

Whey allergens: Influence of nonthermal processing treatments and their detection methods

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Abstract

Whey and its components are recognized as value-added ingredients in infant formulas, beverages, sports nutritious foods, and other food products. Whey offers opportunities for the food industrial sector to develop functional foods with potential health benefits due to its unique physiological and functional attributes. Despite all the above importance, the consumption of whey protein (WP) can trigger hypersensitive reactions and is a constant threat for sensitive individuals. Although avoiding such food products is the most successful approach, there is still a chance of incorrect labeling and cross-contamination during food processing. As whey allergens in food products are cross-reactive, the phenomenon of homologous milk proteins of various species may escalate to a more serious problem. In this review, nonthermal processing technologies used to prevent and eliminate WP allergies are presented and discussed in detail. These processing technologies can either enhance or mitigate the impact of potential allergenicity. Therefore, the development of highly precise analytical technologies to detect and quantify the existence of whey allergens is of considerable importance. The present review is an attempt to cover all the updated approaches used for the detection of whey allergens in processed food products. Immunological and DNA-based assays are generally used for detecting allergenic proteins in processed food products. In addition, mass spectrometry is also employed as a preliminary technique for detection. We also highlighted the latest improvements in allergen detection toward biosensing strategies particularly immunosensors and aptasensors.

KEYWORDS

detection, nonthermal processing treatments, WP allergens, WP composition

1 | INTRODUCTION

Whey proteins (WPs) are used in diverse food products and are recognized as a source of various biologically active components with distinctive functional (foaming, gelling, and emulsifying) and physiological attributes that provides

opportunities for the development of functional foods with potential health benefits. Whey is the by-product of milk upon coagulation during the cheese production process (Mollea et al., 2013). Contrary to that, WPs are involved in causing allergy due to the presence of allergenic proteins, such as α -lactalbumin (α -La) and β -lactoglobulin

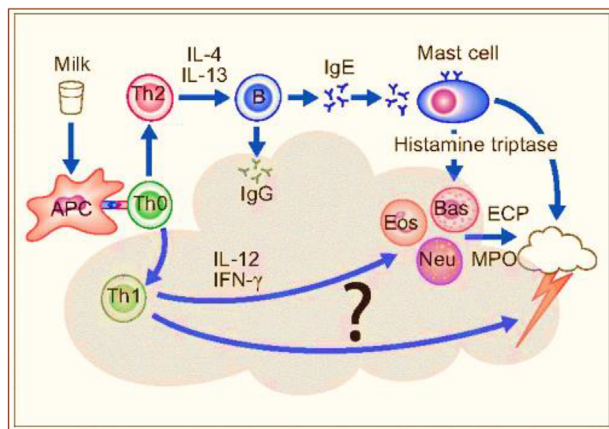


FIGURE 1 Mechanisms of IgE-mediated allergic reaction

(β -Lg), which have several epitopes with affinity to bind specific immunoglobulin E (IgE) from milk-allergic patients. β -Lg and α -La are the most allergenic proteins in the whey fraction (Hochwallner et al., 2010). Allergenic proteins in whey cause IgE-mediated adverse reactions upon binding with allergen-specific antibodies on mast cells, eliciting relatively mild to severe life-threatening allergic reactions (Boyce et al., 2011). Milk allergy is a major public health concern that affects both adults and children, affecting an estimated percentage of 1.9%–4.9% of infants globally (Meyer et al., 2018), 0.6%–2.5% of preschool children, 0.3% of 5–16 years old children, and less than 0.5% of adults (Ramos et al., 2017). The mechanism of IgE-mediated anaphylaxis reaction is illustrated in Figure 1.

Antigenic proteins are primarily characterized by glycoproteins with a molecular mass of 10–70 kDa, which have protein sites called epitopes, which are easily recognizable and bind with allergen receptors. Taking into consideration the structure and molecular configuration, any process that elicits changes in protein conformation can also affect the structural characteristics of epitopes and thus influence the binding ability of antigens. In particular, some processing treatments such as heat processing can induce the development of new allergenic compounds, often referred to as neoallergens, through interactions between constituents (Kumar et al., 2012).

Conventionally, food products are typically processed for sensory, nutritional, preservation, and detoxification purposes. Generally, thermal approaches are employed to attain such targets. The temperature rise progressively declines the native structure of proteins. It can have a positive, negative, or sometimes even no effect on allergenicity at all. Heat treatment generally modifies protein through denaturation, disulfide bond restructuring, and new intra- and intermolecular bonds generation (Ekezie et al., 2018). The thermal treatments may

affect the physicochemical characteristics and nutrient balance of food products, such as protein denaturation, caramelization resulting from sugar dehydration, and inhibition of enzymes (Meda, Orsat, & Raghavan, 2017). In the case of milk, WPs are more susceptible to heat treatment unlike caseins; therefore, conventional techniques have often presented difficulties in detecting whey allergens accurately, especially from highly processed foods (Ramachandran, Yang and Downs, 2020). In contrast, non-thermal processing has shown numerous advantages over thermal processing, as these methods preserve the sensory properties and enhance the nutritional properties along with the mitigation of immunoreactivity (Meinlschmidt et al., 2017).

Nonthermal processing techniques are innovative approaches that use the mechanism other than conventional heating. Nonthermal processing corresponds to the systems that are comparatively novel and utilize mechanisms with less energy and maintain superior quality attributes in comparison with those involved in conventional processing. These nonthermal treatments can affect protein surface hydrophobicity by inducing conformational changes such as unfolding, formation of hydrophilic and hydrophobic groups, breaking disulfide bonds, and aggregation (Esteghlal et al., 2019). Several nonthermal processing technologies have been suggested to lessen the severity of conventional thermal treatments on food matrices such as physicochemical composition, taste, color changes, and so forth. High-pressure processing (HPP), pulsed electric field (PEF), microwave irradiation (MWI), ultrasonication, cold plasma (CP), electrohydrodynamics (EHD), fermentation, and enzymatic hydrolysis are the most investigated nonthermal food processing methods, with some being already industrialized (Shao, Zhang, Zhu, Liu, & Tu, 2020; Yang et al., 2020; Kurpiewska et al., 2019; Misra et al., 2018). Researchers, therefore, examined the influence of nonthermal processing techniques on the allergenic properties of proteins and the possibility of developing these methods to produce hypoallergenic foods.

To date, milk allergy has no proper treatment; therefore, the most useful control strategy for allergic patients is to avoid allergenic foods once diagnosed (Bu, Luo, Chen, Liu and Zhu, 2013). Meanwhile, the complete exclusion of milk proteins (MPs) and their products is extremely difficult to achieve and may cause many nutritional deficiencies with adverse effects on infant and child growth (Mousallem & Burks, 2012). Therefore, an effective and authentic labeling system is obligatory to ensure the safety and life quality of sensitized individuals (Shoji, Adachi, & Akiyama, 2018). Legal measurements have been taken by a majority of the countries to enforce laws related to food products. Despite mislabeling, cross-contamination

incidences may occur during food manufacturing, resulting in an accidental occurrence of hidden allergens. A minor amount of allergenic food can cause allergenicity, so the method for detection and quantification of allergens should be sensitive enough to detect trace amounts (Taylor & Baumert, 2015). Therefore, industrial management is intended to follow appropriate, reliable, and highly sensitive analytical techniques and encourage the authorities to control/monitor allergens.

Currently, several analytical approaches based on protein or DNA and mass spectrometry (MS) tools have been established to detect and quantify food allergens to ensure proper labeling of food items and to protect susceptible individuals. Conventional protein-based analysis techniques that rely on antigen–antibody interactions include primarily enzyme-linked immunosorbent assays (ELISA), dipstick, and lateral flow devices (LFDs), whereas DNA-based methodologies include real-time polymerase chain reaction (PCR), ligation-dependent probe amplification (LPA), and genosensors/microarrays. Furthermore, it is important to note that numerous processing conditions may substantially change the allergen's physical and structural properties, thus increasing or decreasing allergenicity (Cabanillas & Novak, 2019). It could make a substantial contribution to unintentional false results and influence the operation of allergen assessment methods. To address this limitation, various analytical methodologies have been designed and analyzed that can be used in processed foods. Choosing an appropriate technique for detecting allergic ingredients in processed foods would be the most important step in selecting a good marker because such selection would affect the efficiency and performance of the analytical technique employed.

The present work presents an updated overview of the existing information on WP allergens and mitigation of their allergenicity using different nonthermal processing approaches. Besides, this review will also focus on the influence of different processing and matrix effects on detection, various aspects of recent progress in allergen detection methodologies, performance criteria for available methods, as well as emerging technologies and future trends in the field of detection.

2 | WHEY ALLERGENIC PROTEINS AND THEIR DISTINCTIVE FEATURES

Recently, the development of databases providing genetic, biochemical, and clinical data on allergens has allowed an enormous increase in sequencing information and allergen detection. Cow's milk comprises approximately 30–35 g of proteins/L and includes over 40 different types of proteins, but among them, only a few have been rec-

ognized as allergenic (Villa, Costa, Oliveira and Mafra, 2018; Wal, 2002). The acidification of milk (pH 4.6; ~20°C) results in the extraction of protein fractions: Lacto-serum (whey) making up almost 20% of cow's milk (CM) and coagulum (curd) forming about 80% of CM in the form of casein (D'Urbano et al., 2010). The amount of casein in milk is high, but cow milk allergy (CMA) induced by WPs affects many individuals. WPs and their properties as per the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) allergen nomenclature are shown in Table 1.

WP is a group of globular proteins extracted from whey, a CM by-product of cheese. WP is generally a combination of β -Lg (~65%), α -La (~25%), bovine serum albumin (BSA), (~8%), immunoglobulin, and traces of lactoferrin (LF) (Haug, Høstmark and Harstad, 2007). The structure of WP is maintained by intermolecular disulfide bonds and is not phosphorylated, whereas the most allergenic proteins among the whey fraction are β -Lg and α -La (Hochwallner et al., 2010).

2.1 | Beta-lactoglobulin

β -Lg is the major portion of whey that comprises 50%–60% of the overall WP in bovine milk (Liang et al., 2016). It belongs to the lipocalin family with a molecular weight of 18.3 kDa and contains 162 amino acid residues. β -Lg is a small water-soluble protein and its isoelectric point is around 5.2 (Yadav et al., 2015). Allergies associated with β -Lg are reported in approximately 80% of the population (Miciński et al., 2013). This peculiar type of protein is absent in human milk, but present in bovine milk (Krunić, Rakin, Bulatovic and Zaric, 2018). It has an essential role in the transportation and binds to several molecules such as saturated and unsaturated fatty acids, vitamin D₂, and cholesterol as well as it has an antioxidant activity (Adams, Anderson, Norris, Creamer and Jameson, 2006). β -Lg is classified into two genetic variants: β -lactoglobulin A (β -LgA) and β -lactoglobulin B (β -LgB) (Wal, 2004). β -Lg consists of one free cysteine with two disulfide bridges, which may induce lactoglobulin dimerization. Disulfide bridges are linked with high proteolytic cleavage stability in the molecule. These disulfide bonds are responsible for maintaining high stability against acidic hydrolysis and proteolytic enzymes. At low ionic strength and pH, β -Lg forms stiff long fibers under continuous heating. A destabilized gel is formed from a β -Lg solution under these conditions. This property of β -Lg allows its stability to be maintained after digestion, allowing it to be absorbed and further exposed to immunocompetent cells with high allergenic activity via the intestinal mucosa (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014).

TABLE 1 Whey allergenic proteins and their characteristics according to the World Health Organization and International Union of Immunological Societies (IUIS) allergen nomenclature (Hochwallner et al., 2014)

Fraction	Protein superfamily	Allergen nomenclature	Allergen	g/L	%Total protein	MW (KDa)	Number of aa	Isoelectric point	Functions
Whey proteins (20%) (~5 g/L)				~5.0	20				
	Lysozyme	α -Lactalbumin	Bos d 4	1–1.5	5	14.2	123	4.8	Involves in synthesis of lactose
	Lipocalin	β -Lactoglobulin	Bos d 5	3–4	10	18.3	162	5.3	Lipid binding property
	Immunoglobulins	Immunoglobulins	Bos d 7	0.6–1.0	3	160.0	–	–	Defensive property
	Serum albumin	Bovine serum albumin	Bos d 6	0.1–0.4	1	67.0	583	4.9–5.1	Transportation and antioxidative property
	Transferrin	Lactoferrin	–	0.09	Traces	800.0	703	8.7	Fe-chelating and antimicrobial properties
Total proteins				36.0	100				

Abbreviations: aa, amino acids residues/molecule; MW, molecular weight.

2.2 | Alpha-lactalbumin

α -La is acidic in nature and is the second most dominant globular calcium-binding metalloprotein of whey. The sequence and tertiary folding of α -La are homologous to that of proteins in the lysozyme family, and its primary structure comprises 123 amino acids with a molecular weight of 14.2 kDa (Villa et al., 2018). It consists of four disulfide bridges and achieves stability in its secondary structure via a high-affinity calcium linking position. α -La binding to calcium causes pronounced changes in its tertiary structure. Calcium plays a crucial role in maintaining the stability of the well-arranged secondary and compact tertiary spherical structure, but any changes in the allergen conformation would reduce the thermal stability of the protein (Goff, 2016). The incidence of α -La-specific IgE in milk-sensitized individuals varies from 27.6%–62.8% among the participants surveyed (Matsuo, Yokooji, & Taogoshi, 2015). Amino acid sequence analysis showed approximately a 75% degree of similarity between bovine and human α -La (Hochwallner et al., 2014).

2.3 | Bovine serum albumin

BSA is a serum protein fraction that contributes about 5% of the total content of WP. It has a stable tertiary structure containing 66.3 kDa molecular mass with 582 amino acids. The biological function of BSA is the distribution and transportation of a number of elements such as hormones and ions and plays a vital part in the

inhibition of free radicals. In amino acid composition, BSA closely resembles human blood serum albumin (Villa et al., 2018). BSA is hypersensitive because of the involvement of disulfide bonds that maintain native antigenic features. This special type of protein found in milk and beef is capable of causing allergy in both cases. It was recently reported that BSA-sensitized beef allergy patients experience milk cross-reactivity (Sánchez, Pérez, & Parrón, 2020). Research shows that cooking destroys BSA's allergenic activity (Vicente-Serrano et al., 2007).

2.4 | Immunoglobulin

Immunoglobulins (Ig), commonly known as antibodies, are components of glycoprotein generated by plasma cells. The biological function of this protein is to provide immune protection against particular antigens. It represents approximately 6% of the WP and 3% of the total milk protein with a molecular mass of 64 kDa (Mehra, Marnila, & Korhonen, 2006). In its configurational structure, it is more similar to human origin, existing as a polymer or protomer. Limited data are available on the allergenic potential of bovine Ig, whereas its IgE-binding epitopes have not been identified yet. Nevertheless, some research findings have revealed that IgG is another milk allergen because IgE from patients with CMA binds bovine IgG proteins specifically (D'Urbano et al., 2010). This protein is supposed to be a minor allergen in milk because nearly 10% of patients are IgE positive to cow IgG (Matsuo et al., 2015).

2.5 | Lactoferrin

LF, also referred as lactotransferrin, is a globular glycoprotein with a molecular weight of 80 kDa. It is a multifunctional protein of the transferrin family and is limited in the milk of many animals (Van Neerven, Knol, Heck, & Savelkoul, 2012). Folded into two globular lobes, this protein has a single polypeptide chain, each of which has a strong iron-binding affinity. In addition to its main biological functions of binding and transporting iron ions, it has antibacterial, antiviral, anticancer, antiparasitic, and antiallergic properties. The role of LF in triggering allergenicity is still not clear, because some milk allergic individuals possess LF-specific IgE, however, these individuals also possess IgE against other major milk allergens (Negaoui et al., 2016).

3 | APPLICATIONS OF WHEY AS A VALUABLE INGREDIENT FOR THE FOOD INDUSTRY

The utilization of whey and WPs has received considerable attention due to their exceptional nutritional and functional properties (Deeth & Bansal, 2019). WP is rightly considered as one of the most important and valuable ingredients, and currently performs a major role in the production of distinctive food products. WPs can also be added to a variety of food items to boost their nutritional content, and also have a number of beneficial effects on human health. WP is an easily soluble digestible protein that is quickly assimilated by the body, granting it the nickname “fast protein” for its ability to quickly provide nutrients to the muscles (Hoerr & Bostwick, 2000). Functional characteristics of WPs have encouraged food technologists in replacing certain traditional ingredients including milk powder and egg albumin with a broader range of food products (Kumar, Chauhan, Shinde, Subramanian, & Nadanasabapathi, 2018). In addition to certain nutritional and therapeutic effects, WPs have excellent functional properties such as solubility, foaming, emulsifying, gelling, and water retention (Sánchez et al., 2020; Rathour, Chauhan, & Bhatia, 2004). Proteins in foams help to ensure the homogeneity of fine air cells in food structures. The body and fluffiness of food foams are attributed to the formation of air bubbles, which allows for flavor volatilization and improved food palatability (Kumar et al., 2018). WPs in food emulsions help to form and stabilize the system by adsorbing on the oil droplet surface and forming a film that serves to protect the droplets from agglomeration (Kulmyrzaev, Bryant, & McClements, 2000). WP concentrate is used as a binding material and emulsifying agent in a comminuted meat mixture. It has been recognized that purified β -Lg is

more efficient than the other WPs in emulsification. WPs have exceptional gelling properties, notably when pH is over 7.0. Protein, minerals, lipid composition, and pH all have an effect on whey protein concentrate (WPC) gelling properties. Rathour et al., 2004 evaluated the thickening features of WPC and confirmed that WPC has the capacity to establish gel and that multiple gels can be produced with fruits while WPC maximizes the nutritional value of food products. Denaturated proteins are poorly soluble, but they have an exceptionally strong water binding potential. WPs have the lowest viscosity at an isoelectric point pH (4.5). As a result, the water retention ability and viscosity of WPC are significant functional properties that can be better utilized in the production of different food products such as meats, fish, dairy, bakery, soups, gravies, and so on, where viscosity is an impactful aspect.

The manufacturing companies of dairy products have commenced searching for relatively cheap replacements of milk solids and WP is an appropriate choice. Nowadays, myriad of formulations for ice cream blends have been used to replace milk solids nonfat (Yilsay, Yilmaz, & Bayazit, 2006). WPC with a relatively high protein content (over 80%) is used to make low-fat ice cream without compromising the mouth feel (Akalin, Karagözlü, & Ünal, 2008). According to Hemaprabha, Sudha, & Athmaselvi, 2003, skim milk powder in ice cream replaced by WPC at a 25% level was better in terms of flavor, low melting, and strong texture. Fermented dairy products perform an important role in human nutrition around the globe. Milk enriched with WPs is used to significantly boost the value of fermented foods including kefir, yogurt, and dahi. The application of WPC with good sensory qualities is currently of particular interest in the cheese industry.

4 | MITIGATION OF WP ALLERGENICITY VIA NONTHERMAL FOOD PROCESSING METHODS

Consumers for several reasons prefer ready-to-eat processed foods, perhaps the most important being convenience. These processed foods can be treated for a wide range of purposes, whether domestically or industrially, such as to enhance their quality, preservation, safety, and adequacy for particular product purposes. In addition, food processing can modify the allergenicity of foods through different ways. The extent of these modifications is dependent upon several factors, such as method of processing (thermal or nonthermal), time duration, and so forth. Food processing can lead to a number of protein structural modifications, such as aggregation, denaturation, oxidation, glycation, and crosslinking. Such modifications can influence the IgE/IgG-binding ability

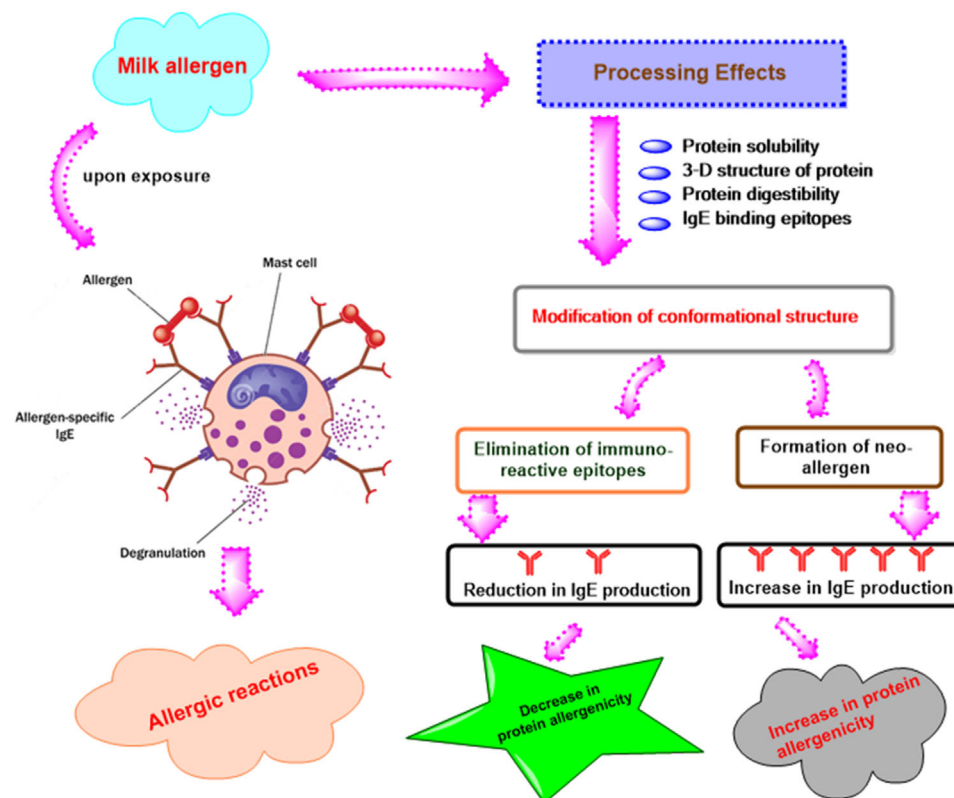


FIGURE 2 Nonthermal processing-induced conformational changes and impacts on the allergenicity of milk proteins

and thus significantly influence the allergenicity of proteins (Ahmed et al., 2018; Liu et al., 2017). Thermal processing may contribute to the formation of neoepitopes, which can potentially increase the allergenicity of proteins. Due to health concerns, in recent decades, minimally processed products have received more attention from food experts to investigate other methods of preservation as alternatives to thermal treatment. Foods processed entirely by heating can irreversibly alter the sensory, organoleptic, and nutritional properties. In industrial and research fields, considerable attempts are being taken to develop novel nonthermal processing technologies to reduce the detrimental thermal effects on foods.

Nonthermal processing is a novel technology that owns a mechanism other than the heat treatment. Nonthermal processing has indeed emerged as the most effective field of research and operation in food engineering. Nonthermal operations can be more aptly characterized as indirect-thermal-energy-input processing. It is due to the fact that nonthermal techniques lead to an increase in thermal vitality in the product (Ezeh, Yusoff, & Niranjan, 2018). The most investigated nonthermal food processing techniques are HPP, PEF, MWI, ultrasonication, CP, EHD, fermentation, and enzymatic hydrolysis with some already being industrialized (Shao et al., 2020; Yang et al., 2020; Kurpiewska et al., 2019; Misra et al., 2018). The

underlying mechanism of each technique plays a crucial role in the effects they have on food systems. The enacted mechanisms include protein denaturation, structural modifications, extraction of allergens facilitation, enzymatic hydrolysis while ultimately modifying food proteins. When either one of such techniques is utilized in food processing, the overall focus is to enhance the food quality and ensure its safety for consumption. Food quality includes a wide array of food characteristics from sensory attributes to stability and nutritional value. These qualities are closely connected with food microstructure, a complex system that includes self-assembled structures found in processed food formulations (Ezeh et al., 2018). The influence of nonthermal processing on allergenic protein is mainly based on the type of food, its conformational arrangement, and allergenic epitopes (Dong, Wang, & Raghavan, 2021). Several food allergens have been identified as labile to a certain type of food processing methods in that context (Cabanillas & Novak, 2019). Nonthermal processing induces conformational changes in allergenic proteins as illustrated in Figure 2.

Nonthermal green techniques, along with the preservation of sensory and organoleptic properties of foods, address the demands of the 21st century by protecting consumers' health and the environment while also enhancing the competition on industrial sector in order to be

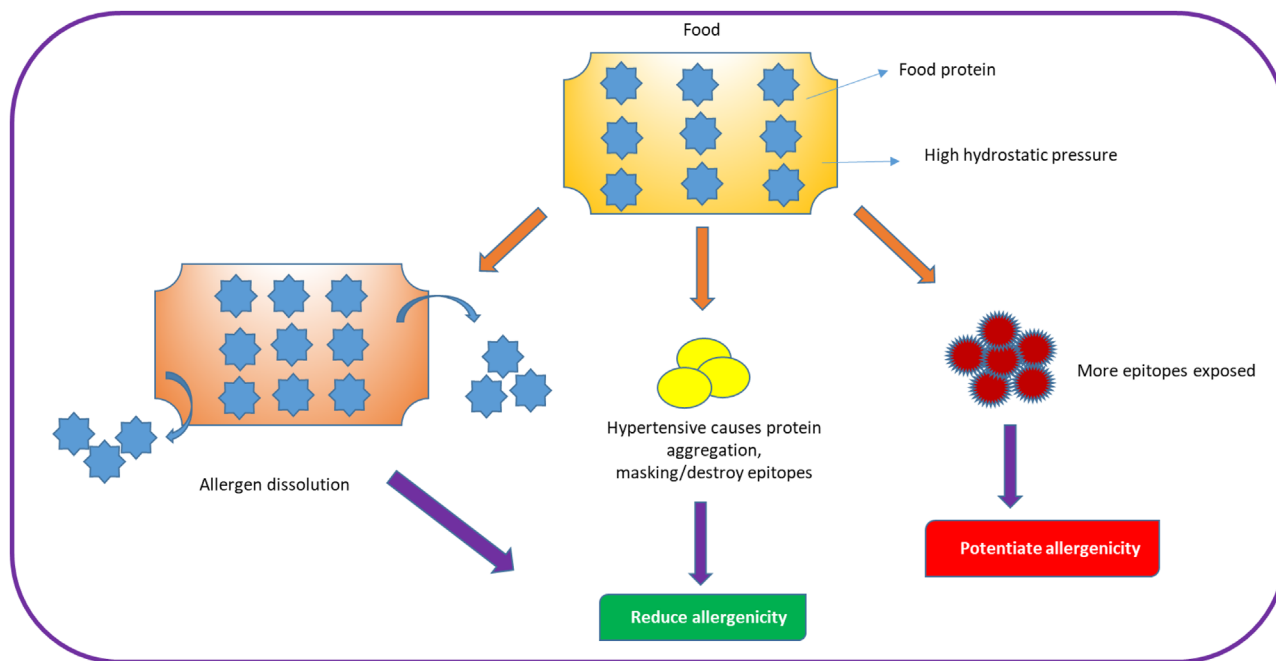


FIGURE 3 Schematic diagram of HPP-induced changes in allergenic proteins

more innovative, economical, and eco-friendly (Chemat et al., 2019). Nonthermal techniques could be used as viable approaches in compliance with the United Nations (UN) Sustainable Development Goals (SDGs) and Agenda 2030 (Režek Jambrak, Nutrizio, Djekić, Pleslić, & Chemat, 2021). Consequently, most of the novel nonthermal processing approaches showed significant effect in reducing the immunoreactivity of allergenic proteins. The most promising technologies for mitigating allergenic WP in food and their underlying mechanisms are addressed in this review.

4.1 | High-pressure processing

HPP is an emerging nonthermal green processing technique, also known as high-pressure hydrostatic processing, which involves the use of 100–800 MPa pressure to extend the shelf life of food products by ensuring their characteristic flavor and nutritional value (Khan et al., 2019). This modern technology is extensively used in the food industry to minimize food loss and to enhance the quality and safety of various food products. While designing, several processing parameters need to be kept in mind such as time of exposure, pressure, and temperature (Kaur et al., 2016). In high-pressure conditions, the structure of the binding protein can be altered. Probably, protein conformational alterations take place when the pressure exceeds 200–300 MPa. This type of processing is better than thermal processing, as the pressure is equally distributed throughout the

foodstuff in all dimensions (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015).

Recently, considerable attention has been paid to the effect of HPP on allergenic foods. HPP has showed efficiency in minimizing WP immunoreactivity by causing denaturation, unfolding, and aggregation of allergenic proteins (Gharbi & Labbafi, 2018). Figure 3 illustrates a schematic diagram of HPP on milk allergenic proteins.

4.1.1 | Effect of HPP on β -Lg and α -La

Considerable variations in the sensitivity of WPs to pressure (β -Lg > LF > Ig > BSA > α -La) and temperature (LF > Ig > BSA > β -Lg > α -La) were reported (Patel, Singh, Anema, & Creamer, 2006), suggesting that β -Lg is the most responsive in WPs. β -Lg has two disulfide linkages and one free SH group; therefore, it is less stable than α -La, which contains four disulfide bonds. The processing of raw milk to 100 MPa will not denature β -Lg and will remain in the native monomeric form (Zobrist, Huppertz, Uniacke, Fox, & Kelly, 2005). β -Lg starts to unfold and detach at pressure over 100 MPa, and the SH group may interact with k-casein or other unfolded β -Lg molecules. It causes an upsurge in the size of casein micelle and a slight aggregation between β -Lg molecules will take place. A high-pressure treatment leads to a significant β -Lg denaturation, which is 80% after 400 MPa (Scollard, Beresford, Needs, Murphy, & Kelly, 2000). It was noted that temperature

has a synergistic influence on the denaturation of β -Lg. About 100% denaturation was observed at 60°C (300 MPa) or 40°C (400 MPa). It unfolds between 100 and 450 MPa and forms dimers via disulfide linkages. It forms polymers through disulfide bonds between 450 and 800 MPa, which is irreversible (Arias, López Fandiño, & Olano, 2000).

The denaturation level of α -La was considerably lower compared to β -Lg. At 600 MPa, 10% of α -La was denatured, but at 800 MPa, about 50% of α -La was denatured. The concentration of α -La in bovine milk ranges from 1.3 to 1.6 g/L; it is the second-largest constituent after β -Lg in the WP fraction by concentration. α -La has four intramolecular disulfide attachments without a free thiol group, which is highly stable and is, therefore, an ideal model for analyzing protein folding (Rodiles-López et al., 2010). α -La is more resistant to pressure denaturation, as it has four disulfide attachments. Its denaturation starts only at >400 MPa. Subsequently, α -La has no free thiol groups in the molecule, and only a minor portion of the protein aggregates even at pressures of up to 1000 MPa. Therefore, thiol-induced oligomerization of this HPP protein can only be accomplished using low-molecular-weight reduction agents including cysteine, 2 mercaptoethanol, or dithiothreitol. Small α -La aggregates were, however, detected at ~1000 MPa due to the bonding between Cys 6 and Cys 120, which was more prone to cleavage due to its environment (Huppertz, Fox, & Kelly, 2004).

Meng, Bai, Gao, Li, & Chen, 2017 treated bovine β -Lg with HPP at 100–500 MPa and analyzed the IgG- and IgE-binding capacity by ELISA with rabbit antibodies and milk allergic patient's sera. The authors found that the IgG-binding potential of β -Lg enhanced exponentially with maximizing pressure and the IgE binding achieved the highest value at 400 MPa. Such an increase in β -Lg immunoreactivity could be due to the contact of IgG linear epitopes after HPP application that led to tertiary structural modifications, which further influenced the allergenicity of β -Lg. Another analysis done by Kurpiewska et al., 2019 confirmed that HPP has the ability to modify β -Lg conformation and physicochemical properties. Their observations have shown that high-pressure protein crystallography can potentially recognize the pressure-sensitive molecules in allergens, act as an indicator of protein allergenicity management, and thus facilitate the production of hypoallergenic food items (Kurpiewska et al., 2019).

High-pressure treatment of WP allergens is also unable to eradicate allergenicity alone, whereas combinations with other techniques can proceed to alternative approaches, such as HPP along with enzymatic hydrolysis. Besides that, changes in protein conformation induced by high pressure can facilitate enzymatic digestion. Dif-

ferent enzymes under high pressure will hydrolyze β -Lg effectively (Chicón, Belloque, Recio, & López-Fandiño, 2006). The enzyme-treated hydrolysates β -Lg at higher pressures may display reduced antigenicity and IgE binding. Treatment with trypsin, chymotrypsin, and pepsin has reported to affect the hydrolysis of WPs with high pressure (100–300 MPa) (Bonomi et al., 2003). The results showed that high pressure augmented WP hydrolysis and the overall antigenicity of hydrolysates, based on the selection of enzymes. Treating allergens with pepsin and trypsin enzymes in conjunction with high pressure (200 or 300 MPa) exhibited maximum decrease in immunoreactivity relative to the enzymatic method alone (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012). Another analysis revealed that the antigenicity of bovine WP hydrolysates was significantly decreased during enzymatic hydrolysis when combined with a high-pressure (300 MPa) treatment (Peñas, Préstamo, Luisa Baeza, Martínez-Molero, & Gomez, 2006).

4.1.2 | Effect of HPP on BSA, IG, LF, and lysozyme

Studies have revealed that BSA in raw milk is highly resistant to high pressures around 400 MPa, as BSA contains one sulfhydryl group and 17 disulfide bonds. The power generated under pressure treatment is not enough to modify the molecular structure of BSA by breaking all the disulfide bonds (Patel & Huppertz, 2014). With the application of 500 MPa pressure treatment, only 35% denaturation occurred in the immunoglobulin of caprine milk; however, it showed resistance at 300 MPa. He, Mao, Gao, & Yuan, 2016 investigated that bovine LF under HPP changes its structural and functional properties. In this study, LF was treated for either 30 or 60 min with HPP at different pH (4.0 and 6.0) and 300–700 MPa. The findings indicated a major shift in the LF tertiary structure with an increased extent of HPP, showing slight denaturation and aggregation. The decrease in the unordered structure suggested that HPP resulted in the loosed secondary structure of LF (He et al., 2016).

4.2 | Pulsed electric field

PEF is an emerging nonthermal green technology approach for preserving or even enhancing product quality in the food sector. In this practice, the electrical field intensity applied to the food items generally ranges from 0.1 to 80 kV/cm, which is delivered between two electrodes for nanoseconds to milliseconds (Bhat, Morton, Mason, & Bekhit, 2019). This emerging technology is not

only applicable to the inhibition of microbes and enzymes but is also widely used today to change the structure of proteins and to improve their physical characteristics (Han, Cai, Cheng, & Sun, 2018). Although the actual processing dynamics of PEF is still not clear, PEF has explained the changes in tertiary and secondary protein structures caused by ionization of certain chemical groups or interference with electrostatic interactions. This innovation has received increasing attention due to its diverse benefits, which include energy-saving features, minimal nutrients loss, and accessibility of fluid supply chains and extend its utilizations in the food industry (Chemat et al., 2020).

The tertiary structure of WP was altered by the application of PEF treatment (Michalac, Alvarez, Ji, & Zhang, 2003). The researchers observed that β -Lg, which is the primary protein of milk, was denatured by PEF treatment (Xiang, Ngadi, Ochoa-Martinez, & Simpson, 2011). They were analyzed in various solvents (water and buffers) as individual proteins. Sui, Roginski, Williams, Versteeg and Wan, 2010 noted that PEF operation had no influence on aggregation, sulfhydryl groups, surface hydrophobicity, emulsification, and thermal properties at 30–35 kV/cm at 30°C (Sui et al., 2010). Perez and Pilosof (2004) found that the sensitivity of β -Lg with high electrical fields (12.5 kV/cm, 1–10 cycles) was slightly denatured, but the thermal stability declined substantially. They also indicated that any further PEF treatment could significantly cause alterations in protein structure, fragmentation, and detachment of noncovalent subunits, whereas covalent bonds or hydrophobic interactions may result in aggregation when subjected to the electrical field for a sufficiently prolonged duration. However, Barsotti, Dumay, Mu, Diaz and Cheftel, 2001 reported that PEF operation for β -Lg-stabilized oil-in-water emulsion and the β -Lg solution did not result in substantial aggregation in continuous operation at 21–36 kV/cm. The changes identified in WP stability may be caused by the variations in pH values and other parameters used during the experiments (Sharma, Oey, & Everett, 2014).

4.3 | Microwave irradiation

This technology is recently introduced in the food sector and has received an overwhelming response. Microwave operation is introduced as an alternative to heat processing by the dairy sector (El Mecherfi et al., 2015). The effects of the MWI have been examined on the structural properties of MPs and were found to affect the kinetic transformation and denaturation of MPs (Grar et al., 2009). It has been noticed that microwave-irradiated α -La and β -Lg have diminished a portion of their IgG-binding potential,

but they have not eliminated their antigenic involvement. However, MWI alone could not completely eliminate or reduce β -Lg antigenicity and thus MWI should be combined with other methods such as enzymatic treatments and intrinsic conditions such as pH (Kaddouri, Mecherfi, Kheroua, & Saidi, 2009).

β -Lg irradiated with 200 W along with pepsin for 3 min enhanced protein hydrolysis and significantly reduced its immunoreactivity. However, hydrolysis with trypsin, chymotrypsin, and their mixtures did not significantly influence the IgE binding potential of microwave-treated β -Lg (El Mecherfi et al., 2015). Hydrolysates produced by the combination of MWI and enzymatic techniques are expected to be used as an extremely high-frequency peptide source. However, irradiation especially by MWI is an efficient way to decrease the allergenicity of β -Lg and develop hypoallergenic formulations (Golkar, Milani, & Vasiljevic, 2019). Further research should be performed in the future to assess the influence of MWI on the immunoreactivity of WPs.

4.4 | Ultrasonication

Ultrasonication is designated as a “green” technological nonthermal eco-friendly processing technique, with a large number of operations incorporating low ultrasound energy with high power. This treatment is used in the food processing sector to improve food quality and develop new products with particular characteristics (Chemat et al., 2017; Chemat, Zill-E-Huma, & Khan, 2011; Musielak, Mierzwa, & Kroehnke, 2016). The treatment of liquid media with ultrasonication has been effective in terms of rheological properties. Ultrasonication can be used to treat a variety of liquids, including milk (Ashokkumar et al., 2010). Ultrasonic frequencies (>22 kHz) are typically used to move across a liquid medium, which causes a significant number of microbubbles to emerge, develop, and crumble in a matter of microseconds (Wang, Xie, Wang, & Fu, 2020).

The mechanism induced by nonthermal techniques such as in the case of ultrasonication produces a switching of high-pressure and low-pressure waves in liquid, resulting in the formation of intense breakdown with small vacuum bubbles. Such process is known as cavitation, and it results in high-speed intruding fluid jets with powerful hydrodynamic shear forces generated by the propagation of ultrasonic waves (Khadhraoui et al., 2018; Bittmann, Hauptert, & Schlarb, 2009). Cavitation generates high shear forces in the liquid media. Microjetting is caused by the implosion of cavitation bubbles on an item's membrane, which causes erosion and particle dissolution (Mason & Lorimer, 2002). Furthermore,

micro-mixing and macro-agitation are prompted by the disintegration of cavitation bubbles in a liquid media. Such influences are utilized to de-agglomerate nanometer-sized substances. It has been used to dissipate nanoparticles in a variety of mixtures. Ultrasonic treatments enhance the foaming and emulsion properties of milk and can be accomplished by using either a bath or an ultrasound probe (Yang et al., 2020). When comparing two choices, there are two major differences: first, the ultrasonic probe is submerged directly into the solution, where the sonication occurs; and second, the probe's ultrasonic power is much greater than the bath. The use of high intensity-based ultrasound allows a faster protein digestion. Ultrasonication is particularly important in the context of allergenicity. Varying degrees of hydrolysis can be obtained depending on the frequency. Colloidal formation has been shown to reduce allergenicity in β -Lg and casein (Wang et al., 2020; Liu et al., 2018). However, this approach has some downsides because it tends to cause the formation of free radicals (Santos & Capelo, 2007).

4.5 | Cold plasma

CP technique is an expanding nonthermal food processing approach that has attracted significant interest of many researchers. It uses strong, reactive gases to inhibit infecting microbes on meat, poultry, dairy, fruits, and vegetables. UV light and the reactive chemicals used in the CP ionization method are responsible for the primary mechanisms involved. According to a modern research pattern, CP technology is an effective and commercially viable innovation for the food processing industry (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017; Misra, Pankaj, Frias, Keener, & Cullen, 2015). Interestingly, recent research shows that CP application has recently participated in the hydrogenation of plant oils to produce trans fat-free cooking oil, and also in the control of food allergens (Yepez & Keener, 2016). Atmospheric CP has emerged as a viable novel technique for causing protein structural changes (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). CP treatment causes structural changes and alters the antigenic response of CM allergens. Ng et al., 2021 recently investigated the influence of plasma treatment on the structure and allergenicity of β -Lg and α -La. Their findings revealed that the secondary structure of proteins was altered following plasma treatment, along with the reduction in the antigenicity of β -Lg, whereas α -La exhibited enhanced antigenicity (Ng et al., 2021). Thus, it can be assumed that atmospheric CP can be tailored to minimize the risk of CM allergens in the dairy processing and ingredients segments.

4.6 | EHD processing

EHD is also recognized as electro-fluid dynamics as a nonthermal green processing approach that recently takes into consideration the dynamics of electrically charged fluids. This processing treatment is an energy-efficient approach best suited for heat-sensitive foods. It meets both the challenges well, notably good quality of the processed food product and minimal consumption of energy (Bajgai, Raghavan, Hashinaga, & Ngadi, 2006). The main framework for EHD is the generation of electric winds, known as corona wind, with a relatively small curved radius applied to an electrode. This corona wind interrupts the outer surface from which the biological material is positioned, thus increasing the mass conversion around the biological outer surface and ambient air. The application of EHD is therefore regarded as an appropriate substitute to traditional food processing approaches such as thermal treatment, steaming etc. (Charry, 2018).

The effect of EHD has been so far observed mainly on food quality on a laboratory scale in many cases. In most cases, the EHD technique is used for drying, freezing, thawing, refrigeration, and shelf life extension of various processed foods (Martynenko & Zheng, 2016; Xanthakis, Havet, Chevallier, Abadie, & Le-Bail, 2013; Palanimuthu, Rajkumar, Orsat, Gariépy, & Raghavan, 2009). Industry-friendly installation and operational costs make it a perfect technique, but a reliable comprehensive EHD processing platform for diverse biological matrixes has yet to be planned, developed, and evaluated. A thorough understanding of the methodology, as well as statistics on the cost and energy required for industrial-scale application, will be required for the configuration of an EHD system. A life cycle evaluation of the EHD mechanism should be conducted to achieve this, with feedback from all shareholders, including those from the social, industrial, and ecological realms. Due to its enormous potential for use in multiple disciplines, a good evaluation of these attributes requires a great deal of investigation in this field. Many sectors, such as pharmaceutical, food related, mechanical, and chemical, can offer research and development opportunities for using EHD in main parts of the processing to address environmental and economic issues associated with traditional approaches.

4.7 | Fermentation

Fermentation is still considered one of the most popular methods of food preservation in the food sector. Some protein-rich fermented foods, such as dairy products (yogurt and cheese), alcoholic drinks, cured

vegetables (pickles and sauerkraut), and meat products (salami and sausages), are the most important fermentation products (Soccol, Pandey, & Larroche, 2013). Products manufactured by fermentation have a positive effect on health as the fermented products have probiotic strains produced by microbial enzymatic hydrolysis of food proteins. Specific microorganisms acting on food substances induce fermentation. In fermentation, the strains of microorganisms and underlying conditions (temperature, pH, and enzyme concentration) have a measurable influence on the different products manufactured (Pérez-Díaz, Altuntas, & Juneja, 2017).

Milk protein hydrolysis has an important effect on the digestion of milk and the synthesis of bioactive peptides during fermentation by lactic acid bacteria (LAB). An interesting approach of the LAB is the reduction of food allergy and its beneficial effects are linked with the consumption of probiotic strains. Clinical reports have shown that probiotics can promote the development of T regulatory (Treg) cells and the rebalancing of T helper (Th) responses (Th1/Th2), stimulating the Th1-dominant state. However, the mechanism is not fully understood and the immunomodulatory effects are strain dependent (Fu et al., 2019). Additionally, the consumption of probiotics and fermented products can minimize some atopy symptoms and allergy expansion through the immune-regulation mechanism. Recent research shows that fermentation can alter the linear and conformational structures of allergenic proteins and plays an important role in the mitigation of allergenicity.

A study conducted by Bu, Luo, Zhang, & Chen, 2010 found that the combined effect of *Lactobacillus helveticus* and *Streptococcus thermophilus* strains is efficient in minimizing WP antigenicity. Both the strains reduced the antigenicity of β -Lg and α -La after 6 hr of fermentation and cold storage. Yao et al., 2014 investigated the IgE-binding ability of α -La and β -Lg that were fermented by *Lactobacillus rhamnosus*. It was found that the IgE-binding ability of these proteins in fermented milk was considerably reduced compared to untreated milk, which may result from alterations of allergens or cleavage of allergenic epitopes (Yao et al., 2014).

Recently, Pescuma, Hébert, Font, Saavedra, & Mozzi, 2019 studied the immunomodulatory ability of β -Lg hydrolysate developed with *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL65 (H656). In this research, serum from mice gavage with H656 displayed reduced relevant β -Lg-IgE values than allergic mice. Interleukin (IL)-4 secretion was highly reduced, whereas IL-6 IL-10 and IL-17A enhanced, when H656 was given orally. Such findings showed that by inducing cytokine secretion of type Th1, H656 had an immunomodulation effect, balancing the allergic response to Th2. They proposed that

the α -La and β -Lg denaturation enhanced the aggregation and accessibility of the peptic cleavage sites. Hydrolysates can help to promote oral tolerance of β -Lg in infant foods (Pescuma et al., 2019).

4.8 | Enzymatic hydrolysis

The enzymatic hydrolysis technique has attracted considerable attention of researchers in recent years due to its efficiency, specificity, and environment-friendly features (Zhang, He, & Simpson, 2018). Many research studies have shown that enzymatic hydrolysis strategies have shown a substantial decrease in the immunoreactivity of allergens or their derivatives by modifying the structure of allergenic proteins. The technique involved in the treatment of enzymatic hydrolysis is the alteration or degradation of linear epitopes by proteolytic hydrolysis. Upon hydrolysis, the complex proteins are fragmented and divided into peptides or amino acids (Ekezie et al., 2018).

The enzymatic hydrolysis process has been used for decades in the production of easily digested foods for infants. To manufacture hydrolyzed formula milk, proteases are used, for instance, for hydrolyzing the MP, thus boosting the digestibility of milk powder. In addition, several studies have shown that treatment with enzymes can reduce the risk of food allergies. Duan et al., 2014 isolated and subcutaneously injected BALB/c mice in three groups on days 0, 7, and 14 with native bovine β -Lg and its hydrolysates. Results demonstrated that mice sensitized by β -Lg-mediated hydrolysates had a lower degree of Th2 proliferation in splenocytes than untreated mice, suggesting that the enzyme hydrolysis had an immunosuppressive influence on β -Lg-induced allergy. The reason for this may be the alteration in the β -Lg configuration induced by hydrolysis, involving specific epitopes that have ultimately been excluded (Duan et al., 2014). The remaining antigenicity of WPs was assessed by the activity of the enzyme, not by the extent of hydrolysis or concentration of molecular weight. Therefore, to achieve a hydrolyzed substance with decreased allergenicity, it is important to choose the best effective enzyme keeping in mind the potency of the antigenic epitopes. Many enzymes were examined individually or in combination to sort out the enzymatic reaction conditions and best dosages for mitigating the allergenic potential. The simulation of digestive processes was based on digestive enzymes such as trypsin, pepsin, papain, chymotrypsin, neutrase, alkalase, and protease (Aguiar, Veloso, Fernando, & Franco, 2017). Enzymatic hydrolysis technique has shown potential for developing hypoallergenic food products.

4.9 | The combined effect of processing treatments

Using a single method of processing does not have a major influence on immunoreactivity mitigation, whereas the combination of multiple processing technologies offers a modern strategy for reducing food immunoactivity and appears to be successful approach rather than alone (Ekezie et al., 2018).

Kleber, Maier, & Hinrichs, 2007 observed that under the combined treatment of HPP (200–600 MPa) together with high temperature (60–68°C), the antigenicity of β -Lg was reduced significantly. However, no major changes were witnessed when β -Lg was treated with high pressure or thermal treatment alone (Kleber et al., 2007). The combined effect of chymotrypsin hydrolysis and MWI increased the hydrolysis of WPs and decreased the relative antigenicity of hydrolysates. Prioult, Pecquet, & Fliss, 2005 examined the impact of hydrolysis with *Lactobacillus paracasei* and *Bifidobacterium lactis* enzymes on the allergenicity of acidic peptides of bovine β -Lg. Their findings showed that the ability to bind IgE was decreased by β -Lg peptide hydrolysis and lymphocyte activation (Prioult et al., 2005). The combined effect of enzymatic hydrolysis with heat treatment significantly improved the tryptic and peptic hydrolysis of β -Lg. The combine treatment exposes regions of thermal denaturation and enhances resistance to proteolysis, thus minimizing the allergenicity of milk allergens (Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006).

Structural, digestibility, and allergenicity changes were observed following cross-linking of bovine α -La with *Agaricus bisporus* polyphenol oxidase. The enzymatic cross-linking technique is mostly used in the bioprocessing of milk products, as it can modify organoleptic and functional properties. The polymers were examined for their structural changes by circular dichroism spectroscopy, fluorescence spectroscopy, and ultraviolet absorption spectroscopy. The allergenicity of α -La polymers was assessed using an animal model to detect particularly IgE-binding ability (Dong et al., 2021). The findings showed substantial differences in the secondary and tertiary structures of different α -La polymers than those of untreated α -La while α -La cross-linking was less prone to digestion. In particular, the allergenicity of cross-linked polymers reduced considerably. It showed a strong connection between the IgE binding of α -La and the loss of α -helix indicating that enzymatic cross-linking may be an effective approach for reducing bovine α -La allergenicity (Li et al., 2020). The use of ultrasonication in conjunction with glycation considerably decreased IgE-/IgG-binding ability and the discharge of β -hexosaminidase, histamine, and IL-6, whereas some modifications were also observed in the secondary and ter-

tiary structures. The reduction in allergenicity of β -Lg was not only dependent on glycation within linear epitopes, but also on conformational epitope denaturation, which was supported by glycation-induced secondary and tertiary configuration modifications. It showed that ultrasonication in combination with glycation is a promising method for minimizing allergenicity of allergenic proteins (Shao et al., 2020). The allergenicity of MPs is more difficult to mitigate compared to other food allergens, such as wheat, soybeans, and peanuts. Because the combined effect of high pressure and enzymatic hydrolysis has shown great efficiency in minimizing immunoreactivity in foods, combined approaches usually show considerable effectiveness compared to a single method of processing to mitigate food allergenicity.

5 | INFLUENCE OF PROCESSING AND FOOD MATRIX ON ALLERGENS DETECTION

The complexity of processing and the effect of embedded constituents of the food matrix can affect the way allergens are introduced into the immune system and thus contribute to the severity of an allergic response. Moreover, both processing and matrix effects play a key role in allergens detection, particularly when food challenges are included in the diagnostic process. Food processing may alter the structure of epitopes, but there is no general rule as how proteins react to these processing effects. The structure of a protein defines protein solubility, so modifications in the structure of proteins induced by processing will have a general impact on the efficiency of detection assay. Due to the processing effects, the sensitivity of selected detection methods may decrease. In assessing protein allergies, modifications in IgG-binding epitopes in response to processing are important factors to note (Verhoeckx et al., 2015). Methods that reduce IgG epitope detection can cause false-negative results. Similarly, conformational epitopes are modified by different treatment parameters (temperature, enzymatic digestion, low pH) resulting in reduced IgG detection during ELISA detection. Linear epitopes are less efficient and remain intact in food processing applications except for hydrolysis (Nowak-Węgrzyn & Fiocchi, 2009).

The effect of processing technologies on protein structure is commonly one of the biggest issues associated with the detection of allergenic proteins by immunological methods in processed food products (Platteau, De Loose, De Meulenaer, & Taverniers, 2011). Numerous studies have reported detrimental effects on the detection of DNA following extreme heat application (Iniesto et al., 2013). Several other processing methods may lead to the

fragmentation or depletion of genomic DNA (Prieto et al., 2014). Similarly, many studies have been conducted to assess the effects of heat treatment on the detection of peanuts, hazelnut, walnut, and almonds, with and without the application of pressure (boiling, HPP, autoclave, baking, and roasting) (Sanchiz et al., 2018). The method of MS for allergens to be analyzed in processed foods is currently the best choice because heat treatment triggers denaturation of proteins and structural changes, which might lead to the failure to identify target proteins, or at least to the misjudgment of the allergen element (Khuda et al., 2016).

Food matrix is a complex system of proteins, carbohydrates, fats, minerals, and other constituents that interact with each other and affect allergenic properties. According to the International Union of Pure Applied Chemistry (IUPAC), the influence of matrix is defined as “the combined effect on the measurement of the quantity of all the constituents of the sample other than the analyte” (Guilbault & Hjelm, 1991). Matrix effects on target analyte quantification *in vitro* cannot be underestimated. At present, a limited number of studies have addressed matrix effects during *in vitro* immune detection on the extractability of the target analytes (Montserrat et al., 2015).

The overall detection of allergens may be affected by factors such as matrix structure, preprocessing conditions, and processing of induced interactions. This knowledge would further improve the development of detection methods and make analytical tools more sensitive and accurate. Furthermore, the understanding of protein interaction in food can be enhanced. Food products with known amounts of allergens are the most effective way to analyze food matrix interactions and evaluate recovery using ELISA or other methods. The influence of food matrix interactions on processing outcomes is not well studied yet, which deserves ample interest to be centered by the researchers. The types of food matrix also increase with the diversification of food processing and food products. Additionally, diverse types of matrices may have substantially different effects on allergens detection (Taylor, Nordlee, Niemann, & Lambrecht, 2009).

Matrix variations can affect the detection of allergens. Solid matrices are typically more complicated to separate proteins because of their complex shape and nature. On the other hand, it is less challenging to recover allergens from aqueous matrices in minimally processed products. Limited research has been conducted on the recovery of allergens from liquid matrices, but this is highly necessary, and further research is required in the future. Allergens are evenly dispersed in liquids unless they are found as particulates (Taylor et al., 2014). Milk extracted in a phosphate-buffered saline solution was analyzed using a model of liquid food matrices to assess the processing results. The recovery of milk from a liquid matrix before

processing noted was >80% following analysis by nine different milk ELISA kits. ELISA kits were still able to detect milk allergens after heat processing, but heat treatment decreased the final recovery by around 40%–50% of the original milk concentration (Bly, 2014). The modifications in proteins induced by processing treatments comprise solubility, reduced extraction, degradation of conformational epitopes, and aggregation (Faeste, Lovberg, Lindvik, & Egaas, 2007).

All these factors are known to reduce the performance of the assay and possibly cause false-negative results. In addition, a combination of processing methods and matrix effect further magnify these implications and complicate the detection process.

6 | ANALYTICAL METHODS FOR THE DETECTION OF WP ALLERGENS

Despite the implementation of regulations requiring mandatory labeling of allergenic foods, the possibility of allergenic individuals suffering from an anomalous immune response remains a fact, even though a restrictive diet plan is adopted (Khan et al., 2019). The increasing awareness of the public health consequences of food allergies has led to the establishment of analytical approaches to monitor the existence of potential allergenic components in processed foods, allowing labeling guidelines to be implemented. In December 2014, new legislation (EU Food Information for Consumers Regulation 1169/2011) was implemented for all food businesses to make sure the availability of food information to consumers. To protect the health of certain individuals, two different instructions for the presentation and promotion of foodstuffs along with nutrition labeling have unified this legislation. However, the occurrence of unintended allergens by cross-contamination in raw material processing, storage, or the finished product is still a major risk factor for sensitized individuals (Nehra, Lettieri, Dilbaghi, Kumar, & Marrazza, 2020).

In recent years, several guidelines have been issued to diagnose and treat CMA; among them Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) by the World Allergy Organization (WAO) contributed the most (Fiocchi et al., 2018). Various food safety organizations, especially the World Health Organization (WHO) and US Food and Drug Administration (FDA), are working on standardizing detection methods for allergens, as well as developing food-labeling standards. Milk processing affects various MPs and can enhance the allergenicity because of pasteurization, reduced enzyme-binding ability due to sterilization, and degradation of existing epitopes (Verhoeckx et al., 2015).

However, suitable dilution using different buffer solutions facilitates the detection of allergenic proteins.

Choosing the best approach for analyzing WP allergens depends on different parameters such as target analyte (DNA or proteins), detection basis (biological or chemical), total charges, chances of cross-reactivity, skillful operator, and the possibility of multi-target detection (Neethirajan, Weng, Tah, Cordero, & Ragavan, 2018). The integrity of the target parameters, such as pH, temperature, and chemical interventions, may considerably affect the extraction. The thresholds for allergenic reactions can be used as a standard for the determination of limit of sensitiveness of the sensors. The right models of dose distribution showed the minimum reactive doses to which the largest proportion of people reacted (Alves, Barroso, González-García, Oliveira and Delerue-Matos, 2016). Methods for the detection of WP allergens differ in term of

- a. detection source (chemical or biological),
- b. target analyte (proteins or DNA),
- c. cross-reactivity phenomena of proteins,
- d. multi-target detection,
- e. the need for expertise, and
- f. sensitivity to the specific allergens (Neethirajan et al., 2018).

Assessment of allergens demands easy, quick, precise, and economical analytical methods for detecting and quantifying WP allergens. A minor amount of allergens and the food matrix may present a challenging situation for food allergen detection. The most applied analytical methods for the detection of WP allergens are protein- and DNA-based methods (Martinez-Esteso et al., 2020; Holzhauser, 2018). These approaches vary in the type of marker used to detect allergens. Protein-based approaches are based on specific proteins such as enzyme and antibody as markers for the detection of allergens, whereas DNA-based approaches are based on particular DNA sequences that specify the existence of an allergen (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017).

6.1 | Protein-based methods

The most widely known protein-dependent approaches used for the detection of allergens are based on antigen-antibody interactions and are accessible in various formats such as ELISA (Zeng et al., 2019; Orcajo, Lavilla, & Martínez-De-Marañón, 2019), LFDs (Chen et al., 2017), dipstick tests (Samsonova, Safronova, & Osipov, 2018), and immunoblotting. At present, special attention has been given to pioneering technologies for the analysis of aller-

gens, specifically immunosensors and MS platforms (Costa et al., 2017).

6.1.1 | Enzyme-linked immunosorbent assay

ELISA is the most broadly used platform for qualitative and quantitative detection of allergens in food. Presently research labs and food manufacturers incline toward ELISA to track foodstuffs due to its high sensitivity and ease of perceived usefulness for the presence of allergenic residues (Zeng et al., 2019). ELISA utilizes monoclonal or polyclonal antibodies produced in species, which identify selected proteins as markers for the involvement of allergenic foods. Monoclonal antibodies are appropriate for a single protein epitope, whereas polyclonal antibodies can identify multiple epitopes on a single protein or a combination of proteins. Food products encompass a wide variety of nonallergenic and allergenic proteins (Diaz-Amigo, 2010). ELISA is an appropriate platform for the analysis of food allergens; it usually examines allergenic proteins. The sensitivity and durability of the antibodies produced to the specific analyte are essential for the establishment of a sensitive and dependable ELISA.

There are different ELISA formats including sandwich ELISA (sELISA), direct ELISA, indirect ELISA, and competitive ELISA (cELISA). The choice of an ELISA design relies on different factors comprising the food matrix, the optimal sensitivity, and specifications of the target analyte and the chosen antibody. sELISA is the widely used tool for the analysis of food allergens, where allergens in the sample are bound on the microwell plate by an immobilized antibody, which is detected by a second enzyme-labeled allergen-specific antibody. The intensity of the color development after the addition of the substrate is proportional to the concentration of allergens in the sample. cELISA is a technique in which the intended allergen in the sample binds to the relevant antibody in a solution and interacts with the immobilized allergens on the plate. Here, it is different from sELISA because, in this method, the intensity of the colored product in the food sample is inversely proportional to the allergen concentration. These two evaluation platforms may be either direct, indirect, or improved (Montserrat et al., 2015). The antibodies are tagged via a hydrogen peroxidase enzyme for direct ELISA and not tagged with indirect ELISA, where antibodies are attached to a different immunoglobulin-specific, tagged antibody. The detector antibody is bind to a molecule (i.e., biotin) for enhanced ELISA formats, which binds with enzyme-conjugated streptavidin molecules, thereby boosting sensitivity.

TABLE 2 Detection of whey allergen and quantification in foods via commercially available ELISA and LFD kits

Commercial kit (brand)	Food Matrix	Assay type (cat nr)	Analytical range	LOD (mg/kg)	Performance time
Veratox [®] for Total Milk (Neogen [®] , Lansing, MI, USA)	Milk deficient products (juice, wine, sauces)	Sandwich quantitative ELISA (8470)	2.5–25 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)	2.5–67.5 mg/kg	0.7	30 min
ELISA Systems Casein (ELISA SystemsTM, Queensland, Australia)	Non-dairy products	Sandwich semi quantitative ELISA (ESCASPRD-48)	1.0–10 mg/kg	0.5	45 min
ELISA Systems BLG (ELISA SystemsTM, Queensland, Australia)	Nondairy products	Sandwich semi quantitative ELISA (ESMRDBLG-48)	0.1–1 mg/kg	0.05	45 min
BioKits BLG Assay Kit (Neogen [®])	Food products	Sandwich quantitative ELISA (902061Y)	2.5–40 mg/kg	2	120 min
RIDASCREEN [®] Fast β -LG (r-Biopharm AG, Darmstadt, Germany)	Whey protein, whey protein containing products	Competitive quantitative ELISA (R4901)	0.5–13.5 mg/kg	0.19	30 min
AgraQuant [®] β -LG (Romer Labs Div., Getzersdorf, Austria)	Food products	ELISA (COKAL1048)	0.01–0.4 mg/kg	0.015	NR
Reveal [®] 3-D for Total Milk Allergen (Neogen [®])	Liquid products (juices, sorbets)	LFD (8479)	5–1000 mg/kg	5–10	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, USA)	NR	LFD (MILK-1004)	1–1000 mg/kg	1–2	10 min
AgraStrip [®] Caseins (Romer Labs Div., Getzersdorf, Austria)	Food products	LFD (COKAL1210AS)	1–10,000 mg/kg	1	NR

The specificity and sensitivity of all ELISA models are indeed largely reliant on the chemical and biophysical parameters (e.g., solubility, shape, conformation, and chemical modification) of target allergens. Food treatment performs a pivotal part in the modification of the conformational structures of the allergen, denaturation, aggregation, chemical modification, or reactions with food matrix constituents. Such modifications affect protein extraction efficiency and antibody detection of allergenic proteins (Parker et al., 2015). Thermal and nonthermal treatments have major effect on the detection of allergenic components in a food product. For instance, hydrolysis can modify the target protein epitope-binding region, influencing the antibody interaction that is essential for accurate quantification (Panda et al., 2015). ELISA uses many denaturing agents to maximize the extraction performance of processed and diverse food products for bet-

ter allergen extraction (Matsuda et al., 2006). The complete removal of allergenic protein from diverse treated food matrices is a challenging task. The proper selection of detection antibodies and target analytes along with appropriate extraction techniques is the key aspect for enhancing the validity of immunochemical techniques. Table 2 provides a wide range of currently accessible ELISA and LFD allergen detection kits for WP.

6.1.2 | LFD/dipstick

LFDs are user-friendly devices effectively used in detecting pregnancy and drug abuse for years. Recently, LFDs are widely used to identify immunological allergens that depend on particular antibody–antigen recognition. The principle behind the LFD method is the same as

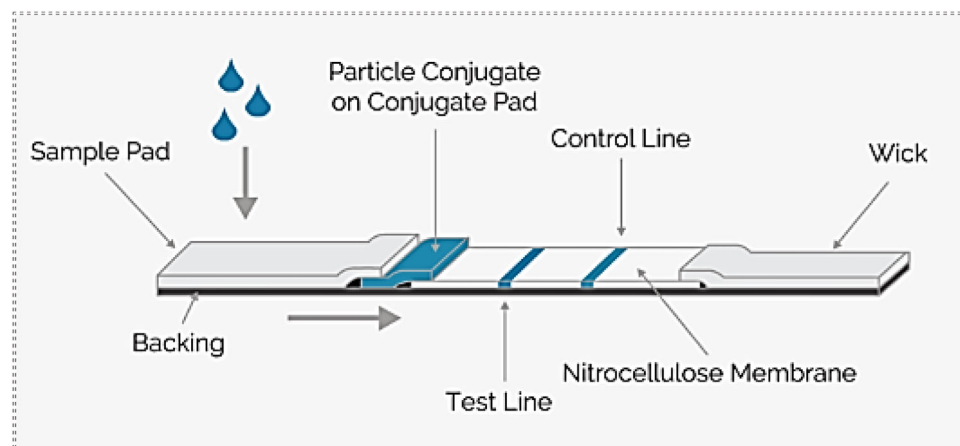


FIGURE 4 Configuration of lateral flow device test strip

that of ELISA, but in the case of LFD, qualitative or semiquantitative findings can be clearly demonstrable. The LFD utilizes a membrane (nitrocellulose, polyvinylidene difluoride, or nylon) upon which the antigen and antibodies samples are attached. According to Walker (2019), the ELISA method can monitor allergen origins in the service for validating cleaning operations or to monitor raw products and final products, as quantifiable results may be provided (Walker, 2019). However, lateral flow immunoassays (LFIAs) are used by the food industry for regular cleaning tests as well as for manufactured goods due to ease of use, rapid response, and affordable cost (Courtney, Taylor, & Baumert, 2016). Figure 4 shows the configuration of the LFD test strip.

The LFIA technique allows the proper identification of allergens without the need of well-equipped testing laboratories and trained operators, ensuring it is an easy approach and can be implemented in the hazard analysis critical control point (HACCP) system (Dzantiev, Byzova, Urusov, & Zherdev, 2014). In particular, because of its rapid reaction (5–10 min), this technique facilitates in situ analysis and hence takes preventive steps in a short period (Galan-Malo et al., 2019). Presently, multiple LFIA kits have been established to determine WP allergens, and their limitations and strengths have subsequently been assessed. Masiri et al., 2016 developed an LFD kit that can detect β -Lg residues of up to $0.01 \mu\text{g}$ per swab and 0.1 ppm of a specific protein. This LFD kit is specific to CM products and utilizes both sandwich and competitive formats. Selectivity experiments using a matrix panel, including widely using cleaning products, have demonstrated a decrease of one to four times the detection limit (Masiri et al., 2016). In certain instances, test producers claim that maximum MPs are present in certain items. However, most of them cannot detect WP, but only casein. Galan-Malo et al., 2019 recently established a new lateral-flow

immune chromatographic assay that incorporates independent and simultaneous analysis of each protein (β -Lg and casein) in a single test. The test has confirmed to detect β -Lg (0.5 ppm) and casein (2 ppm) in compliance with AOAC guidelines (Galan-Malo et al., 2019). Based on this approach, no cross-reactivity was identified among food products.

6.2 | Immunosensors-based detection

Immunosensors are advanced tools for allergens analysis and are assumed as one of the most effective methods for easy, reliable, and cheaper multi-target detection. Besides that, it has a high-speed execution, automation feasibility, and quick operation, which may signify a step forward toward its potential industrial application to detect allergens directly and real-time monitoring of production lines (Pilolli, De Angelis, & Monaci, 2018). The concept of biosensors is focused on the specific identification of biochemical interaction between a target molecule and a receptor by a transducer that generates a detectable message (protein or DNA). The biological receptor is generally incapacitated on the sensor chip layer, with various types of transducers monitoring interaction and generating a message, which is subsequently examined with a message-processing device, which combines the intensity of a given target with relative performance (Prado et al., 2016). The signal amplitude is in proportion to the concentration of the analytes.

Based on the changes of the transducer layer (Nehra et al., 2020), different tools have been documented using bioreceptors (enzymes, antibodies, and DNA) (Zhang, Zhou, & Du, 2019), covalent and noncovalent immobilization techniques (Rashid & Yusof, 2017), and the detection system (electrochemical and optical) (Vasilescu, Nunes,

Hayat, Latif, & Marty, 2016). A key factor that influences the efficiency of biosensors (immunoreactants and aptamers) is the cross-reactivity of biomolecules with homologous proteins in mammalian milk. Antibodies' cross-reactivity occurs among cow, goat, buffalo, horse, and ewe milk proteins, although not with camel milk proteins (Restani et al., 1999; Mehennaoui, Poorahong, Jimenez, & Sijaj, 2019). However, the use of different aptamers can solve the problem of cross-reactivity with other similarly patterned proteins.

Immunosensors are used to detect immunochemical antibody–antigen interactions. The sensitivity of the immunosensors is strongly reliant on the nature of the antibody and the transduction mechanism. In particular, the ability of genetically modified recombinant antibodies offers various advantages including fast immobilization, high sensitivity, and selectivity. Biosensors developed on the concept of a solid-phase immunoassay with magnetic beads (MBs) are suitable platforms for proteins or nucleic acids to be incapacitated. In several biotechnological and analytical applications, the immunoassays are recognized as reliable, versatile, and powerful approaches (Cho et al., 2018). Sandwich-based electrochemical magneto immunosensors are made from the Pingarron group to detect allergens β -Lg and α -La. To capture the targets, the modified MB antibodies were therefore oven dried on the sample solutions. Both β -Lg and α -La were detected in immunosensors, with a detection limit of 11.0 and 0.8 $\mu\text{g/ml}$, respectively (Ruiz-Valdepeñas Montiel, Campuzano, Torrente-Rodríguez, Reviejo, and Pingarrón, 2016). In the analysis of the milk samples, the magneto-immunosensors were efficiently implemented and commercial ELISA spectrophotometric kits subsequently verified their findings. There is no doubt that the limit of detection (LOD) by magneto immunosensors for detecting β -Lg was found to be 10 times lower ($20 \times 10^{-6} \mu\text{g}$ in a 25 μl sample) compared to the commercial ELISA kit (i.e., $195 \times 10^{-6} \mu\text{g}$), but the immunosensors displayed a relatively quicker detection procedure (approximately 60 min) than the ELISA kit (4 hr) (Ruiz-Valdepeñas Montiel et al., 2015). The immunosensors provided a quick detection method (30 min) relative to the ELISA system in the case of α -La analysis. Consequently, immunosensors are particularly efficient in detecting low protein content ($10^{-6} \mu\text{g}$) in milk samples. The protein content was reduced relative to unprocessed milk samples (3–4 g/L) in the context of treated milk samples (pasteurized milk). Due to thermal techniques, this decline can be associated with native protein structural modifications. Furthermore, permanent structural modifications in proteins will influence their detection through the processing of particular biomolecules (Rynne, Beresford, Kelly, & Guinee, 2004). To date, different biosensing platforms for

the detection of WP allergens have been summarized in Table 3.

6.2.1 | Electrochemical biosensor

Electrochemical biosensors have several merits than optical biosensors in terms of possible cost, accessibility, miniaturization, capacity, and diagnostic sensitivity of diverse food products (Campuzano, Ruiz-Valdepeñas Montiel, Serafín, Yáñez-Sedeño, and Pingarrón, 2020). Besides, signal detection depends on electrochemical responses, and electrochemical biosensors are fast, flexible, and appropriate for portable electrical instruments (Lin et al., 2017). Electrochemical biosensors are now regarded as of particular importance in the area of allergen testing. Electrochemical biosensors are integrated tools that enable the generation of detailed quantified or semiquantified analytical data relying on the detection of biological interactions (antibody–antigen) following direct interaction with the transduction devices (Pilolli, Monaci, & Visconti, 2013). The electrochemical biosensor records the variation in the electron flux produced by the oxidation or reduction of the test solution upon the working electrode (detection electrode) when exposed to a static or dynamic potential. The electrochemical biosensor's sensitivity, specificity, and durability are highly affected by the form of the operating electrode that can be made from various substances (e.g., carbon and gold) (Alves et al., 2015). Depending on the type of transducer utilized, electrochemical biosensors may be classified as potentiometric, voltammetric, amperometric, and impedimetric. Voltammetry and amperometry are the most important electrochemical biosensors used for the detection of allergens (Jiang et al., 2015; Čadková et al., 2015).

6.2.2 | Piezoelectric biosensors

Piezoelectric biosensors, commonly recognized as quartz crystal microbalance (QCM), are capable of detecting electrical impulses from vibrating particles that interact with a mass-sensitive device at a fundamental frequency. As an effective instrument for the detection of biomolecular connections and bioanalytical techniques, these sensors draw significant interest (Jackman et al., 2018). For such instruments, a quartz crystal immobilization in a bioreceptor is accomplished with the use of an extrinsic alternating electric current (Yun et al., 2019). Furthermore, the biospecific reaction works together between the two molecules, one free in solvent and the other stationary over the top layer. Therefore, in light of its label-free identification, real-time monitoring, and convenience, such biosensors are

TABLE 3 Different biosensing platforms (immunosensors) for detection of whey allergens

S. No.	Type	Target analyte	Biosensing platform	Transduction mechanism	Detection limit	References
1	Immunosensors	β -Lactoglobulin	Horseradish peroxidase labeled antibody immobilized activated carboxylic-modified magnetic beads/carbon	Amperometric	$0.8 \times 10^{-3} \mu\text{g/ml}$	Montiel et al. (2015)
2		α -Lactoglobulin	Horseradish peroxidase labeled antibody immobilized activated carboxylic-modified magnetic beads/carbon	Amperometric	$11 \times 10^{-6} \mu\text{g/ml}$	Ruiz-Valdepeñas Montiel et al. (2016)
3		β -Lactoglobulin	Anti- β -lactoglobulin antibody immobilized graphene/carbon	Electrochemical	$0.85 \times 10^{-6} \mu\text{g/ml}$	Eissa et al. (2012)
4		β -Lactoglobulin	Anti- β -lactoglobulin antibody immobilized gold sensor chip	Surface plasmon resonance	$0.164 \mu\text{g/ml}$	Ashley et al. (2018)
5		β -Lactoglobulin	Double-antibody sandwich immunoassay	Surface plasmon resonance	$5.54 \times 10^{-3} \mu\text{g/ml}$	Wu et al. (2016)
6		α -Lactalbumin	CdSe/ZnS quantum dots conjugated with monoclonal antibodies	Fluorescence-linked immunosorbent assay	$0.1 \times 10^{-3} \mu\text{g/ml}$	Yang et al. (2014)
7		Casein and immunoglobulin G	Integrated lab-on-a-membrane foldable device using Pb- and Cd-quantum dot tags	Electrochemical	0.04 and 0.02 $\mu\text{g/ml}$	Kokkinos et al. (2016)

valuable. Ashley et al., 2018 discovered that β -Lg analyzed using a flow-based QCM sensor and monoclonal antibodies offers a LOD of up to $1 \mu\text{g/ml}$. Indeed, QCM biosensors are subjected to sensitivity, precision, and unnecessary interferences (Marrazza, 2014).

6.2.3 | Surface plasmon resonance

Surface plasmon resonance (SPR)-based immunoassays are mostly employed to determine where biomolecules are capable of binding to a particular ligand (antigens or antibodies) and primarily incapacitated on the surface of a thinner metallic film (typically gold) (Alves et al., 2015). This approach provides distinguishing advantages such as flexibility, versatility, quick, automatic, and real-time assessment of different molecular attachments varying from protein to protein, peptide to peptide, and cell interactions. Many optical biosensing techniques were used for multiple purposes such as medical, ecological, and food assessment tests (Zhou et al., 2019). SPR-based immunoassays have recently been acknowledged for milk allergen analysis in rinse water samples during the on-site cleaning of food production (Ashley et al., 2017). The SPR sen-

sor showed enhanced sensitivity, relative to ELISA, with a LOD of $57.8 \times 10^3 \mu\text{g/ml}$. Additionally, the immunoassay-based SPR biosensor was also designed for β -Lg detection with an exceptional sensitivity of $0.164 \mu\text{g/ml}$ LOD (Ashley et al., 2018). The SPR technique was also documented in raw and processed milk comprising six samples per assay for continuous and quantitative detection of various milk allergens, such as β -Lg, α -La, LF, Ig, and BSA (Billakanti, Fee, Lane, Kash, & Fredericks, 2010).

Today, multiple research studies have directed their emphasis on the usage of digital sensors in biosensing devices, following recent up-to-date digital techniques and smartphone inventions (Hernández-Neuta et al., 2019). The effectiveness of these digital sensors for target detection in biological fluids has intensively addressed in recent research publications (Hosu et al., 2017). The implementation of smartphone technology with immunosensors detects various food allergens, but little research work is documented on the detection of milk allergens (Ross, Bremer, & Nielen, 2018). Improvements in immunosensors for slightly quicker detection of allergens, antibody-based approaches either (ELISA or immunosensor) have challenges in analyzing processed foods. Food matrix treatments lead to changes in denatured proteins, hydrolysis,

and protein conformation; therefore, antibodies might not detect them accurately (Castillo & Cassola, 2017). Furthermore, in the development of ultrasensitive immunosensors, the potency of the antibody is also quite significant.

6.3 | MS-based proteomics methods

MS has played a pivotal role in biological proteomics, enabling peptides and proteins to be detected, characterized, and quantified on a mass level (Yates, 2011). Due to the massive innovations in MS technologies and progresses in data informatics, food allergenomics appears as a potential approach in genomic and immunochemical-based methodologies for detecting allergens in complex food samples. MS method comprises discovery-based proteomics and targets analyte strategies to provide a platform for highly multiplexed allergen detection. Proteomics is a large-scale protein analysis in a specific biological system. Allergenomics uses proteomic approach for the detection of immunogenic proteins, whereas proteomics encompasses the analysis of protein sequences, interactions, modifications, functional activities, and their positions. Therefore, proteins can serve as biomarkers of many food properties in different areas of the food chain during food processing and distribution. Recent developments in proteomics are highly dependent on MS-based assays to take advantage of research on food structure, quality, safety, and biological activity of food components (Pandey & Mann, 2000).

MS systems provide several advantages, such as precision, flexibility, specificity, and robustness. Its sensitivity is higher than ELISA and PCR for multi-target detection with high accuracy in a single run (Picariello, Mamone, Addeo, & Ferranti, 2011). In comparison, cross-reactivity complications often associated with immunoassays are exempted because the target protein identification will not involve biological receptor (antibody) interaction, allowing the target analytes to be directly and clearly identified. Proteomic analysis of the samples typically consist of one or more isolation processes (e.g., electrophoresis and liquid chromatography [LC]) followed by MS assay at protein or peptide concentrations. Two main methods are employed to detect, quantify, and characterize food allergens, which are as follows:

- a. Bottom-up method, also recognized as peptide-based proteomics, which helps to digest proteins through enzymes such as trypsin and assess the subsequent peptide segments via MS (Pandey & Mann, 2000).
- b. Top-down method, in which the protein is segmented within the spectrometer, ignoring the protein's variable digestion phase (McClafferty et al., 2007).

For both methods, peptides and proteins are classified and identified by a comparison of experimental MS results with computed theoretical mass values obtained from the protein database; the proteins better matched are allocated to scientific results. Nowadays, the bottom-up method is most frequently used due to the recent low efficiency of the top-down methods (Brockmeyer, 2018). Such methods can be further subdivided into proteomic approaches based on gel and shotguns. Within gel-dependent proteomics, proteins are isolated from the gel and digested in the MS-analyzed peptides utilizing two-dimensional electrophoresis of gel. A better description of this method is peptide mass fingerprinting, where matrix-assisted laser desorption ionization time-of-flight MS collects and allocates the peptide mass to a protein depending on the tryptic digestion of the isolated protein (Perkins, Pappin, Creasy, & Cottrell, 1999).

A high-resolution quadrupole-orbitrap mass spectrometer is used to detect allergens in skim milk, egg, soya meal, and hazelnuts in highly processed matrices (Pilolli et al., 2018). The researchers examined specific acquisition mechanisms: full MS, selective data-dependent fragmentation monitoring of chosen ions (t-SIM/dd2), and synchronized monitoring of reaction. The results obtained highlighted t-SIM/dd2 as a preferred alternative based on multiple allergen detection parameters. This method permitted the quantification of 17 peptides, which belonged to the five allergens with a detection limit between 17 and 30 $\mu\text{g/g}$ depending on the allergen entity.

The detection of WP allergens in food items with MS technologies has indeed documented at a phenomenal speed. Multi-target approaches for specific WP allergens (β -Lg and α -La) with the reactivity of 0.1 to 5 mg/kg can be identified in multiple food matrices including infant formulations, baking products, snacks, and beverages. Ji et al., 2017 developed LC-tandem MS (LC-MS/MS) to validate and detect several milk allergens, in cookies, pancakes, and pastries, with a reported 0.2 mg/kg LOD for α S1-casein and α -Lg and 0.39 mg/kg for α -La (Ji et al., 2017). It is noteworthy that LOD is highly influenced by food processing, matrix effect, and target peptides while evaluating the same allergen (Costa et al.,). MS-based approaches are more effective for raw matrices, as is the case for ELISA and immunosensors than for processed matrices (Bhat et al., 2019). The demand for uniformity between approaches and validity by inter-laboratory research will eventually enable the development of rigorous experimental methods that facilitate allergen management in the food industrial sector. Despite all the well-documented advantages and numerous effective MS techniques, it demands high equipment costs, sophisticated tools, complex operations, and time-consuming and expensive data analysis.

6.4 | DNA-based approaches

At present, DNA-based allergen detection approaches have gained considerable attention due to higher sensitivity, accuracy, independence from potential biological implications linked with the development of antibodies, and increased heat stabilization of DNA molecules, primarily important for analyzing processed foods. Subsequently, DNA-based approaches have proved viable options than protein-based approaches, especially when examining highly treated food products (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017). For the analysis of allergens, DNA-based approaches usually focus on amplifying gene sequences encoding allergenic proteins while some other segments may also be used. Due to more reliable number of copies of cells of various tissues, single-copy nuclear DNA targets are known as the best choice for reliable quantitative approaches (Prado et al., 2016). Most of the data reported utilizing DNA-based approaches involves extending PCR to the initial target DNA sequences, using particular primers, which provide high precision assessments (Martinez-Esteso et al., 2020). A wide range of approaches are used for the detection of allergens, such as multiplex PCR, end-point PCR, PCR-ELISA and real-time PCR. However, recent promising innovative technologies have received considerable attention, such as real-time PCR combined with high-resolution melting (HRM) assessment, single-tube real-time PCR, DNA arrays, and LPA. Furthermore, recently genosensing and aptasensing methods also have been used to detect DNA.

6.4.1 | Real-time PCR

PCR is the well-known amplification method based on nucleic acid and is extensively used in the field of detection, due to its higher sensitivity, accuracy, and reliability. The PCR amplification method consists of three main phases: high-temperature denaturation (95°C), low annealing temperature (55–60°C), and mild temperature (72°C) (Čadková et al., 2015). PCR method is favorable as it can magnify smaller amounts of target DNA fragments by millions times if the freshly isolated double strands of DNA are employed as models for the next reaction cycle. The detection of food items by PCR is usually carried out by agarose gel electrophoresis. It is dependent on PCR technology and is capable of activating the amplification response in real-time with transparent functionality. The fluorescence signal aggregation can represent the magnification of its target DNA. The reliability of the PCR system for detecting DNA in food products is based on several parameters that need to be enhanced such as specificity

and sensitivity of DNA sample, priming, and probe configuration. By testing appropriate target DNA fragments and modifying reaction parameters, sensitivity and specificity of detection have improved.

Multiplex real-time PCR enables the concurrent amplification of numerous DNA fragments by incorporating a variety of primers. This approach was used to determine the DNA of major eight allergens: milk, hazelnut, peanut, celery, soya, egg, almond, and sesame. It showed high precision and specificity within the 0.01% range, lower for milk and egg due to low DNA content. The detection of WP allergens in food matrices is addressed in limited experiments, with real-time PCR being the primary technique. The potential benefit of real-time PCR methods is reliable with appropriate operational costs, good operating times, and minimal provisions for special facilities and professionals. Krunić et al., 2018 developed two hexaplex and tetraplex real-time PCR models with TaqMan samples for a combined analysis of a diverse range of food allergens including milk allergens having a sensitivity up to 0.64 µg/ml (Krunić et al., 2018). In another experiment, Yang et al., 2014 developed a real-time PCR method with a TaqMan specific groove binder sample to detect α -La in food products. It demonstrated a high sensitivity of bovine DNA 0.05 ng to monitor milk as an ingredient in 42 commercial products (Xiao and Qin, Wenju, and Qin, 2016).

Villa, Costa, Oliveira, & Mafra, 2020 developed a highly efficient and reliable PCR method for the identification and quantification of milk allergens in processed meat products, attempting to target the 12S rRNA mitochondrial gene of domestic cattle (cow). This method was capable of achieving LOD of 6 fg of milk DNA. A systematic approach was used in ham (fresh and cooked) and autoclaved sausage for the detecting milk protein concentrate to achieve sensitivity of up to 0.01% (w/w) and in fresh sausage mixtures 0.005%. This model specifically provided sufficient PCR output values by effectively validating blind samples and commercial samples while comparing them with immunochemical samples (Villa et al., 2020). With this research, innovative, highly specific, and efficient real-time standardized PCR methods have been designed for the detection and characterization of milk components in meat products, which could make an active contribution to better control WP allergens by the food industry, with a view of protecting the health of sensitized consumers.

6.4.2 | Real-time PCR in conjunction with HRM analysis

HRM technique is an advanced alternative approach to detect and classify closely related species, pathogenic microorganisms, genetically modified organisms, and food

allergens (Druml & Cichna-Markl, 2014). HRM typically involves a post-PCR closed tube analysis based on the real-time melting mechanism of PCR products. New progress in high-resolution equipment and processing of DNA-binding fluorescent dyes have supported such analysis (EvaGreen). In particular, such dyes are capable of reacting with double-stranded DNA because they are used at high levels and are less likely to induce nonspecific amplification or inactivation of PCR, allowing minor fluorescent shifts to be detected during incremental melting of PCR components. It enables minor differences, including the shift of one base between two nucleotide segments of the same size and composition (Madesis, Ganopoulos, Bosmali, & Tsaftaris, 2013).

The application of HRM technology can be functional to food sector in two main areas including DNA barcoding and microsatellites (Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013). Currently, HRM techniques are operated to authenticate foods because their ingredients differ from the species according to the melting profile of certain PCR products. In case of detecting allergens, HRM approach is recently used to detect and differentiate almonds in food-stuffs from other *Prunus* fruits (Costa, Mafra, Kuchta, & Oliveira, 2012) while detecting and differentiating mollusk (*Crassostrea* oysters) (Jin, Li, Kong, Yu, & Zhong, 2015).

6.4.3 | End-point PCR and PCR–ELISA

End-point PCR is the easiest and probably most economical method for DNA amplification. The only limitation is that the end-point PCR method needs an extra step to determine the amplification material typically performed through gel electrophoresis, which is simply a qualitative technique. PCR–ELISA is an immune detection method, which integrates PCR amplification with ELISA to quantify the PCR material. The immunosorbents are the products of polymerases in the chain of immunosorbent enzymes (Slowianek & Majak, 2011). The DNA can be assessed by changes in color that develops from an enzyme–substrate reaction due to the interaction of the protein to a particular enzyme-labeled antibody. It is a highly precise, sensitive, effective, consistent method, but can only be considered semiquantitative when measuring target DNA within the PCR amplicon because it can be quantified at the completion of the PCR reaction and not during the exponential point of growth, such as in real-time PCR. Digoxin-labeled amplified samples of peanut, soybean, and hazelnut DNA were detected due to the hybridization with biotinylated probes that were incapacitated on a streptavidin-modified interactive (Tortajada-Genaro et al., 2012). To compare PCR–ELISA with sELISA, an analysis was conducted for detecting hazelnuts and it

was observed that both approaches have similar sensitivity while PCR–ELISA offers better specificity (Holzhauser, Stephan, & Vieths, 2002). Moreover, the sensitivity and specificity of PCR–ELISA was comparatively higher compared with real-time PCR without specific arrangements and costly tools (Hadrach et al., 2011).

6.4.4 | Quantitative real-time PCR

Quantitative real-time PCR enables DNA amplification to be monitored as generated by supervising fluoresce signals during the amplification of the target DNA (Higuchi, Dollinger, Walsh, & Griffith, 1992). The signal increases significantly as the amount of product builds up, and its signal rate declines as the core components of the reaction are vanished (Kubista et al., 2006). Currently, numerous organizations have developed qPCR-based techniques for food analysis including food-allergenic ingredients. This approach is well applied to highly processed samples with fragmented DNA and shows better results because of gel visualization (Prado et al., 2007). The qPCR offers a broad variety of food analysis applications including the likelihood of utilizing short fragments relative to the endpoint PCR (Hird et al., 2006). In qPCR technique, two detection substances are commonly used. The first is a binding DNA dye, typically a cheaper substitute called SYBR-Green, while the second one is a hydrolysis test technique, which typically uses TaqMan probes to boost the test accuracy. In addition to binding the PCR primers, a successful identification requires an efficient binding of a certain probe (López-Andreo, Lugo, Garrido-Pertierra, Prieto, & Puyet, 2005).

qPCR has a quantitative potential for food allergenic components, but it is important to specifically determine the quantitative analysis of these ingredients to have accurate findings. First, a relative quantitative approach cannot be used as in many situations, like the quantification of GMOs, the shift in the concentration of target DNA cannot be correlated with the original reference gene. However, many of the approaches are focused on absolute calculation. These approaches are dependent on the standard curve generated in a parallel series of reactions by amplifying the target DNA. Such methods demand that both the plasmid or genomic DNA and the observed DNA be amplified with the equivalent efficacy to achieve reliable quantitative results (Sivaganesan, Seifring, Varma, Haugland, & Shanks, 2008). Therefore, the quantitative results are generally represented as the DNA concentration or the DNA copy number; therefore, the methods established will determine the resulting percentage of allergenic components to compare the findings with the amount displayed on the product. To tackle these challenges, researchers

TABLE 4 Different biosensing platforms (aptasensors) for detection of whey allergens

S. No.	Type	Target analyte	Biosensing platform	Mechanism of transduction	Detection limit	References
1	Aptasensors	β -Lactoglobulin	Aptamers immobilized graphene/carbon	Electrochemical	20×10^{-6} $\mu\text{g/ml}$	Eissa & Zourob (2017)
2		β -Lactoglobulin	23-nucleotide aptamer-amphiphile	Enzyme linked apta-sorbent assay	10 nM	Clemente et al. (2019)
3		β -Lactoglobulin	Aptamer coupled poly(aniline-co-anthranilic acid)/graphite	Electrochemical	0.053 $\mu\text{g/ml}$	Lettieri et al. (2020)
4		Lactoferrin	Bivalent aptamer linked to fluorescein isothiocyanate dye and silver decahedral nanoparticles	Fluorescence polarization	0.1×10^{-3} $\mu\text{g/ml}$	Chen et al. (2017)
5		β -Lactoglobulin	Microfluidic paper-based device with aptamer conjugated gold nanoparticles/graphene	Colorimetry	12.4 nM	Tah et al. (2018)

conducted a food product calibration graph, which was spiked at different levels of allergens to simulate the identification of actual food samples (López-Calleja et al., 2013).

6.4.5 | Geno-sensors

Geno-sensors or DNA-biosensors are widely accessible to detect individual cDNA fragments, mostly because they are quick, automatic, cheap, and highly selective. Geno-sensors are analytical instruments that are dependent on the hybridization of two different complementary strands: a singular sequence of oligonucleotides and a complementary sequence (RNA or DNA) (Cugia, 2011). In case of immunosensors, a transducer that generates a detectable signal achieves the known interaction.

6.4.6 | Aptasensors

Aptamers are nucleic acids that act as ligands and emerge as modern analytical reagents for the use of various biosensing transduction systems. Aptasensor is an affinity bio-sensor that immobilizes aptamers on the surface of the electrode and acts as a bio-receptor for selectively binding the marked analyte. The binding induces a shift at the transducer layer in the electrical or optical analytical signal, which can also be easily checked. It provides several advantages with regard to their high affinity and binding target specificity, and simple labeling with multiple reporter substances (Amaya-González, De-Los-Santos-Álvarez, Miranda-Ordieres, & Lobo-Castañón, 2013).

At present, aptamers are substituting certain components of bio-recognition, particularly antibodies with similar functionality due to remarkable advantages of increased stabilization, low development costs, and more flexible options available (Khedri, Ramezani, Rafatpanah, & Abnous, 2018). Though antibodies are commonly used as biological reagents, however, there are still a lot of antibody-related complications, such as ethical concerns of animal usage, lengthy methods, and increased production costs. Aptamers have a greater affinity and specificity for binding targets compared to antibodies. Besides, these can be easily modified with new functional groups owing to the potential for modifiable denaturation (Sas-solas, Blum, & Leca-Bouvier, 2009). The detection of WP allergens using various bio-sensing platforms (aptasensors) is presented in Table 4.

The systematic evolution of exponential development ligands (SELEX) demonstrated substantial work on the development of aptamers against specific targets in the last few decades (Acquah et al., 2020). SELEX based on milk allergens, fluorescence-activated cell sorting (AgNPs-FACS), and silver decahedral nanoparticles were recognized particularly for reactive and efficient screening of aptamers against major allergens in milk (Yu et al., 2019). Shi et al. (2018) developed a fluorometric aptamer based on carbon dots and magnetic nanoparticles as a signal marker for the detection of β -Lg. The primary objective of this technique was on aptamer hybridization, magnetic nanoparticle immobilization, and the complementary sequence of carbon-dotted oligonucleotides. Aptamers generally bind with proteins in the presence of β -Lg, which results in a gradual release of the

complementary sequence into the fluid. As the nanoparticles were magnetically separated, the solution supernatant produced emitted carbon dots, which were subsequently evaluated by fluorometry. β -Lg was observed in the range of 0.25×10^{-3} to $50 \times 10^{-3} \mu\text{g/ml}$ and a LOD of $37 \times 10^{-6} \mu\text{g/ml}$. In hypoallergenic formulas, this process for detecting β -Lg was successfully tested. Furthermore, a highly specific β -Lg-23 aptamer binding affinity for β -Lg was developed through enzyme-linked apta-sorbent assay (Clemente et al., 2019). Within 20 min, the proposed bio-sensor provided successful identification of β -Lg with a $0.18 \mu\text{g/ml}$ or 10 nM LOD. The results were similar to the widely available Crystal Chem ELISA kit with LOD of 0.30 to $10^3 \mu\text{g/ml}$ with a 3 hr analysis duration.

Chen et al. (2017) developed a novel bivalent aptasensor for the detection of LF, which was based on fluorescence polarization. In this technique, high rotation sensitivity can be accomplished by conformational or structural modifications caused by the target. In aptasensor, two split aptamers in silver decahedral nanoparticles and isothiocyanate dye molecules that function as stimulators have examined and modified to react with fluoresce. The aptamers develop a split aptamers-target complex after binding to LF, which reduces a gap within the silver nanoparticles and dye. In this case, an improved fluorescence effect and mass variation can be observed because of silver decahedral nanoparticles. The bivalent aptasensor gave three times more sensitivity with LOD of 1.25 ppm in milk powder compared to conventional aptamer-based homogeneous assays.

In addition, paper-based microfluidic systems receive significant recognition for point-of-care (POC) monitoring (Kumar et al., 2019). To perform the diagnostic test, these tools were based on the normal capillary action of the cellulose substrate. Tah et al., 2018 used a paper-based microfluidic tool to conjugate aptamers with gold nanoparticles and graphene oxide. In this case, functional gold nanoparticles provided a bridging role of different graphene oxide layers by pi-pi stacking, which can be initiated by the combination of sDNA aptamers of gold nanoparticles (Tah et al., 2018). The microfluidic platform provided a high colorimetric detection sensitivity for β -Lg having LOD of 12.4 nM . Such platforms are simple, cheap, and accurate while designed to detect WP allergens in less time.

7 | CONCLUSION AND FUTURE PROSPECTS

WPs are among the sustainable products of milk processing (often neglected at present) that might have multiple uses in the food and healthcare sectors owing to their significant nutritional content and unique functional quali-

ties. However, WPs are a major source of food allergens and trigger adverse reactions in sensitized individuals. β -lg, α -la, and caseins are the primary allergens from CMP sources. To eliminate WP allergenic reactions, researchers all over the world are seeking strategies to overcome or modify these allergens. Nonthermal processing technologies have demonstrated potential in minimizing the allergenicity of WPs, as well as many noticeable benefits such as consumer safety, low energy consumption, and eco-friendly. Processing causes modification in WPs that can have a direct effect on their vulnerability to gastrointestinal digestion and ultimately on their immune response. Consequently, the allergenic potential of WPs can be reduced by selecting suitable parameters while processing. Although reduction in allergenicity has been reported with certain processing methods, however, no method is fully efficient.

Several regulations and legislation have enacted to safeguard public health and to ensure the life quality of individuals vulnerable to WP allergens. Therefore, to enforce the labeling regulations and track the existence of hidden allergens, it is important to establish highly efficient, selective, and reliable methods for detecting and quantifying milk allergens. ELISA method has proven to be successful and reliable among the available techniques for detecting milk allergens in food items. However, no commercially available kit has yet confirmed in a collaborative study, with most being limited to detecting intact protein in raw foods. Recently, in conjunction with innovative nanotechnology and sensing devices, biosensors (immunosensors and aptasensors) offer rapid, cheaper, simpler, and multiplex detection of WP allergens compared to traditional methods such as real-time PCR, ELISA, and LC-MS. The MS platforms have demonstrated their effectiveness in the identification of allergenic WPs that can be used as a validator tool to identify multiple allergens. While DNA-based method could be a successful alternative to other analytical methods for the detection of WPs in foodstuffs, due to their lesser vulnerability to food processing.

To what extent the existing methodologies can safeguard allergic consumers or even detect allergens in food remains uncertain. The approaches used for detecting low-concentration allergens in foodstuffs are dependent on various factors, including extraction practices, food matrices, food processing methods, and the accessibility of allergens. Despite certain shortcomings, the existing approaches available to detect WP allergens play an important role in informing allergic consumers, which is crucial to protect their health from the exclusion diet. We assume that in the future the detection of food allergens will be affordable and more reliable. After enacting and optimizing these innovative detection methods, it would be possible to detect allergens easily not only in food

manufacturing sectors but also in families using commercial food products.

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AUTHOR CONTRIBUTIONS

Mati Ullah Khan—Conceptualization; methodology; writing original draft. Zhenxing Li—Conceptualization; investigation; supervision.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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