

# PROTEOMIC APPROACH TO DAIRY PRODUCTS PROCESSED WITH INNOVATIVE TECHNOLOGIES

## Abstract

The thermal processes used in milk pasteurization lead to undesired chemical, physical, and sensory changes, such as browning, a cooked taste, and loss of essential vitamins or amino acids, depending on the applied heat parameters and conditions. Interest in new technologies has begun to increase to minimize the negative effects of conventional heating methods and to meet consumers' demand for milk with ideal properties. These technologies can be classified as thermal and non-thermal processing methods. Non-thermal processing involves techniques that are effective at ambient temperatures and do not involve lethal heat treatment. They offer advantages such as energy savings, pathogen destruction, longer shelf life, and better preservation of nutrients compared to traditional approaches. Some new technologies studied in laboratory and pilot scales for milk processing include ohmic heating, microwave heating, radio frequency heating, high-pressure treatment, irradiation, pulsed electric field, and ultrasonication methods. Generally, these new technologies are used with fermentation and enzymatic hydrolysis combination as they are less disruptive to proteins than conventional methods. They have the potential to increase yields, modify peptide configurations, and release a high rate of bioactive peptides compared to existing heating technologies.

These innovative methods focus on enhancing microbial food safety and enzyme inactivation in food research and have shown positive and reliable results in reducing the microbial load in various food products, including milk. Besides decreasing microbial load, these techniques also influence food compounds in physical,

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chemical, and sensory properties. Among the components in milk, milk proteins with technological and functional properties are most affected. Technologically important properties of milk proteins, such as foaming, gelling, emulsification, and solubility, play a significant role in stabilizing desired characteristics in the final product. The gelling properties of milk in yogurt and cheese processing, the potential for bioactive peptide formation in fermented milk products, and the foaming, emulsification, and solubility properties in ice cream and gastronomy applications are noteworthy.

In addition to the technological impact of milk processing, its health implications are also crucial. Changes in the biochemical structure of milk during processing can reduce the bioavailability and digestibility of milk proteins. While new techniques effectively reduce the microbiological load, it is essential to ensure that proteins retain a structure that can be assimilated by the digestive system in the body. The food industry and researchers should evaluate the efficacy of food products in terms of both food safety and bioavailability. This review emphasizes denaturation, functional and structural changes in milk proteins from a proteomic perspective. Additionally, it investigates the potential of bioactive peptide formation through applied innovative processes, their impact on protein digestibility, structural changes in proteins, and their acid and rennet gel formation potentials. This comprehensive review can guide researchers and industry leaders in evaluating green and sustainable technologies and developing new applications.

**Keywords:** milk proteins; bioactive peptide production; digestibility; gelling properties; physicochemical changes

## I. INTRODUCTION

Milk has a high nutritional value due to its high content of lactose, protein, fatty acids, vitamins, minerals, and essential trace elements. However, the presence of these components creates an ideal environment for the growth of microorganisms, including pathogens, leading to rapid milk spoilage. Aside from its nutritional benefits, dairy products have been shown to possess functional food properties, effectively contributing to the prevention of cardiovascular diseases, osteoporosis, metabolic disorders, and cognitive issues [1]. Milk proteins also led to their utilization in various pharmaceutical formulations due to their high functional and nutritional properties [2]. It is crucial to reduce or eliminate the microbial load and enzymes present to ensure the safe consumption of dairy products [3].

One key aspect to consider is that milk and dairy products have different physicochemical structures. For instance, milk is in liquid form, yogurt is in gel form, and cheese is in solid form [4]. The physicochemical properties of dairy products, such as the fat-to-protein ratio, hardness, and elasticity, can be altered during processing, affecting the interactions and digestibility of milk proteins in the body [4,5].

Thermal processes, including pasteurization and sterilization, are commonly employed worldwide for milk processing [3]. However, these traditional heating methods may lead to undesired chemical, physical, and sensory changes, such as browning, the development of a cooked flavor, and the loss of essential vitamins or amino acids, depending on the heat parameters and conditions applied [6,7,8].

Heat treatments are implemented to eliminate pathogens and spoilage-causing microorganisms to combat milk's perishability and enhance its shelf life [9,10]. Researchers are increasingly focusing on novel technologies to minimize the aforementioned negative effects and meet consumer demands for milk with ideal properties [11]. These new techniques can be categorized as thermal and non-thermal processing methods. Non-thermal processing refers to techniques that are effective at ambient temperatures and do not involve lethal heat treatment. They offer advantages such as energy conservation, pathogen destruction, extended shelf life, and better preservation of nutrients compared to traditional approaches [12,13]. Some of the new technologies explored in laboratory and pilot scale studies for milk processing include ohmic heating, microwave heating, radio frequency heating, microfiltration, high-pressure treatment, irradiation, pulsed electric field, and ultrasonication methods [14]. Interest in innovative process methods has risen recently due to large-scale processing and continuous system operations. It is also considered sustainable and environmentally friendly due to its energy efficiency. On the other hand, innovative processes have disadvantages such as high installation costs, continuous control of microbial effectiveness, affecting the sensory properties of food, and inability to use all systems in every food depending on the physical structure of the food.

Researchers initially focused on preventing microbial growth in milk; however, studies on the physicochemical structure, bioavailability, and digestibility of proteins using the applied methods started later. Even though new techniques perform the microbiological load reduction completely, it should be considered that the proteins must be in an appropriate structure that can be utilized by the digestion system in the body. The food industry and researchers must evaluate the food products' effectiveness in terms of both food

safety and bioavailability. Therefore, this section emphasizes the denaturation, functional, and structural changes of milk proteins by proteomic approaches.

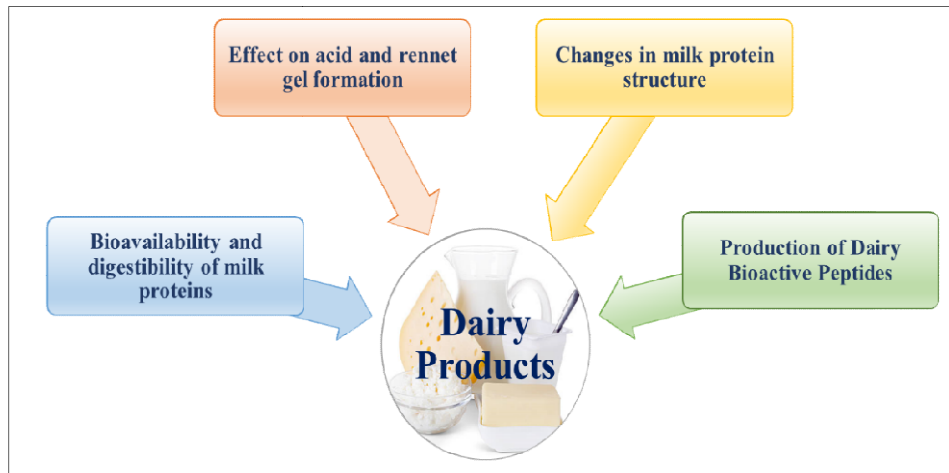
## II. PROTEOMICS TO ASSESS THE QUALITY OF MILK AND MILK PRODUCTS

Heating milk is the most common preservation method applied before consumption or during the production of various dairy products. The process involves inactivating microorganisms and enzymes through heat treatment to ensure the milk is safe for consumption or further processing. In many countries, heat treatment of raw milk at specific temperatures and durations is legal enforcement. However, low-temperature treatments may not fully achieve the desired microbial safety, while high-temperature applications can lead to undesired changes in the protein structure of milk components. In this section, the impact of innovative processes on milk proteins has been discussed by emphasizing four main aspects: changes in protein structure, formation of bioactive peptides, digestibility, and the properties related to acid and rennet gel formation (Figure 1).

Processes applied to milk cause conformational changes in milk proteins. Most of the time, these changes are described as the unfolding of the tertiary and secondary structures of proteins, the breaking apart of proteins, an increase or decrease in the number of free sulfhydryl groups, the formation of hydrogen bonds, a decrease in the number of  $\alpha$ -helix and  $\beta$ -sheet forms in protein solutions, structural changes, and interactions between hydrophobic groups [15]. Physicochemical changes in protein structure are desirable in certain dairy product processing. Heat treatment can cause reactions between milk proteins, leading to the denaturation and aggregation of whey proteins and the formation of complexes with caseins [16-19]. This protein interaction is a desired reaction for improving viscosity and texture in yogurt and ice cream. However, these physicochemical changes are undesirable in cheese production. Furthermore, depending on the milk composition, heat treatment may adversely affect flavor and color properties, resulting in issues like browning and a cooked taste [20-26]. Whey proteins, more heat-sensitive than casein, can form disulfide bonds and non-covalent interactions between casein micelles and  $\beta$ -Lactoglobulin at high temperatures [27,28]. The high-temperature heating, such as ultra-high-temperature (UHT) treatment, causes changes in the rheological properties of proteins. While heat treatment improves protein hydrolysis and digestibility compared to raw milk, UHT treatment may also result in structural modification, denaturation, fragmentation, and aggregation of proteins, including immunoglobulins [29]. Moreover, through the consumption of UHT milk by infants, only a small number of peptides have been generated from alpha-lactalbumin, and no immunoglobulin peptides were found in the coagulum after digestion [30].

On the other hand, milk proteins are an excellent source for producing bioactive peptides. However, the high temperatures applied during conventional heat treatment cause protein aggregation, limiting subsequent microbial fermentation and enzymatic hydrolysis [31].

Except for a short brief of conventional heat applications, innovative process technologies, such as ohmic heating, microwave heating, radio frequency heating, microfiltration, high-pressure treatment, pulsed electric field, and ultrasonication, have been explored for their effects on milk proteins, and will be discussed following in details.



**Figure 1:** Pros and cons of novel processing technologies on milk proteins

### III.EFFECT OF IINNOVATIVE PROCESING ON DAIRY PROTEINS

- 1. Proteomic Approach in Cold Plasma Treatment on Milk Proteins:** *Cold Plasma Treatment*; Apart from solids, liquids, and gas, there is also a state of matter known as "plasma." While solids, fluids, and gases are composed of atoms and molecules, plasma consists of atoms, molecules, positive-negative ions, radicals, excited atom molecules, electrons, and photons. Additionally, it produces significant amounts of ultraviolet (UV) radiation and ozone. These various particles existing in plasma have led to their versatile use in many aspects of our daily lives. Plasmas find application in lighting technology due to the light they emit, in coating and electronic technology due to the presence of ions, in several fields including medicine, food, and agriculture due to the radical particles, and plasmas are also used for sterilization purposes using UV and ozone [32].

Cold plasma is an innovative technology belonging to the non-thermal group, and its applications in the food industry are relatively new [32]. Like other innovative technologies, cold plasma has been extensively studied, primarily in the prevention of microbial growth and enzyme inactivation, especially in milk and milk-based beverages, and its positive effects have been established [33,34,35]. It is considered an approved technique for ensuring microbial food safety in its food applications. The reaction mechanism involves the oxidation of microorganisms' cell membranes and electrostatic disintegration, resulting from the effect of the formed radical particles [36]. Meanwhile, the primary target is to influence the protein structure of DNA [37]. However, it may also affect the proteins in the food.

- 2. Changes in Protein Structure:** Atmospheric cold plasma can cause structural changes in milk proteins, although there have not been found remarkable effects on organoleptic properties. For instance, when cold plasma is generated using nitrogen and oxygen gases simultaneously, it leads to protein clusters on the  $\beta$ -sheet structure. Due to their high sensitivity to nitrogen-oxygen plasma, whey proteins like  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin are denatured, and their ratios change after processing. The primary reason behind this is the partial denaturation of proteins, which leads to the interaction between

whey proteins and casein. The existence of oxygen also supports protein oxidation, affecting amino acid side chains and causing non-covalent bonds to unfold. The protein  $\alpha$ -helix structure generally has a more stable protein structure than the  $\beta$ -sheet, and this structural state is related to the status of intermolecular H-bonds.

Also, during the process, the sulfhydryl groups in the whey proteins unfold and form disulfide structures with other proteins [33]. This conformational change supports the notion that  $\beta$ -sheets are relatively increased. On the other hand, when cold plasma is applied to sheep's milk, which has a high-fat content, an increase in  $\beta$ -turn structures and a decrease in helical conformation, similar to cow's milk, have been observed [38]. Additionally, cold plasma application alters the microstructure of protein drinks and affects their fluidity properties [39].

- **Gelling Properties:** From an industrial perspective, cold plasma treatment of milk offers many advantages. Partial denaturation of whey proteins by plasmas obtained from different gases is a positive step in developing gelling and foaming properties. Again, it results in the desired increase in viscosity during beverage production.

Cold plasma treatment, with its partial denaturation effect, causes coalescence and aggregation in other proteins through disulfide bonds. When milk is treated with N<sub>2</sub>-O<sub>2</sub> cold plasma for 2 minutes, the free SH level goes down. This is because the sulfhydryl groups bind to each other in the beta layer to make disulfide bonds. This phenomenon significantly affects the viscosity and hydrophobicity, which play a crucial role in the hardness of acid gel in products like yogurt that require a thick and firm texture [40].

By treating milk proteins with cold plasma to make their beta layers less water-loving, they can stick together with other proteins to form a protein gel structure that is good for dairy products. However, the same reaction is undesirable for the required interaction between rennet and casein, such as in cheese. For this reason, while the effects of cold plasma are desired in products with yogurt or gel properties, they are undesirable as they will cause a low yield in cheese production [32].

- **Bioactive Peptide Formation:** Processes applied to milk have been found to affect the nutritional properties of the product, as mentioned earlier [34,5,41]. Research has shown that cold plasma application results in ACE and antioxidant activity. Therefore, an average processing time of 15 minutes is recommended [41]. In addition to drinking milk, cold plasma application to flavored milk also increased the total phenolic content, resulting in a product with ACE activity. However, when the processing time is prolonged, the phenolic component and ACE activity level decrease [42]. Also, it causes protein oxidation by increasing the total aldehyde content in milk. It highlights the significance of cold plasma parameters in determining the final quality and functional properties.

### 3. Proteomic Approach in High Hydrostatic Pressure Treatment on Milk Proteins:

- **High Hydrostatic Pressure Treatment:** High-pressure treatment (HPP) is a non-thermal technology used for solid and liquid foods, applying pressures ranging from

100 to 1000 MPa to deactivate all microorganisms. HPP is applied for various purposes, including reducing allergen effects, inducing bioactive components, homogenization, enzyme inactivation, meat tenderizing, and microbial inactivation in the food industry [43].

HPP is considered advantageous as it has minimal negative effects on the organoleptic properties of food, such as taste and appearance, as well as on bioactive compounds, vitamins, and nutrients [44]. However, one of its disadvantages is that when heat and pressure are applied simultaneously during high-pressure treatment, it can cause changes in protein structure and alter physicochemical properties [45]. The HHP process can be applied using both continuous and batch methods. In the batch system, the desired pressure is applied to the packaged product, followed by a waiting period, and then the pressure is reduced to remove the product. In the continuous system, pressure is applied by pushing the product through space using a piston [46,47]. In both methods, HPP can lead to desired or problematic changes in milk proteins.

- **Changes in Protein Structure:** The application of high hydrostatic pressure causes irreversible decomposition or shrinkage of casein micelles [48]. At the same time, increasing the pressure leads to elevated protein hydrophobicity, volume reduction, and dissolution of calcium and phosphorus from the colloidal phase into the aqueous phase of milk [49]. On the contrary, whey proteins undergo reversible molecular unfolding at low pressures. Beta-lactoglobulin, among milk protein fractions, is more sensitive to high-pressure treatment, while alpha-lactalbumin exhibits substantial resistance. Notably, even the most sensitive protein, lactoglobulin, remains undenatured at pressures up to 150 MPa [50]. As pressure levels rise, whey proteins denature, interacting with non-casein nitrogen present in milk [51]. Besides, high pressure induces the liberation of thiol groups in beta-lactoglobulin, conferring antioxidative properties upon it [52]. High-pressure treatments can modify the hydrophobic groups of milk proteins, influencing their gelling, emulsifying, foaming, and water-binding capacities, providing advantages in the production of yogurt-like products [53]. It is evident that high pressure positively affects the formation of bioactive peptides in high-pressure-treated milk or dairy products. For instance, it has been noted that ACE inhibitor activity increases in cheese subjected to high pressure. This increase is attributed to proteolysis and the generation of free bioactive peptides resulting from high-pressure-induced changes in the natural enzymes of milk [54, 55,56]. The bioactive peptides primarily formed through pressure mainly consist of high molecular weight and hydrophobic peptides due to  $\beta$ -LG hydrolysis [57,58]. Arguably high pressure has a limited impact on the formation of bioactive peptides. This limitation arises from high pressure only being insufficient to break covalent bonds and unfold proteins. To achieve superior results, combining high pressure with enzyme additions through enzymatic hydrolysis is necessary [54].
- **Bioactive Peptide Formation:** High-pressure pretreatment of milk proteins facilitates the formation of bioactive peptides, reducing the allergenicity of whey proteins and enhancing their antioxidant activity [60]. Additionally, high-pressure pretreatment combined with peptic enzymes leads to the hydrolysis of almost all  $\beta$ -lactoglobulin, producing bioactive peptides with heightened antioxidant capacity [61]. As mentioned

earlier, high pressure applied to cheeses fosters the formation of peptides with antioxidant activity and ACE-inhibitory potential [54]. Proteolytic enzymes such as chymotrypsin and pepsin can be employed as alternative methods to generate hydrolysates from high-pressure-treated milk proteins for hypoallergenic infant formulas [55]. Structural changes in milk proteins caused by high-pressure applications are crucial factors affecting functional properties and digestibility [62]. Generally, high pressure impacts the structural and functional properties of milk protein fractions, particularly whey proteins [54,63], and these changes can contribute to protein digestibility treated with high pressure. Protein denaturation and aggregation are two critical reactions influencing digestibility, and high pressure has been suggested to induce these reactions [63]. High protein-containing dairy products, such as cheese, are affected by high-pressure treatments by increasing ripening and proteolysis. This effect primarily results from the augmentation of enzymatic activities, particularly enzymatic hydrolysis, rather than the direct breakdown of proteins by high pressure. While it enhances plasmin activity in cheese made from raw milk, it induces the enzyme activities of starter cultures in other cheeses [54,64].

- **Digestibility of Milk Proteins:** By intensifying high pressure, it may affect the charge balance in milk, especially the electrostatic and hydrophobic interactions, leading to the unfolding of casein and the formation of casein sub-micelles [65]. The increase in casein submicelles enhances the surface area of casein, thereby facilitating their digestion [66,67]. While milk proteins, such as casein and  $\alpha$ -lactalbumin, exhibit substantial resistance to high-pressure applications [63,68].  $\beta$ -lactoglobulin is more sensitive and forms thiol-disulfide bonds with caseins under the influence of pressure [69,70]. In most cases, the preferred approach involves using trypsin or chymotrypsin enzymes in conjunction with high pressure [71].
- **Gelling Properties:** Studies have shown that acid gels generated by high-pressure-treated milk exhibit superior hardness and fracture resistance. The exposure of hydrophobic regions in acid gels made from milk with reduced fat content leads to an 8-9 fold increase in gel hardness. Additionally, these gels show increased resistance to syneresis during storage, which is associated with a decrease in the pore size of the gel [51]. When yogurt is made from high-pressure-treated milk, the gel structure changes due to alterations in the water-binding capacity of milk proteins, resulting in decreased sensitivity to syneresis during storage [72]. However, it has been discovered that the viscosity of yogurt made from high-pressure-treated milk is lower [73]. The acid or rennet gelling structures can be improved by treating high-pressure-treated milk with a cross-linking enzyme such as transglutaminase [74,75].
- **Formation of Rennet Gel:** High pressure has been found to increase the curd hardness and decrease the coagulation time in the generation of rennet gel. However, this structural change did not adversely affect protein digestibility. Cheese produced from pressurized milk has a higher moisture content, resulting in a pasty structure. The increase in moisture is a result of denaturation in whey proteins and casein, which also leads to an increase in water-holding capacity [53].



#### 4. Proteomic Approach on Microwave Heating (MWH) and Radio Frequency Heating (RFH) of Milk Proteins:

- **Microwave Heating (MWH) and Radio Frequency Heating (RFH) Treatment:** Microwave and radio frequency heating are innovative pasteurization techniques to ensure food safety and increase shelf life by providing rapid and volumetric heating. These methods fall under electromagnetic heating techniques, along with other non-thermal methods like high pressure, pulsed electricity, and ultrasonication. They are being developed as alternative approaches to conventional processing methods to avoid quality loss in heat-sensitive foods [76]. The steam heating methods heat food from the outside to the inside, which can cause denaturation, burns, and nutrient loss in the outer parts before the core temperature reaches the desired level. Moreover, the heat transfer in steam-based methods is relatively slow [77]. However, electromagnetic heating systems' efficiency depends on the dielectric properties of the material being heated. Most food products are dielectric materials, meaning they have poor electrical conductivity, often associated with poor thermal conductivity [9]. Direct interaction between food and electromagnetic energy significantly reduces the time needed to reach the target temperature, resulting in improved organoleptic qualities, appearance, and nutritional values of the product [78].

Microwaves operate within 300 MHz–300 GHz, while radio frequency waves range from 1 to 300 MHz [79]. In both electromagnetic systems, polar molecules try to align themselves with their charge in response to the applied electric field, causing friction losses as they rotate based on the electric field charge. This internal intermolecular friction, resulting from the polarization of dipolar molecules by the electric field, generates heat. Additionally, heat is produced due to friction between charged ions in the food during ionic polarization [80].

Microwave ovens are used in homes due to their fast and efficient heating, low energy consumption, and easy maintenance. In the food industry, microwave treatment is preferred for pasteurization, enzyme inactivation, and sterilization of food [81,82]. However, radio-frequency heating treatment is mostly employed in industries as an alternative for drying, post-harvest treatment, and fumigation [79]. While the thermal effects of electromagnetic waves result from molecular friction, non-thermal effects, such as protein denaturation and unfolding, are caused by molecular rearrangement.

- **Changes in Protein Structure:** Microwave processing is a preferred method in the food industry to reduce microbial load, increase shelf life, and improve the functional properties of food [83,84]. In the dairy industry, microwave treatment is performed for dissolution, rapid concentration, drying, and as an antibacterial function.

In the microwave heating method, the way molecules interact (through ionic conduction and dipolar rotation) doesn't change the structure of proteins much, except that proteins become more hydrophobic and slightly lose their shape. Most of the time, microwave heating helps enzymes work by denatured and unfolding proteins, which makes proteolytic activities possible [85]. Microwave heating is more effective in denaturing milk proteins, especially whey proteins, than conventional heating [86].

- **Bioactive Peptide Formation:** Microwave heating is used for extracting bioactive compounds from plants due to its rapid heating capabilities. It facilitates rapid proteolysis and the production of bioactive compounds when used with proteolytic enzymes [87]. It is often used as a pretreatment to denature proteins and open regions for interaction with fermentative enzymes [88]. In cheese production, applying microwaves to cheese milk leads to antioxidant activity and the formation of ACE inhibitor peptides [54]. Microwave as a pretreatment contributes to enzymatic proteolysis of whey proteins, making them more susceptible to pepsin digestion [89]. The concomitant use of immobilized enzymes with microwave treatment increased proteolysis in whey proteins [90]. Microwave applications are preferred due to their fast operation, cost-effectiveness, simplicity, convenience, and repeatability [91].
- **Digestibility of Milk Proteins:** The functional and structural properties of milk protein fractions, which can influence protein digestibility, can be affected by microwave treatment of milk and milk products. In vitro, gastrointestinal studies using pepsin and trypsin on microwave-treated protein solutions show an increase in digestibility and a significant increase in the rate of hydrolysis in milk proteins [92]. Microwave application for cheese produced from treated milk causes an increase in proteolysis by supporting enzymatic activities, similar to high-pressure treatment [54]. Microwave processing can promote the hydrolysis of  $\beta$ -lactoglobulin to reduce its allergenicity, with the support of pepsin and chymotrypsin. The denaturation of proteins, unfolding of secondary and tertiary structures, and providing structural changes due to microwave processing make them suitable substrates for enzymes [93].
- **Gelling Properties:** Microwave-treated whey proteins can produce better-structured gels than the conventional method. And these gels show a more homogeneous structure, while steam heat applications result in more heterogeneous rheological properties. The gel structure also varies with pH, becoming tighter and more homogeneous at higher alkaline values (higher pH) and forming a fine thread network structure at lower pH values [94]. The heating rate also affects the gel structure, with higher heating temperatures reducing process time and changing protein kinetics, denaturation degree, and mechanical properties. Slower heating leads to slower intermolecular interactions and denaturation, resulting in a tighter and more homogeneous gel structure [95].

## 5. Proteomic Approach of Ultrasound Treatment on Milk Proteins

- **Ultrasound Treatment:** Ultrasound treatment, also known as ultrasonication, involves the application of sound waves to food at frequencies above the hearing range of humans [96]. It is considered a non-thermal technology, but its effectiveness in microbial inactivation in dairy products is limited at low temperatures [97,98]. Ultrasonication can cause changes in physicochemical properties when heating is involved [99,100]. In the dairy industry, it is commonly used for lactose crystallization, hydrolysis, microbial inactivation, homogenization, and emulsification [100].

The basic principle of ultrasonication is the creation of cavitation and vibration by ultrasonic waves. Mechanical vibrations affect solid particles, while microbubbles formed by cavitation in liquids merge, grow, and burst, creating a mechanical effect on cells or molecules [101]. When ultrasound is used with heat treatment simultaneously, it induces structural, conformational, and physicochemical changes [101]. Ultrasonication is favored due to its ease of use, controllability, support for bioactive peptide production, and applicability at low or high temperatures in the food industry.

- **Changes in Protein Structure:** Ultrasonic processing applications on milk proteins do not generally affect the amino acid chain or hydrolysis of milk proteins. Casein and alpha-lactalbumin are more resistant to these processes, while beta-lactoglobulin is more sensitive. The application of ultrasound is mainly used as a pretreatment to unfold the tertiary and secondary structures of proteins and induce structural changes such as casein micelle shrinkage, increased hydrophilicity, conformational changes of  $\alpha$ -helix and  $\beta$ -layers, formation of random spirals and layers, and decreased free sulfhydryl content in milk proteins after processing [103].

In long-term ultrasonication of casein micelles at pH values above eight, casein micelles shrink, and this reduction continues with increasing alkaline values. Ultrasonication applied to full-fat and skimmed milk at neutral pH values causes a decrease in casein micelles, but when EDTA is added to milk, no change is observed in casein micelles [104,105]. Ultrasonication provides an advantage to the industry by preventing protein aggregation without altering the natural structure and mineral balance of milk proteins. When ultrasonic treatment is applied to whey protein concentrate instead of milk, proteins are more affected, leading to increased water-holding capacity due to the exposed hydrolytic ends of amino acids [106].

- **Bioactive Peptide Formation:** Ultrasonication causes structural changes in milk proteins, particularly in whey proteins, leading to increased sulfhydryl content, structural changes in  $\beta$ -layers, reduced casein micelle structure, increased surface area, and enhanced hydrophobicity. While ultrasonication does not directly affect proteolysis, it is commonly used as a pretreatment to make proteins suitable for enzymatic proteolysis and peptide formation [107,108]. For instance, the collision of whey proteins with the alkaline enzyme after ultrasonication can contribute to ACE inhibitory and immunomodulatory activities of proteins [109].

When bromelain enzyme is added to whey proteins that have been ultrasonified, peptides with high ACE inhibitory activity are made [110]. Also, using pepsin and neutral protease in goat milk after ultrasonication makes peptides with bioactive antioxidant and ACE-inhibiting activity [111]. By adding neutral protease to milk proteins after ultrasonication [103], you can make peptides that block ACE. However, the emergence of ACE inhibitory activity in peptides requires the addition of herbal protease or neutralizing whey proteins after ultrasonication.

- **The Digestibility of Milk Proteins:** The digestibility of milk proteins can be affected by ultrasonication, which has the potential to induce various structural changes in milk proteins, such as alterations in hydrophobicity, free sulfhydryl groups,  $\alpha$ -helix

structure,  $\beta$ -layers, and particle size [112,113]. Structural changes can have implications for the activities in the digestive system [114].

Studies have shown that ultrasonication of casein does not significantly affect its digestibility properties [115]. However, it has been observed that ultrasonication applied to concentrated casein micelles results in a decrease in particle size and increased interaction with enzymes, reducing interactions between casein micelles with each other and intramolecularly [116]. Similar changes were observed in whey proteins [117,118].

When ultrasound is used on milk proteins, both casein and whey proteins can change in their structure and chemistry. This exposes hydrolytic sites, which makes the proteins easier to digest. One effect of ultrasound is the reduction in the disulfide content of milk proteins, leading to a decrease in water-binding capacity [112]. When making cheese, ultrasonication of milk increases proteolysis by helping other natural proteolytic enzymes, like coagulant and plasmin, do their jobs, which makes the cheese easier to digest.

Overall, the effect of ultrasonication on milk proteins unfolds whey proteins, making them more amenable to enzymatic peptic hydrolysis. These configuration changes lead to the formation of peptides and an increase in digestibility both during the cheese ripening period and during digestion [119].

- **Gelling Properties:** Gelling properties can also be influenced by ultrasonication. For example, ultrasonication can reduce the viscosity of condensed milk by reducing the particle size of milk components [120]. The solubility and foaming properties of whey proteins can also be enhanced by ultrasonic treatment [121,122].

In cheese production, continuous system ultrasonication can be applied to reduce syneresis, decrease gelation time, and increase the firmness of cheese protein gels [120,123]. When ultrasonication is applied uncontrolled to milk, the milk temperature can reach 95°C, achieving a tight gel structure by denaturing the whey proteins [104]. On the other hand, when heat and ultrasonication are used simultaneously, the gel strength of yogurt gels improves. The reduction in fat globule diameter was cited as a potential reason for the increase in surface area and viscosity [124,125,126].

## 6. Proteomic Approach of Ohmic Heating Treatment on Milk Proteins

- **Ohmic Heating Treatment:** Ohmic heating is a new heat treatment technology that involves the treatment of alternating electric currents directly to generate heat in foodstuffs. It offers several advantages over conventional methods, such as rapid and homogeneous heating, short processing time, minimal impact on sensory properties, and preservation of nutritional quality [129]. It generates better quality products than high-pressure processing and has higher energy efficiency than microwave heating [130,131].

Early studies in the dairy industry on ohmic heating were conducted in the 19th century. However, its development has been postponed due to supply problems and the high processing cost of inert materials for electrodes. Contamination of electrodes during the process and decreased heat exchange efficiency caused defects in the product, resulting in reduced productivity [132,133]. Therefore, the number of studies in milk science on ohmic heating applications is relatively limited.

- **Changes in Protein Structure:** In the ohmic heating method, whey proteins are more affected than casein, which has a more water-soluble structure. Diverse electric field intensities and heat mechanisms induce morphological changes and reactions with other compounds in whey proteins. Denaturation of whey proteins can lead to the formation of more viscous structures with the provided aggregates, and it can also help reduce the allergenic effect of beta-lactoglobulin [124]. Beta-lactoglobulin undergoes denaturation and conformational changes at temperatures above 60°C, resulting in the exposure of hidden free sulfhydryl groups, which can then form non-covalent bonds with other protein molecules [135].

Generally, ohmic heating induces denaturation, aggregation, and unfolding mechanisms in whey proteins. Low electric field strength and rapid heating capacity jointly may result in protein aggregate generation. While aggregates may be undesirable in some dairy products, they can be advantageous for products requiring thickening or gel formation and can be used for encapsulating bioactive components. By developing a cold gel-like emulsion, the functionality of whey proteins is improved with a decrease in allergenicity [136].

- **Bioactive Peptide Formation:** The direct mechanism of action that enables protein hydrolysis and the formation of bioactive compounds through ohmic heating has not been fully determined. Ohmic heating is used to reduce the microbial load in foods. However, it has been observed that ohmic heating treatment at low electric field strength with high protein vanilla flavored milk improved health effects such as antioxidant, anti-diabetic, antihypertensive, and advanced rheological properties [31].

In summary, ohmic heating is a promising technology with various applications in the food industry, including the dairy sector. It can induce changes in whey protein structure and enhance the functional properties of these proteins. However, further research is needed to fully understand its potential for forming bioactive peptides and their specific effects on various food products.

- **Digestibility of Milk Proteins:** Ohmic heating affects proteins thermally and through electrochemical effects. It can induce changes in the physicochemical properties of proteins, including their structure, conformation, aggregation, size, and expansion [127]. These changes can increase the digestibility of milk proteins.

In milk, the effect of ohmic heating on proteins may be less noticeable due to its low protein content. However, in products with higher protein ratios, such as whey concentrate, the denaturation of proteins can be more evident. Studies on whey protein concentrate subjected to ohmic heating and subsequent digestion simulation with pepsin showed that digestibility increased as the processing time increased. This

increase in digestibility is caused by the unfolding of tertiary and secondary protein structures, the effect on covalent bonds, and the exposure of hydrolytic sites that can react with pepsin [128]. By being more accessible and ready for the active sites to react with the enzyme, the proteins undergo increased hydrolysis and become more digestible.

- **Gelling Properties:** In untreated milk, whey proteins are soluble, and they do not significantly affect the consistency of the gel structure when forming an acid gel. However, with heat treatment or other processes applied to the milk, denaturation of whey proteins occurs, leading to the unfolding of the proteins' structures. This activation of hydrogen and disulfide bonds and interactions with other proteins results in a tighter texture in the gel. Acid gels produced by applying ohmic heating at a high temperature (85°C) to milk can provide a firmer and more stable texture compared to acid gels generated by conventional heating at the same temperature. The temperature application in ohmic heating plays a role in the textural and structural forms of the gel. The intermolecular bonds are weaker at low temperatures, and the gaps in the acid gel matrix are larger, resulting in a structure with lower gel strength. However, the inter-matrix cavity is reduced by raising the end temperature during ohmic heating, leading to a tighter and more homogeneous gel structure obtaining increased gel strength [137].

Overall, ohmic heating offers opportunities to modify protein structures and enhance their digestibility. It also contributes to the generation of improved texture and stability gels, making it a promising technology in the dairy industry for producing high-quality products.

## 7. Proteomic Approach of Pulsed Electric Field Treatment on Milk Proteins:

- **Pulsed Electric Field Treatment:** Pulsed electric field (PEF) is a technique that involves applying short pulses of high voltage to food in microseconds or milliseconds. The effects of PEF on proteins can vary depending on the strength of the electric field, the number of pulses, and the processing time.

Milk proteins must have functional properties when processed into fermented milk products. These technologically important properties are foaming, gelling, emulsification, and solubility which provide the desired properties in the final product [3]. The clarity of the hydrophilic and hydrophobic ends of the amino acids provides solubility and emulsification, the emergence of the sulfhydryl groups ensures gelling, and the amount and clarity of whey proteins provide foaming. Depending on the product generated, milk protein fractions may need to undergo some preliminary processes for structural and conformational changes [93].

- **Changes in Protein Structure:** Caseins are the major proteins in milk, and they are generally more resistant to heat and other processes than whey proteins. Low-intensity pulsed electric pulse treatments typically do not affect milk proteins; However, increasing the intensity of the pulses can cause important changes in the structure of whey proteins. High-intensity pulsed electric pulse treatment can change the

secondary structure of sodium caseinate and open protein molecules, facilitating the interaction of hydrophobic regions with the surface of protein molecules [138, 139].

When milk proteins are exposed to an electric field, their polar groups absorb energy and form free radicals. These free radicals can negatively affect the existing bonds of proteins, such as Van der Waals, hydrophobic, hydrogen, and disulfide bonds. The electric field also affects the dipole moments of the polypeptide chains, leading to protein denaturation and the exposure of active and hydrophobic surfaces. Prolonging the processing time can increase the interactions between protein molecules, leading to the formation of aggregates [140,141].

- **Bioactive Peptide Formation:** PEF applications do not directly lead to the formation of bioactive peptides in milk proteins. However, the disruption of hydrogen bonds and hydrophobic interactions in the quaternary structure of proteins, as well as the liberation of sulfhydryl groups, can support the formation of bioactive compounds by revealing the parts of the proteins that can be hydrolyzed [142].
- In other food products, such as plant proteins, fish, and meat, high-intensity PEF treatments have been shown to hydrolyze proteins, leading to the formation of both large and small peptides with antioxidant activity [143,144]. Overall, PEF treatment can induce changes in protein structures and support the formation of bioactive compounds. It is a promising technology for various applications in the food industry, including milk and other protein-rich products, to improve functional properties and generate value-added bioactive peptides.
- **Digestibility of Milk Proteins:** When discussing the digestibility of milk proteins, the focus is primarily on  $\beta$ -lactoglobulin. This protein fraction contains a highly stable  $\beta$ -sheet core that is resistant to gastric pH and structurally complex. The monomer size is approximately 18.4 kDa and highly resistant to pepsin digestion [145]. However, the resistance of  $\beta$ -lactoglobulin to pepsin hydrolysis can be reduced by thermal and chemical treatments that expose pepsin-sensitive peptide regions [146].

To enhance the digestibility of  $\beta$ -lactoglobulin, its aqueous solutions were subjected to pulsed electric field treatment and in vitro gastric digestion using pepsin. Pre-digestion SDS-PAGE analysis revealed the formation of  $\beta$ -lactoglobulin protein aggregates due to disulfide bond formation. Besides, a monomeric portion of  $\beta$ -lactoglobulin, a thin strip, was observed alongside low molecular weight proteins in SDS-PAGE gel [147,148].

The increased digestibility of  $\beta$ -lactoglobulin is attributed to protein unfolding and aggregation, exposing their hydrophobic ends. These conformational changes allow the enzyme pepsin to hydrolyze peptide bonds adjacent to light-sensitive aromatic amino acids such as tyrosine, phenylalanine, and tryptophan. The conformational and structural changes induced by pulsed electric field treatments, as well as the liberation of sulfhydryl groups and pepsin active sites, enhance digestion [145,149].

- **Gelling Properties:** Regarding gelling properties, they are vital quality factors in products like yogurt, cheese, and dairy desserts (114). Water holding capacity and gelling properties of acid gels are increased via the denaturation of whey proteins and enhanced interprotein interactions. Pulsed electric field treatment to milk proteins unfolds the whey proteins, revealing sulfhydryl groups, and facilitates the formation of disulfide bonds, thereby improving gelling properties in products like acid gel. Another mechanism that supports gel formation and development is the formation of bonds between polar molecules through electrostatic forces [142]. An important consideration in gel formation is the applied process parameters. When a low-intensity pulsed electric field is applied to whey protein isolates, gelation improves, while increasing the electric pulse results in lower gel strength [150,151].

When cheese is produced from milk treated with a pulsed electric field, a firmer curd is obtained compared to pasteurized milk, and the coagulation time is shortened [92].

In conclusion, Pulsed Electric Field (PEF) treatment holds great potential for enhancing the digestibility and gelling properties of milk proteins and other protein-rich products, providing opportunities to improve their quality and functionality.

## 8. Proteomic Approach in Irradiation Treatment on Milk Proteins:

- **Irradiation Treatment:** Irradiation technology is increasingly being applied in the food industry. Generally, irradiation is a non-thermal technique used for food safety and shelf life extension [151,152]. In this process, foods are exposed to ionizing radiations such as high-energy electron beams ( $\beta$  particles), X-rays, or gamma ( $\gamma$ ) rays from radioactive isotopes like cobalt-60 ( $^{60}\text{Co}$ ). The irradiation of protein-rich foods has gained interest as it can lead to the breakdown of large molecules, potentially enhancing digestion. Moreover, irradiation-induced changes in protein configurations or protein breakdown do not affect the nutritional value of the product negatively [153,154].
- **Changes in Protein Structure:** Gamma radiation, part of the irradiation process, causes significant changes in protein structures. Unstable radicals are formed due to the free radicals generated by gamma rays, and they react with proteins and amino acids, leading to denaturation (35). This process can release sulfhydryl groups, form disulfide bridges, and alter the spatial structure of proteins. The covalent bonds within proteins can break, resulting in the formation of smaller proteins or peptides. Gamma radiation also affects the viscosity of protein solutions and the emulsion capacity of proteins [153,154].
- **Bioactive Peptide Formation and Digestibility of Milk Proteins:** While research on the formation of bioactive peptides or the digestibility of milk proteins after irradiation is scarce, similar processes have shown that denaturation of whey proteins can enhance subsequent enzyme activities. This formation can contribute to the production of bioactive compounds during fermentation and improved digestibility when treated with enzymes like pepsin and trypsin during digestion, especially for beta-lactoglobulin.



- **Gelling Properties:** Regarding gelling properties, there are limited studies on the application of radiation to milk, cheese, or yogurt. However, radiation applications have shown positive results in reducing mold and yeast growth in cheese post-production and extending the shelf life of yogurt [154,155,156].

A study examining the gelling properties of whey protein isolates found that gamma radiation has been applied to whey isolates with different solubility levels, and their gelling properties have been monitored over six weeks. The whey isolates with the highest concentrations gelled in about three weeks, while the ones with lower concentration took around six months to gelling structure. The degree of gelation can vary based on factors such as protein content, irradiation rate, and irradiation time [153,154]. Higher beam density causes stronger cohesion by leading to increased cross-linking between proteins, which can form large polymers. In summary, irradiation treatment can induce significant changes in protein structures, potentially improving digestibility and gelling properties. Further research is needed to explore the specific effects of irradiation on milk proteins and its applications in various dairy products.

#### IV. CONCLUSION

Due to the presence of natural enzymes and microbial load in milk, traditional thermal processing methods have been used in the dairy industry. However, these thermal methods, such as hot water or steam-based treatments, have several drawbacks in terms of view heat transfer, processing time, and cost. Additionally, they can lead to undesirable chemical, physical, and sensory changes, such as browning, the development of a cooked flavor, and the loss of essential vitamins or amino acids in milk, depending on the applied heat parameters and conditions.

Non-thermal techniques have been explored in milk processing, and laboratory and pilot-scale studies have been conducted using methods such as ohmic heating, microwave heating, radiofrequency heating, microfiltration, high-pressure processing, irradiation, pulsed electric fields, and ultrasonication to minimize thermal process disadvantages,

Many of these non-thermal methods primarily aim to reduce the microbial load in milk. As a result, research on some technological and functional characteristics of milk proteins has not been extensively pursued. Most studies have focused on the allergenic beta-lactoglobulin from whey protein. There is limited research on enhancing the technological properties of milk proteins. Moreover, innovative approaches have indicated that casein is a resistant protein, but there have been only a few studies in this area.

It is important to note that even simple mixing processes can impact the physicochemical properties of milk, and inevitably, methods such as radiation, high pressure, electromagnetic waves, or heating will also influence milk proteins. Despite this, proteins are believed to have positive health effects, and there is a need for a more comprehensive examination of the impacts of innovative approaches on proteins.

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