins was found to release the peptide beta-casomorphin-7 (BCM-7) that has shown to be the primary causative factor for health and digestive disorders associated with A1 milk, such as cow milk protein allergy (Caroli et al., 2009). On the contrary, the presence of a proline residue in A2 β -casein prevent the breakdown of the sequence at position 67 so that another peptide called BCM-9 is generated. Interestingly, no relationship has been found between the presence of A2 β -casein in the milk and cow milk protein allergy (CMPA) or health problems

(Sun et al., 1999). In the light of this the A2 cow milk has emerged and recommended for remedy to cow milk allergy.

A recent investigation of Lisson and others (2014) highlighted that genetic polymorphisms of bovine caseins influence the allergenic potential of some immunodominant epitopes, indeed by peptide microarray-based immunoassay, the authors found variation in IgE binding for peptides AA 103 to 123 and AA 108 to 129 of 3 β-CN variants A1, A2, and B although in some cases the IgE response was patient-dependent (Lisson et al., 2014).

Regarding β - casein allergenicity, on IEDB online platform a total of 126 linear B-cell epitopes are so far reported for bovine. In 2001 Chatchatee and others identified six major and three minor IgE-binding epitopes, as well as eight major and one minor IgG binding regions, on β - casein sequence (Chatchatee et al., 2001). On the other hand, Cerecedo et co-workers (2008) by using peptide microarray–based immunoassay, identified four main sequential epitope, namely ^{136}ESQSLTLTDVENLHLPLPLL^{155}, ^{67}FAQTQSLVYPFPGPIPNSLPQNI^{89}, ^{40}RINKKIEKFQSEEQQQTEDELQDKIH^{65}, and ^{169}TVMFPPQSVLSLSQSKVLPV^{188} which showed differential recognition patterns between patients reactive and tolerant to cow milk allergy (Cerecedo et al., 2008). In **figure 2** the sequential linear epitopes covering the whole protein are listed, while in **table 1** the most relevant information about these epitopes location along with the substring/overlapping epitopes are reported.

4.2.4 κ-casein (Bos d 12)

κ-casein counts for approximately 13% of the cow milk casein fraction and together with α-casein represents the major allergenic subtypes of caseins in cow milk (Natale et al., 2004). It is the only glycosilated casein conitaining galactose, galactosamine and sialic acid and, on the base of the degree of glycosilation, multiple isoform of κ-casein can co-exist in milk (Villa et al., 2018). To date, 11 variants of these proteins have been observed (Fox et al., 2001, Farrell et al., 2004). Regarding its allergenicity, in a first report describing the allergenic epitopes of bovine β- and κ-casein recognized by allergic individuals, eight major IgE-binding epitopes, as well as two major and two minor IgG-binding epitopes, were detected on kappa-casein (Chatchatee et al., 2001). Specifically, by using overlapping synthetic peptides and sera from cow milk allergenic patients, the authors identifed 3 major epitopes recognized by 93% of patients' serum samples, namely ^{9}IRCEKDERFFSDKIAKYI^{26}, ^{21}KIAKYIPIQYLLSRYPSYGLNYY^{44}, and ^{47}KPVALINNQFLPYPYYAKPAAVR^{68}), and 6 epitopes by the majority of older patients (Chatchatee et al., 2001). 2 additional dominant epitopes were identified by Cerecedo and others (2008), namely ^{16}RFFSDKIAKYIPIQYVLSRY^{35} and ^{34}RYPSYGLNYYQQKPVALINN^{53} (Cerecedo et al 2008). Interestingly, Järvinen et co-workers in 2002 observed that there is an IgE-binding epitope of κ-casein, namely ^{155}SPPEINTVQV^{164}, typic of patients with persistent cow milk allergy, indeed no IgE signals were observed when children affected by transient cow milk allergy were tested, thus suggesting the possibility to use this allergenic epitope as a screening instrument for persistent cow milk allergy (Järvinen et al., 2002). Moroever, taking into account that the allergenicity is strictly related to the aminoacid sequence and that any eventual residue substitution of the native residues by others could affect the overall loss/decrease of IgE-binding, Han and others (2008) identified 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.

Indeed, by testing several synthesized peptides representing the IgE-binding epitopes of κ-casein with single AA substitutions at each position, they found that for 10/11 allergenic peptides, one to five different single AA substitutions resulted in elimination of IgE-binding of pooled patient sera (Han et al., 2008). Finally it was reported that cow milk κ-casein show a 61% homology with human milk, with the three most frequently recognized Ig-E epitopes, namely AA 9-26, AA 21-44 and AA 47-68 showing a 53%, 67% and 64% homology, respectively, with human κ-casein. Taking into account that the degree of homology between certain food antigens and human proteins may have some influence on the sensitization and development of allergy to certain food proteins, the high degree of homology between cow and human κ-casein could explain the high allergic potential of these antigenic peptides for human (Chatchatee et al., 2001). By searching on the IEDB on-line platform, a total of 125 B-cell epitopes are currently listed and as observed for other casein proteins, approximately all the protein sequence retains sequential epitopes (**figure 2**). In **table 1** all the information about the sequential epitopic peptides and other relevant information are reported.

5. Milk processing strategies to mitigate casein allergenicity

Allergenicity reduction is currently attracting the interest of the reasearch activity in order to produce foods with a decreased allergenic potential that should undergo appropriate clinical assays (e.g. oral food challenge tests) before claiming to be safe for allergic individuals.

The processing applied to mitigate allergenic potential is divided in two subcategories: thermal and non-thermal treatments, depending if heating (moist or dry) is applied or not (Shriver and Yang, 2011). Thermal processing can be effective on the food protein but may also impact the sensory and nutritional value of the food. Growing consumer demands for minimally processed and fresh-tasting foods have therefore driven the rapid development of novel non-thermal food processing technologies (Dong et al., 2021). **Figure 4** illustrated the potential paths/ mechanisms of attenuation of food allergenicity by means of thermal and non-thermal methods. Non-thermal processing technologies such as cold atmospheric pressure plasma, pulsed ultraviolet light, gamma-irradiation or high pressure, which can induce changes in proteins and potentially mitigate allergenicity while retaining the organoleptic properties of food, are currently being investigated (Ekezie et al., 2018). Some of them (such as HPP, short-wavelength electromagnetic gamma (g)-irradiation) also turned out to be an effective preservation method, extending the shelf-life of perishable foods (Kasera et al., 2012).

5.1 Heat based treatments

Depending on whether water is used to convey heating, thermal food preparation methods could be split in two sub-groups, “moist heat treatments” and “dry heat treatments” with pasteurization and sterilization being the two temperature ranges generally adopted for these treatments. Considering the impact of thermal treatments on the native structure of proteins, the effects of these methods on the finally allergenicity of foods could be very different, varying from a mitigation of allergenicity to a significant increase of it (Cabanillas and Novak, 2019). As known, proteins have a native structure which stability depends on hydrogen bonds, disulphide bonds, electrostatic, and

hydrophobic interactions (Davis and Williams, 1998). When the heat energy is applied, the molecular bonds that hold the allergen protein structures together tends to modify themselves thus the proteins slowly starts to lose its native structure.

Taking into account that conformational epitopes are associated to secondary protein structure, it is reasonable to assume that they are more labile to this processing methods than linear epitopes and, therefore, more likely to be disrupted under harsh conditions (Cabanillas and Novak, 2019). In the light of this, if a reduction in the final allergenicity of food could be expected after thermal processing (masking or destruction of epitopes), in practice controversial results were reported on the use of heating to alter the immunological potential of some allergens. Indeed, along with the numerous studies proving the efficacy of thermal processing to reduce the allergenicity of foods (reviewed by Cabanillas and Novak, 2019; Vanga et al., 2017), there are some investigations where an increase in the IgE reactivity of a certain proteins (exposure of epitopes or generation of new ones) or no change in it (allergen stability) were observed (Van der Ventel et al., 2011; Carnés et al., 2007; Abramovitch et al., 2013; Pastorello et al., 2010).

The effect of thermal processes on the final allergenicity of food depend on the temperature, type, and duration of the treatment, as well as of the intrinsic characteristics of the protein and of the physicochemical conditions of its microenvironment (Mills et al., 2009; Wal, 2003).

Several studies demonstrated that both the heating and the food matrix e.g. in the baking process could result in a reduction of allergenic potential of milk containing foods demonstrating to be tolerated by patients allergic to milk (Leonard et al., 2016, Bavaro et al., 2019). As reported by Leonard et al. (2016) in baked form, cow's milk isless allergenic and is tolerated by most milk-allergic children. Not only, may including baked milk in the diets of children who are tolerant improve nutrition and promote more social inclusion but there is also evidence that inclusion may accelerate the resolution of unheated milk and egg allergy. In a recent study it has been reported that milk baked within the muffin matrix might promote formation of complexes with food components inducing a modulation of the immunoreactivity towards milk allergens compared to milk baked in the oven at 180 °C for ten minutes. The interactions between milk proteins and some components of the food matrix during heating seemed to play a role in the possible reduction of allergenicity as assessed by in vitro tests (Bavaro et al., 2019).

5.2 Non heat treatments

Among the non heating treatments the following will be presented: 1. High Hydrostatic Pressure High hydrostatic pressure (HHP), 2. Cold Atmospheric Plasma (CAP), 3. Food Irradiation (FI), 4. Ultrasound treatment (UT), 5. Enzymatic Hydrolysis (EH)

High Hydrostatic Pressure High hydrostatic pressure (HHP) processing has shown enormous potential in the application to the food industry to inactivate spoilage microorganisms and enzymes while preserving sensorial properties and nutritional values of foods (Zhu et al., 2022; Aganovic et al., 2021). HHP typically employs pressures ranging from 100 to 800 MPa at moderate or elevated temperatures. Water is used as the pressure transfer medium, and the pressure is transmitted instantaneously and uniformly throughout the food system regardless of its size and shape. Since the first discovery in 1914 (Bridgman, 1914) of coagulation phenomena occurring on egg albumen submitted to HHP, high pressure has been applied as a robust tool for probing changes in the

structure and properties of proteins, in the fields of biophysics, chemistry, microbiology, and food technology (Meersman and McMillan, 2014; Teixeira et al., 2014). It is likely that under pressure treatments, protein chains will unfold and oligomeric proteins will disassociate (Le Vay et al., 2020). Upon decompression, the altered protein structure may not recover as a result protein properties are modified (Roche and Royer, 2018). HHP applied to allergenic food ingredients can induce changes of the noncovalent bonds and for this reason it has been recently investigated as a technique capable of reducing the allergenic potential of different foods (Cuadrado et al., 2020; Huang et al., 2014; Pan et al., 2020). Allergenicity reduction induced by HHP can be due to several reasons through application of high pressure: (1) increase the penetration of surrounding solution into food when the food undergoes the HHP immersed into a liquid medium through the extraction of allergens contained in food; (2) irreversible or reversible modification of protein structures caused by HHP that can also give rise to the gelatinization, aggregation, or denaturation of proteins (Han et al., 2018; Bavaro et al., 2018; De Angelis et al., 2018, 2022). The aggregation of proteins may mask or destroy the critical epitopes, often causing the reduction of allergenicity, although in some cases can also promote production of neo-epitopes. Finally, HHP might cause conformational changes of allergic protein then affecting the overall allergenicity. It should be underlined that HHP only affects the weak bonds including hydrogen, ionic, and hydrophobic bonds thus exerting an influence on the tertiary and quaternary structures maintained by noncovalent interaction. Such changes in the structures might determine changes in the allergenicity of allergenic proteins

5.3 Cold atmospheric plasma (CAP)

Cold atmospheric plasma (CAP) has been used in the past mainly for sterilization of sensitive materials and more recently also extended to food industries as a novel processing technology offering increased safety profiles and extended shelf life for food products (Thirumdas et al., 2015). It is considered as modern non-conventional technique used in the preservation of food and for the preparation of modified starches, altering its physical and chemical properties. Its role is more prevalent for application in microbiology as an alternative microbial inactivation technology due to its germicidal effects while maintaining quality of fresh products (Thirumdas et al., 2015; Schlüter et al., 2013). Given the technology's multimodal action it has the potential to reduce allergens in foods, although data on the efficacy and mechanisms of action are not exhaustive. An hypothesis for its mechanism of action is the ability of CAP to promote reactions in liquids by injecting reactive oxygen radicals, altering the epitope structure. As for milk allergens, Tammineedi et al. (2013) used cold plasma to treat casein, b-Lg and a-La founding no significant difference in the IgE binding values between the control and treated samples. Conceivably, the poor plasma efficiency associated with remote plasmas might be responsible for the absence of attenuation in immunoreactivity (Tammineedi et al., 2013). On the contrary, in a recent study, casein, β-lactoglobulin and α-lactalbumin analyzed before and after plasma treatment revealed alterations in the secondary structure of the proteins with consequent decrease of antigenicity of casein and β-lactalbumin, whereas α-lactoglobulin showed increased antigenicity (Ng et al., 2021).

5.4 Food Irradiation (FI)

572 Recent studies about food irradiation have demonstrated the reduction of allergenicity of foods
573 including shellfish, soy, peanut, milk, tree nut, egg, wheat and fish. Principles of food irradiation,
574 including mechanisms of allergenicity-reduction, irradiation types and characteristics have been
575 addressed and final conclusions that food irradiation is a safety tool to reduce the allergenicity of
576 food effectively, with high nutritional value and long shelf-life, making it a competitive alternative
577 technology to traditional techniques such as heating treatments (Pi et al., 2021). Irradiation was
578 found to improve the quality of milk, although modifications in proteins structure could occur. For
579 example, changes in the secondary and tertiary structure were observed in β-lactoglobulin (in
580 solution) subjected to γ-irradiation with consequent protein aggregation (De La Hoz and Netto,
581 2008). Moreover, a reduction in milk allergenicity due the destruction of human IgE-binding
582 epitopes was reported in treated milk, although the final results were dependent on the dose of
583 irradiation (Lee et al., 2001)

584

585 *5.5 Ultrasound Treatment (UT)*

586 To date, the effect of ultrasound treatment on the allergenicity of several foods has not been
587 extensively investigated. The high mechanical energy produced by high-intensity ultrasound causes
588 a cavitation effect, which results in the destruction of the protein spatial structure with an increase
589 of solubility and emulsifying properties and in the case of a reconstituted milk protein concentrate
590 (Sun et al., 2014). Given the connection between protein structure and allergenicity, it can be
591 inferred that ultrasound treatment could be effective in producing hypoallergenic cow's milk. In
592 another study reported by Wang et al. (2020b), casein (CN) proteins were purified from fresh milk
593 and the effect of ultrasound on the allergenicity of CN was investigated (Wang et al., 2020b). After
594 treatment, the authors demonstrated that IgE-binding capacity of CN was decreased, indicating that
595 the conformational epitopes were destructed. It was suggested that ultrasound treatment not only
596 destructed the epitopes but also decreased the oligomerization level of CN and, therefore,
597 significantly impaired the allergenicity of CN. This paves the way for optimizing a novel method for
598 the production of food with a decreased allergenicity. Similarly, Stanic-Vucinic and colleagues have
599 demonstrated that, when β-lactoglobulin was exposed to high-intensity ultrasound, a
600 conformational change occurred where the β-sheet converted to α-helix (Stanic-Vucinic et al.,
601 2012). However, no remarkable alterations in the sera IgE-binding reaction, basophil activation test,
602 and skin prick test of patients were observed after these conformational changes

603

604 *5.6. Enzymatic treatment*

605 Proteolysis offers an efficient way to destroy allergenic epitopes. To reduce their allergenicity,
606 proteins can be broken down by enzyme hydrolysis into small peptide molecules and amino acids.
607 Some food-grade proteinases have been used to manufacture whey protein hydrolysates with
608 reduced antigenicity (Quintieri et al., 2017). In the process of hydrolysis, the differences in the types
609 of enzyme, hydrolysis model and the degree of hydrolysis may result in some discrepancies in
610 peptide composition and residual antigenicity of the hydrolysate; this occurres due to the non-
611 specific hydrolysis of the epitopes by the proteases. Thus, new method to screen the efficacy of
612 proteases based on the detection of residual allergenic epitopes are currently investigated (Liu, et

al., 2022). Recently, sequential enzymatic hydrolysis catalysed by chymosin and papain with IgG-binding inhibition was effective in reducing αs1-CN-antigenicity; this innovative approach investigating enzymatic hydrolysis for reducing CN-antigenicity based on CN structure was suggested promising to develop hypoallergenic CN hydrolysed infant formulas (Zeng et al., 2023).

Also fermentation induced by microrganisms such as *Lactobacillus spp. (LAB)* have been considered to exhibit proteolytic activity toward milk proteins. During fermentation, proteases and peptidases produced by LAB cleave milk proteins into peptides and amino acids. This proteolysis potentially breaks some epitopes and consequently decreases the antigenicity and allergenicity of milk proteins (Chobert, 2012, Meng et al., 2021, Yao et al., 2014). In particular, *L. plantarum* reduce cow's milk protein allergenicity through the combination of cell-envelope proteinase and peptidase on α-CN. *L. helveticus* is well-recognized for its high proteolytic activity and ability of releasing bioactive peptides during milk fermentation (Gandhi and Shah, 2014). Besides, *L. helveticus* has proved to effectively hydrolyze αs1-casein and β-casein into peptides, and decreased their IgE binding ability (Ahmadova et al., 2011).

Synergistic effects of *Streptomyces* aminopeptidases belonging to the M1, M24, and M28 families on the degradation of the allergen peptides were also demonstrated; in particolar, the combination of M1 and M24 aminopeptidases were effective to hydrolyze VLPVPQK and FFVAPFPEVFGK, from bovine casein-derived allergen peptides (Wan et al., 2019).

Enzymatic crosslinking has the potential to alter the primary, secondary, and tertiary structure of allergens by modification of specific amino acid residues and the formation of high molecular weight polymerized allergens. Studies have shown that enzymatic crosslinking caused the loss of secondary structure of food allergens and unfolded the tertiary structure (Ahmed et al., 2020; Fei et al., 2016). Modification of the secondary and tertiary structures of food allergens are the main factors involved in the reduction of food allergenicity due to the reduced binding propensities to their specific IgE antibodies (Ahmed et al., 2018). Enzymatic crosslinking of caseins has been also shown to affect the allergenic properties has been reported (Stanic et al., 2010). Stanic et al., (2010) investigated in vitro the residual allergenicity of β-CN crosslinked by transglutaminase, tyrosinase, mushroom tyrosinase/caffeic acid and laccase/caffeic acid. Among the assayed peptides, laccase/caffeic acid and mushroom tyrosinase/caffeic acid had the highest potential in mitigating IgE binding and allergenicity of the β-CN out of all investigated enzymes.

Few researches has been published on the impact of different crosslinking enzymes on milk allergenicity (Ahmed et al., 2021).; most reported studies include whey proteins and, however, more clinical trials need to be still performed.

6. CMA prevention, management and therapeutic approaches

Family history of allergy-associated diseases is the most important risk factor for allergy manifestations in the offspring. Primary prevention strategies could be applied in high-risk infants defined as those with a first-degree relative with a history of allergy (Trogen et al., 2022).

Primary prevention of CMA should start from pre-pregnancy with a focus on a healthy lifestyle and food diversity to ensure adequate transfer of inhibitory IgG- allergen immune complexes across the placenta especially in mothers with a history of allergic diseases and planned c-section delivery. For non-breastfed infants, there is controversy about the preventive role of partially hydrolysed

formulae (pHF) despite some evidence of health economic benefits among those with a family history of allergy.

Clinical management of CMA consists of secondary prevention with a focus on the development of early oral tolerance. Preventing disease progression from mild or moderate symptoms to severe symptoms or another allergy phenotype in children with CMA is considered as secondary prevention and management of CMA

The use of extensive Hydrolysate Formulae (eHF) is the nutrition of choice for the majority of non-breastfed infants with CMA as summarized in **Figure 4**; potentially with pre-, probiotics and LCPUFA to support early oral tolerance induction. Future opportunities are, among others, pre- and probiotics supplementation for mothers and high-risk infants for the primary prevention of CMA. A controlled prospective study implementing a step-down milk formulae ladder with various degrees of hydrolysate is proposed for food challenges and early development of oral tolerance. This provides a more precise gradation of milk protein exposure than those currently recommended.

The standard treatment of CMA relies on CM avoidance. Breastfeeding is the best source of nutrition for nearly all infants and at all times it should be supported in children with CMA. Breastmilk contains a series of inimitable molecules with potential immune-modulating activities, including the establishment of gut microbiota, the prevention of overweight and obesity, the development of immunoallergic parameters and the neural development (Agostoni et al., 2009).

Breastfeeding is the first choice for infants with CMA. However, the suggestion of CM elimination diet for mothers breastfeeding allergic infants is a peculiar issue that needs to be confirmed in an evidence-based manner and evaluated on individual basis.

If breastmilk is unavailable, it is required a hypoallergenic formula, i.e. an industrially produced substitute highly controlled for nutritional content and tolerance in CMA infants. In the past 10 years, formulas in the CMA management have been profoundly expanded, with extensively hydrolysed formulas (eHFs) (Strozyk et al., 2020), rice hydrolyzed formula (HRF) (Vandenplas et al., 2014), amino acid formulae (AAF) (Fierro et al., 2020), camel and dromedary milk (Navarrete-Rodriguez et al., 2018), and donkey milk (Monti et al., 2012).

Choosing the most appropriate formula for each infant should be based on clinical presentation, nutritional composition, and residual allergenicity of the proposed hypoallergenic formula.

EHFs and AAFs are valid alternatives. Heed is needed to ensure the formula is nutritionally sufficient.

CM-OIT and/or omalizumab may be effective by itself/themselves in improving the threshold of reactivity to CM. There is currently stronger evidence for CM-OIT because of paucity of data on omalizumab in FA but the current evidence is promising. In particular, omalizumab may be considered if allergy to several food sources and concomitant allergic diseases.

After several decades of ‘passive clinical managements’, based only on avoidance of CM products and the use of epinephrine in the event of anaphylaxis, there has been a switch to active treatment. The most recent evidence-practice guidelines strongly recommend the use of CM-OIT as an effective therapeutic option in persistent CMA (Pajno et al., 2018).

CM-OIT consists of providing gradually increasing doses of CM (up-dosing phase) up to a maintenance dose that will be continued (maintenance phase) with the goal of inducing desensitization (i.e. increasing the threshold of reactivity while in treatment) (Wood, 2017). Several protocols have been evaluated, with the most notable examples being the weekly (Pajno et al.,

2018), or slow up-dosing regimens (Kaneko et al., 2010), the rapid oral desensitization combined with omalizumab (different maintenance feeding regimens (Nadeau et al., 2011; Pajno et al. 2013) and baked CM-OIT (Dantzer et al. 2022; Goldberg, et al. 2015; Kim et al., 2011). Notwithstanding the heterogeneity among studies, children with IgE-CMA seem to tolerate significantly more CM while on therapy (Dang et al., 2016; Nurmatov et al., 2017; de Silva et al., 2022).

Conclusions and future perspectives

Caseins represent the main protein fraction of cow's milk. They have binding capacity, stabilizing and emulsification activities, gelation and water binding capacity making them good candidates as delivery systems for food and pharmaceutical applications.

Despite their valuable properties, caseins are also the major responsible of cow's milk allergy especially in children. Strategies for allergenicity reduction have been put in place in order to produce a final product that can be tolerated by allergic consumers. Baked milk demonstrated to be well tolerated by allergics to be included in the diet of a majority of milk-allergic individuals/children; more research is required to identify new thresholds for this category of patients by planning oral food challenges.

More efforts, in perspective, should also be directed in identifying clinical factors and biomarkers for predicting baked milk tolerability or reactivity as there is a lack of knowledge in this area and more needs to be done. Also, to avoid exposing allergic patients to oral food challenge studies, the use of alternative and less invasive approaches e.g. prick by prick test and allergen specific IgE assays should be also sought for, as new diagnostic predicting tools.

In addition, thanks to the advanced bioinformatics tools and proteomic platforms so far available, further research is needed for the identification of new antigenic determinants spread along casein moyeties and for the characterization of the neoantigens that might be formed upon processing in the allergen containing food.

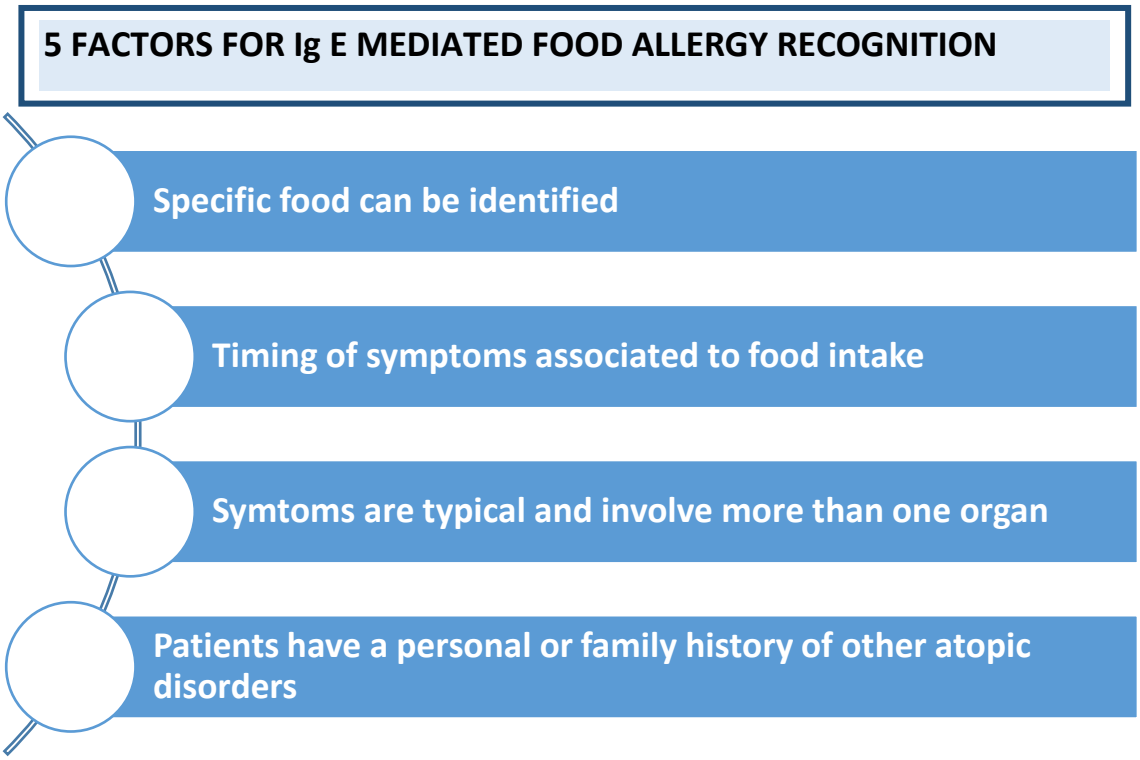


Figure 1. Main factors accounting for IgE mediated food allergy.

Alpha S1 casein bos taurus – Bos d 8/9

>sp|P02662|CASA1_BOVIN Alpha-S1-casein OS=Bos taurus OX=9913 GN=CSN1S1 PE=1 SV=2
MKLLILTCLVAVALARPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTED
QAMEDIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAE
ERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIP
NPIGSENSEKTTMPLW

Alpha S2 casein bos taurus – Bos d 10

>sp|P02663|CASA2_BOVIN Alpha-S2-casein OS=Bos taurus OX=9913 GN=CSN1S2 PE=1 SV=2
MKFFIFTCLLAVALAKNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEE
YSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAV
PITPTLNREQLSTSEENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTV
YQHQKAMKPWIQPKTKVIPYVRYL

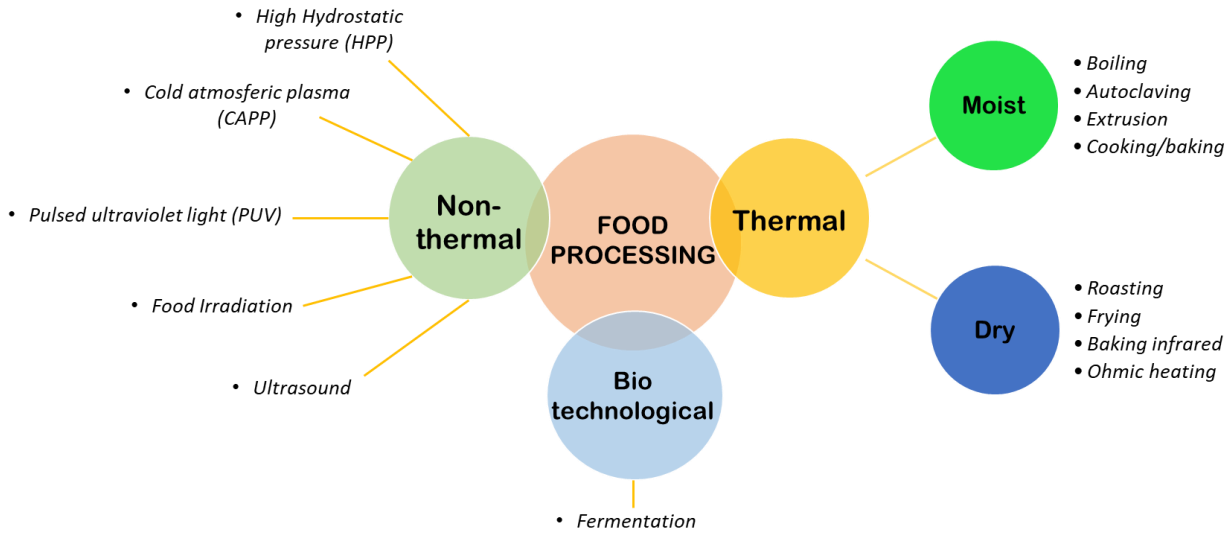
Beta casein – Bos d11

>sp|P02666|CASB_BOVIN Beta-casein OS=Bos taurus OX=9913 GN=CSN2 PE=1 SV=2
MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHP
FAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVE
PFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQR
DMPIQAFLLYQEPVLGPVRGPFPIIV

Kappa- casein – Bos d12

>sp|P02668|CASK_BOVIN Kappa-casein OS=Bos taurus OX=9913 GN=CSN3 PE=1 SV=1
MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQ
KPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPK
KNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV

Figure 2. Linear epitopes located along the entire length of each casein subunit.



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769

770 **Figure 3.** Imported from Monaci, et al., 2023. (Bio)technological Approaches for Reducing
771 Allergenicity of Food Ingredients. In: Ferranti, P. (Ed.), Sustainable Food Science: A Comprehensive
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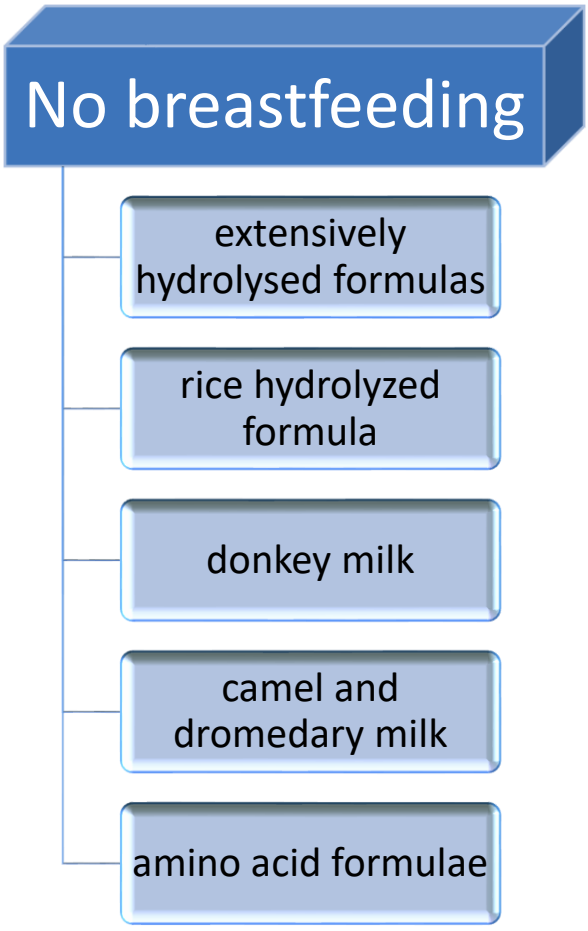

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776 **Figure 4.** Alternative hypoallergenic formula for infants in case of unavailability to breastfeeding

Antigen	Uniprot ID	Regions	Epitopic sequence	Mapped start-end position (aa)	# epitopes substring	# epitopes overlapping two consecutive regions	Overlapping regions
α S-1 casein	P02662	1	RPKHPIKHQGLPQEVLNENLLRFFVAPFPEV	16-46	20	22	1-2
		2	FGKEKVNELSKDIGSESTEDQAMEDIKQMEAES	47-79	17	15	2-3
		3	ISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLR	80-115	20	16	3-4
		4	LKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKE	116-148	20	19	4-5
		5	PMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYV	149-182	25	23	5-6
		6	PLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	183-214	36	\	\
α S-2 casein	P02663	1	KNTMEHVSSSEESIISQETY	16-35	\	1	1-2
		2	QEKNMAINPSKENLCSTFCK	37-56	4	5	2-3
		3	VVRNANEEEY	58-67	\	3	3-4
		4	SEESAEVATEEVKITVDDKH	73-92	\	5	4-5
		5	QKALNEINQFYQKFPQYLQY	94-113	4	9	5-6
		6	YQGPIVLNPWDQVKRNAVPI	115-134	1	6	6-5
		7	TLNREQLSTSEENSKKTVDM	137-156	\	\	\
		8	STEVFTKKTK	158-167	\	4	8-9
		9	EKNRLNFLKKISQRYQKFALPQYLKT	172-197	10	5	9-10
		10	MKPWIQPKTKVIPYVRYL	205-222	1	\	\
β-casein	P02666	1	RELEELNVPGEIVESLSSSE	16-29	5	9	1-2
		2	ESITRINKKI	36-454	\	5	2-3
		3	QSEEQQQTEDELQDKIHPFA	49-68	4	12	3-4
		4	TQSLVYPFPG	70-79	\	7	4-5
		5	PNSLPQNIPP	82-91	\	4	5-6
		6	QTPVVVPPFLQPEVMGVSKV	94-113	\	5	6-7
		7	EAMAPKHKEMPFPKYPVEPF	115-134	3	16	7-8
		8	ESQSLTLTDVENLHLPLPLL	136-155	3	6	8-9
		9	QPLPPTVMFPPQ	164-175	2	5	9-10
		10	SVLSLSQSKV	176-185	\	7	10-11
		11	LPVPQKAVPY	186-195	\	7	11-12
		12	PQRDMPIQAFLLYQEPVLGP	196-215	7	7	12-end
κ-casein	P02668	1	QEQNQEQPIRCEKDERFFSD	22-41	6	11	1-2
		2	KIAKYIPIQY	42-51	\	10	2-3
		3	VLSRYPSYGLNYYQQKPVAL	52-71	5	8	3-4
		4	INNQFLPYPY	72-81	\	8	4-5
		5	YAKPAAVRSPAQILQWQVLS	82-101	7	6	5-6
		6	CQAQPTTMARHPHPHLSFMA	109-128	5	14	6-7
		7	PPKKNQDKTEIPTINTIASG	130-149	8	7	7-8
		8	EPTSTPTTEA	150-159	\	8	8-9
		9	VESTVATLED	160-169	\	6	9-10
		10	SPEVIESPPE	170-179	\	6	10-end

Table 1. Allergenic epitopes and overlapping regions in the casein subunits.

Reference	Pubblication years (N° of papers included in the meta-analysis	Prevalence of CMA				
		Self-reported	SPT alone	sIgE assay alone	symptoms and sensitization (SPT 3 mm or sIgE > 0.35 kU/L)	OFC
Rona et al. (2007)	1990-2005 (51 papers)	1.2%-17%.	0.2%-2.5%	2%-9%,	0%-2%	0%-3%
Nwaru et al. (2014)	2000-2012 (42 papers)	2.3% (95% CI 2.1–2.5),	0.3% (95% CI 0.03–0.6)	4.7% (95% CI 4.2–5.1).		0.6% (95% CI 0.5–0.8)

Table 2. Meta-analysis results for estimating the prevalence of CMA.

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