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REDUCTION OF MILK ALLERGENICITY THROUGH ENZYMATIC AND FERMENTATION HYDROLYSIS

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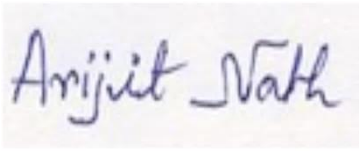

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1. INTRODUCTION

Cow's milk is a crucial component of the diets of infants and children globally, providing a rich array of nutrients such as proteins, lipids, lactose, minerals, and vitamins. These contribute to a robust immune system, normal metabolism, growth, and overall health development, leading to an increasing consumption of cow milk products in many countries (Liang et al., 2021). However, despite its nutritional advantages, cow's milk stands as a primary and prevalent trigger for food allergies in early childhood. Food allergies manifest as abnormal immune responses, with immunoglobulin E (IgE) causing immediate reactions and cell-mediated allergies exhibiting delayed symptoms (Hochwallner et al., 2014). Milk allergy, categorized as IgE-mediated, non-IgE mediated, or mixed, needs differentiation from non-immunological reactions like lactose intolerance. IgE-mediated responses involve a hypersensitivity cascade leading to symptoms like urticaria, respiratory issues, gastrointestinal discomfort, and cardiovascular symptoms. Cow's milk allergy is a significant global concern, affecting 3-8% of young kids and causing severe reactions, particularly in infants (Lajnaf et al., 2022). Current strategies to prevent and manage milk allergies typically involve complete elimination of milk consumption. However, avoiding cow's milk proteins poses challenges as they are commonly found in various processed foods. Eliminating these proteins may result in nutritional deficiencies, impacting the growth of infants and children. Efforts to mitigate milk allergenicity while preserving nutritional value through processing techniques have garnered attention. Efforts in this direction explore food processing techniques that can modify protein structures, either by removing or destroying protein epitopes. However, it's important to note that these techniques may inadvertently create new epitopes or enhance the accessibility of existing ones (Bu et al., 2013). The subsequent sections of the literature review will delve into current research on enzymatic hydrolysis using papain and fermentation with lactic acid bacteria as for their potential in reducing allergenicity, specifically by breaking down milk proteins into peptides and amino acids which are capable of cleaving epitopes and subsequently result in the reduction of allergenicity. Furthermore, fermented products offer not just added nutrients but also supply bioactive peptides. These peptides are released through the breakdown of proteins by bacteria during the fermentation process. Studies indicate that bioactive peptides found in yogurts exhibit functional properties, including ACE inhibition and antithrombotic activity (Huang et al., 2023).

GOAL OF THE THESIS

General Objective

The primary goal of this investigation was to create yogurt with enhanced biochemical properties, specifically focusing on antioxidant capacity and angiotensin-converting enzyme inhibitory activity. This was achieved through a two-step process involving sequential hydrolysis of proteins in UHT (Ultra High Temperature) and ESL (Extended Shelf Life) milk. The first step involved papain, and the second step involved microbial hydrolysis using lactic acid bacteria (LAB). Additionally, the study delved into the antigenicity of milk proteins in UHT and ESL milk. Furthermore, it explored milk protein-derived peptides generated through the sequential process of papain and microbial hydrolysis. The assumption was that this cluster of peptides, each possessing distinct functional activities, could collectively enhance the overall biological activity of the yogurt. Importantly, the investigation deliberately avoided the use of membrane- and chromatography-based downstream processing methods to isolate individual peptides. The emphasis was on studying the combined effects of the peptide cluster, allowing for a more holistic understanding of the biological potentialities without isolating specific components.

Specific Objective

The specific objectives of the study are:

- Understand the hydrolysis of milk proteins by papain and LAB using different concentration ratios of enzymes.
- Understand the texture characteristics, especially the hardness and firmness of yogurt produced through the action of papain and LAB.
- Understand the antigenicity of milk protein hydrolysate.
- Understand the antioxidant capacity of milk protein hydrolysate, shedding light on potential changes in immunogenic properties during the hydrolysis process.
- Understand the angiotensin-converting enzyme inhibitory activity of milk protein hydrolysate.

2. LITERATURE REVIEW

2.1.Characteristics of Cow's Milk Proteins

Cow milk contains around 30 different proteins, some of which might cause allergic reactions. These proteins are labeled as "Bos d" followed by a number indicating their protein type. These proteins are classified into two groups depending on their solubility at pH 4.6 and 20ºC. Caseins, such as αS1-casein, αS2-casein, β-casein, and κ-casein, are precipitating proteins. On the other hand, serum or whey proteins consist of α-lactalbumin (α-LA), β-lactoglobulin (β-LG), immunoglobins (Ig), bovine serum albumin (BSA), and lactoferrin (LF). Caseins constitute 80% of total proteins, while whey proteins make up the remaining 20%. The primary allergens are caseins, β-LG, and α-LA, responsible for allergic reactions in individuals allergic to milk. Consumption of milk or dairy products by allergic individuals can prompt an immune system response to these proteins, leading to allergic symptoms. Although present in lower concentrations, proteins like LF, BSA, and Ig found in milk also contribute significantly to the development of milk allergies (Lajnaf et al., 2022),

Table 1. Characteristics of Cow Milk allergens by Restani et al., 2009

Fraction of protein		**Amount of protein (%)**	**Molecular weight (kDa)**	**Amino acids**	**Isoelectric point**	**Denaturation temp (ºC)**
Casein	α_{s1}-casein	32	23.6	199	4.9-5.0	-
	α_{s2}-casein	8.4	25.2	207	5.2-5.4	-
	β-casein	26	24	209	5.1-5.4	-
	kappa-casein	9.3	19	169	5.4-5.6	-
Whey	β-lactoglobulin	9.8	18.3	162	5.3	71.9
	α-lactalbumin	3.7	14.2	123	4.8	64.3
	Bovine serum albumin	1.2	66.4	583	4.9-5.1	72-74
	Lactoferrin	traces	80	703	8.7	72-85
	Lactoperoxidase	traces	78	612	9.8	70

2.1.1. Whey Proteins

The most allergenic components within the whey protein fraction are the globular proteins Bos d 5, also known as β-LG, constituting 56% of this fraction, and Bos d 4, referred to as α-LA, comprising 21%. Furthermore, there are minor elements present, such as Bos d 6 (BSA) contributing up to 7%, Bos d 7 (Ig) contributing up to 14%, and LF providing 2% (Lajnaf et al., 2022). Whey proteins have complex structural properties such as secondary, tertiary, and quaternary structures. These structures influence these proteins' functional characteristics. Whey proteins also feature intramolecular disulfide bonds, which contribute to their stability. This resilience is critical for the preservation of their structure during food processing procedures. Whey proteins' three-dimensional structure is crucial in influencing their allergenic properties. Conformational epitopes are unique sections or forms of a protein that the immune system recognizes and can cause allergic reactions. The ability of whey proteins to preserve their three-dimensional form is linked to their allergenicity. Changes in this form, whether caused by denaturation or another mechanism, can have an impact on their allergenic characteristics (Monaci et al., 2006).

β-LG. Found in the milk of nearly all mammalian species except for camelids, rodents, and humans. β-Lg is considered as a primary allergenic protein due to its predominance among whey proteins, (about 56% of total whey protein composition) and also due to the fact that it is absent in human milk. β-LG is a small protein with a molecular weight about 18.3 kDa, 162 amino acids (Lajnaf et al., 2022). β-lg possesses a tertiary structure comprising two anti-parallel β-sheets forming a binding pocket, connected by an α-helical strand. This arrangement allows β-lg to interact with various molecules, often existing as dimers, forming pairs of bonded molecules. Additionally, it can adopt other quaternary structures, enabling interactions with different protein molecules (Złotkowska et al., 2021). With one unpaired cysteine and two disulfide linkages, β-lg exhibits resistance to acid hydrolysis and enzyme activity. Due to these unique properties, β-lg can traverse the gastrointestinal tract without significant breakdown. As it encounters immune cells in the gut, undigested β-lg may trigger immunological responses, leading to allergic reactions, especially in sensitive individuals (Wal, 1998). Epitopes for both IgE and IgG have been identified within the β-lg structure, reacting with blood samples from individuals with cow's milk allergy. Food allergies to β-lg may impact up to 80% of the population (Miciński et al., 2013).

α-LA has a lower allergenicity compared to β-Lg since the chemical composition of α-LA in bovine and humans is quite similar. They constitute approximately 22% of the total whey protein content in cow's milk. This protein is a monomeric protein, which means it exists as a single, non-repetitive unit. It has a globular structure, implying that it forms a compact, roughly spherical shape. α-LA participates in the formation of lactose and binds metals, such as cobalt, magnesium and zinc. One distinguishing factor is that α-LA is well soluble in water (Lajnaf et al., 2022). It has 123 amino acids, a molecular weight of approximately 14.2 kDa, and three genetic variations. The protein has four disulfide bridges, which are covalent bonds between two sulfur atoms in the protein's structure. These bridges contribute to the protein's stability (Wal, 2004). α-LA has a high thermal stability and refolding ability, as well as a highly structured secondary configuration, resulting in a compact, spherical tertiary structure. Despite the relatively low denaturation temperature of roughly 64°C, their structural arrangement provides greater resistance to heat-induced protein aggregation (Hochwallner et al., 2014; Villa et al., 2017).

BSA comprises 582 amino acids and has a stable tertiary structure with a molecular weight of 66.3 kDa. They are found in cow's milk, usually in amounts of about 20 mg per 100 mL. Its principal biological role is to transport, metabolize, and distribute chemicals such as fatty acids, ions, and hormones and also protects against free radicals (Hochwallner et al., 2014). BSA is divided into three domains, each of which has nine loops connected by 17 disulfide connections, many of which are well-protected within the protein's core, making them less accessible. These disulfide connections are critical for sustaining the protein's innate antigenic properties, owing to its extraordinary stability in maintaining its three-dimensional structure even when subjected to conditions that normally break proteins (Restani et al., 2004). When exposed to temperatures ranging from 70°C to 80°C, this protein loses its function. Among all the proteins found in cow's milk, only bovine serum albumin is expected to retain its potential to elicit an immunological response even after heat treatment (Miciski et al., 2013). BSA may be a minor allergen in cow's milk, and some individuals who are allergic to beef may also be at risk of being allergic to cow's milk due to the presence of BSA (Fuc et al., 2019).

LF is an iron-binding glycoprotein belonging to the transferrin protein family. It can bind and chelate iron, which is effective at depriving bacteria of this essential nutrient. This iron-binding property makes LF a natural antimicrobial protein found in milk. However, LF has been linked to

allergic reactions. According to research, it can be detected by IgE antibodies in people who are allergic to cow's milk. The proportion of cow's milk allergic patients who have IgE antibodies to lactoferrin might range from 5% to 66%. Regardless of these findings, the clinical significance of lactoferrin in milk allergy remains unknown. While lactoferrin-specific IgE antibodies have been found in the sera of cow's milk allergic patients, the amount of its allergenic activity and its impact on the severity of clinical symptoms has not been completely studied. While lactoferrin may be a possible allergen, additional study is needed to determine its precise role and clinical importance in milk allergies (Linhart et al., 2019).

2.1.2. Caseins

β-casein constitutes 39% of the total casein content, while α-S1, α-S2, and κ-caseins contribute 38%, 10%, and 13%, respectively. Collectively, these proteins form casein micelles, crucial for binding essential minerals like calcium phosphate (Hochwallner et al., 2014). Caseins are phosphorylated and conjugated proteins lacking a three-dimensional structure and disulfide linkages, rendering them resistant to heat-induced changes but insoluble in water due to exposed hydrophobic components. Their distinctive structural features make them unlikely to trigger an immunological response and easily digestible by enzymes. Caseins exhibit high hydrophobicity and surface activity (Wal, 2004; Goff et al., 2016). Genetic polymorphisms present in all caseins result in various protein variants. These modifications, along with those occurring during processing, have the potential to influence allergenicity and the ability to bind with IgE (Fox, 2001).

αS1-Casein is a phosphoprotein that consist of 199 amino acids with a molecular weight of 23.6 kDa. They have a high proline content. Most of its structure is disorganized, with around 70% of its segments being unstructured. Secondary structural features such as α-helices and β-sheets are present in low amounts, while the tertiary structure is less evident because of the lack of disulfide bonds. Caseins' absence of tertiary structure could indicate the presence of mostly linear epitopes (Villa et al., 2017). Breast milk lacks a structural and functional equivalent to animal αs1-casein. This significant variation is a major element in this protein's immunogenicity in humans and the development of cow's milk allergies (Miciski et al., 2013).

αS2-Casein consists of 207 amino acids and contains one disulfide bond in each molecule, resulting in a molecular mass of 25.2 kDa. αS2-casein has four genetic variations named A, B, C, and D, each with a different amount of phosphoryl groups ranging from 10 to 13. One of these versions, variant

A, has 11 phosphoserine residues and has a particularly unstable structure when subjected to pH fluctuations (Miciski et al., 2013). Because of the loss of a cluster containing three phosphoserine residues, variant D has different structural properties. This change has the potential to affect the allergenic characteristics of this protein (Villa et al., 2017).

β-Casein comprises 209 amino acids and contains 5 phosphate groups, resulting in a molecular weight of 24 kDa. It exists in 12 primary genetic variants, each characterized by varying levels of phosphorylation. The molecular structure of β-Casein closely resembles that of αS1-casein. It features a hydrophobic domain at the C-terminal end, a highly hydrated and charged domain at the N-terminal end, an evenly distributed proline content, and notably, it lacks disulfide bonds (Villa et al., 2017). A protein homologous to bovine β-casein, exhibiting a similar structure and physicochemical characteristics, has been identified in human milk. This discovery suggests that this casein in cow's milk is among the least likely to trigger allergies (Lajnaf et al., 2022).

κ-Casein with a molecular weight of 19 kDa and a primary sequence of 169 amino acids, exhibits a distinct structural composition. It comprises a strongly hydrophobic N-terminal region and a highly hydrophilic C-terminal region. This unique protein plays a crucial role in preventing the aggregation of micelles by inducing steric and electrostatic repulsion. Notably, κ-Casein is the only glycosylated casein, incorporating galactose, galactosamine, and sialic acid as tri- or tetrasaccharides attached to threonine residues in the C-terminal region, enhancing its hydrophilicity. There are 11 κ-casein variants, differing in the number of attached oligosaccharides. κ-Casein exhibits robust resistance to calcium-induced precipitation, contributing significantly to the stabilization of other caseins. However, when rennet cleaves the Phe105-Met106 bond, this stabilizing ability is nullified, resulting in two components: a hydrophobic para-kappa-casein and a hydrophilic kappa-casein glycomacropeptide (GMP) (Fox, 2001). O-glycosylation sites in the GMP can vary from 0 to 7, leading to the coexistence of non-glycosylated and glycosylated variations in digested milk. Intriguingly, glycosylated GMP variants undergo less digestion compared to their non-glycosylated counterparts, suggesting a potential link between glycosylation and the immunoreactivity of GMP (Creamer et al., 1998).

2.2. Epitopes in Cow Milk Protein

Managing cow milk allergies is challenging because the human immune system reacts uniquely to the proteins in cow milk. Unlike other allergies, there isn't a specific allergen or clearly defined protein structure identified as the primary cause of milk allergy. About 75% of individuals with cow milk allergies develop sensitivities to multiple proteins, and the specific proteins and the strength of their immune responses can vary significantly. The proteins most commonly recognized as allergens are those most abundant in cow's milk, such as caseins, β-LG, and α-LA. These proteins are often implicated in allergy reactions, triggering immunological responses in affected individuals. Due to the individual variability in allergenicity, healthcare practitioners need to customize allergy management based on each patient's unique sensitivities and reactions to these proteins (Hochwallner et al., 2014). IgE antibodies play a crucial role in allergy responses by targeting specific regions of allergenic proteins. In the context of milk protein allergens, these targeted areas are referred to as epitopes or antigenic determinants. Epitopes are specific amino acid sequences within a protein that can bind to IgE antibodies, initiating an immunological response. Milk proteins exhibit two types of epitopes: linear (sequence) epitopes and conformational epitopes. Linear epitopes are continuous sequences of amino acids along the protein chain, while conformational epitopes arise from the three-dimensional folding of the polypeptide chain. The existence of distinct genetic variants in various bovine milk proteins adds complexity to the IgE and IgG-binding epitopes in milk proteins. These genetic variations can alter the structure and composition of milk proteins, leading to the presence of different immune-recognized epitopes (Abd El-Salam & El-Shibiny, 2019). This complexity results in a wide range of allergic reactions to milk proteins in individuals with milk allergies. A single allergenic protein may contain multiple epitopes, and some are particularly potent, known as immunodominant epitopes, provoking a strong immune response. Allergic reactions can occur not only through the absorption of allergens into the bloodstream but also through direct skin contact with the allergen (El-Agamy, 2007). Patients with IgE antibodies recognizing sequential epitopes, indicated by their ability to bind to synthetic peptides derived from relevant allergen sequences, tend to react to food in any form, even when extensively cooked. This suggests that IgE recognition of sequential epitopes is associated with more persistent and severe cow milk allergies, highlighting the crucial role of these epitopes in cow's milk allergy (Cerecedo et al., 2008).

2.3. Influence of Technological Processing on Cow's Milk Proteins

The process of getting cow's milk from the farm to supermarket shelves in developed countries involves several crucial stages to ensure its safety and purity for consumption. The process commences by rapidly cooling the milk to 4°C which helps preserve the milk's freshness and prevents the growth of harmful microorganisms. The cooled milk is stored and transported in stainless steel tanks to maintain the low temperature. The next step involves centrifugation, a process that separates the milk fat from the remaining skimmed milk. This step is crucial for standardizing the fat content of the final product. Following that, standardization occurs in which precise ratios of milk fat are added back to the milk. The next important step is heat treatment, with the most common methods being pasteurization, where milk is heated to a temperature of 70-80°C for a short duration (15-20 seconds). Pasteurization kills harmful microorganisms while preserving the milk's flavor and nutritional value. Sterilization involves heating milk to higher temperatures, typically between 110-120°C, for a longer period (10-20 minutes), it ensures the destruction of a broader range of microorganisms and spores. Ultra-High-Temperature (UHT) processing is the most aggressive heat treatment method, involving temperatures of 135-145°C for a very short duration (0.5-4 seconds). UHT processing is highly effective in eradicating harmful microorganisms and spores and minimizes the Maillard reaction, which can otherwise affect the color of the milk. This method is particularly popular in Europe for ensuring milk safety and quality. After the heat treatment process, the milk is quickly cooled to a temperature below 4°C. The final step involves packaging the processed milk. Packaged milk is then distributed to supermarkets and other retail outlets for consumers to purchase (Verhoeckx et al., 2015; Goff et al., 2016).

Numerous methods exist in food processing that mitigate the allergenic characteristics of proteins. These processes involve the destruction of conformational epitopes. Some food processing methods aim to break down these epitopes, which can help reduce the allergenicity of the protein. The disruption of these epitopes makes it less likely for them to interact with the immune system in a way that leads to allergic reactions. Unlike conformational epitopes, sequential epitopes are linear sequences of amino acids on a protein. Food processing methods may have little impact on these sequential epitopes, meaning that they remain largely intact. However, sequential epitopes are typically less allergenic compared to conformational epitopes. In addition, chemical interactions can occur between proteins, lipids and carbohydrates found in the food matrix, limiting protein

accessibility to the immune system. Food processing, on the other hand, has the potential to increase protein allergenicity, this may happen because of the development of new epitopes (neoepitopes) and the influence of the food matrix, which may hinder protein digestion in the stomach while preserving allergenic epitopes, allowing them to engage with the immune system in the intestine (Nowak-Wegrzyn & Fiocchi, 2009). We used ESL and UHT milk in our research, which had undergone important heat treatment and homogenization operations. These milk variations were then converted into yogurt through papain and microbial hydrolysis. The following section discusses the effect of Technological Processing on the Allergenic Characteristics of Cow's Milk Proteins.

2.3.1. Effect of Heat Treatment

Heat treatment is critical in the production of numerous dairy products. However, the effect on protein allergenicity is highly complex and depends on various parameters such as protein type, heating temperature, and heating duration. Heat treatment causes substantial structural and chemical changes in proteins, including denaturation, aggregation, and interactions with substances such as sugars via the Maillard reaction. These modifications interrupt the proteins' original shape, altering the exposure and accessibility of allergenic epitopes. Heating, in general, reduces the allergenicity of milk proteins by removing or modifying the allergenic epitopes. However, several studies have shown that heating milk proteins can increase their antigenicity. This could be due to the unmasking of buried allergenic epitopes during heat denaturation or the formation of neoepitopes that could cause allergic reactions in some people. (Bu et al., 2013).

Because caseins lack secondary, tertiary, and quaternary structures that can be damaged by heating, they are considered heat stable. This suggests that heating milk only partially reduces its allergic traits (Verhoeckx et al., 2015). Whey proteins, on the other hand, including β-LG and α-LA, have a complex 3D structure that is critical in retaining conformational epitopes. Heat treatment can cause these proteins to denature or unfold, potentially altering their allergenicity. (Bu et al., 2009).

Heating of whey proteins can lead to denaturation of the protein structure, which can result in the destruction of conformational epitopes and a decrease in IgE-binding capacity. The specific changes in protein structure and their impact on allergenicity may vary depending on the heat treatment method used (Villa et al., 2017). Table 3 shows the effects of the different heating methods on the allergenicity of milk proteins.

Generally, typical heating processes such as pasteurization, sterilization, and UHT processing do not eliminate milk antigens' allergenic potential. Furthermore, there is a lack of data on the effect of UHT processing on milk allergenicity because previous research has focused on heat treatments up to 120°C (Verhoeckx et al., 2015). It is critical to understand that heat treatment alone may not completely eliminate all allergic proteins from milk, and that further methods such as enzymatic hydrolysis or fermentation may be required to effectively minimize allergenicity (Bu et al., 2013).

Table 2. Effects of Heat treatment on milk allergens.

Matrix	Allergen	Findings	Reference
Purified protein, skim milk and sweet whey	β-LG	Temperatures from 80 to 90°C, the allergenicity of β-LG rose. However, it decreased when exposed to temperatures over 100°C..	(Kleber et al., 2007)
Whey protein isolates	α-LA, β-LG	Temperatures ranging from 50 to 90 °C, a significant rise in IgE-binding to α-LG and β-LG. However, the antigenicity decreases at higher temperatures within the range of 90 to 120 °C.	(Bu et al., 2009)
Whole milk and β-LG solution	β-LG	Denaturation and aggregation processes occurred between 90°C and 95°C, the IgE-binding ability of β-LG dropped slightly but significantly.	(Asghar Taheri-Kafrani et al., 2009)
Purified protein	β-LG	β-LG's natural form is resistant to simulated stomach digestion. When subjected to a temperature of 90°C, however, its digestibility improves.	(Peram et al., 2013)
Fresh skim milk	β-LG, α-LA	β-LG and α-LA showed a decrease in allergenicity at 90 to 100°C after 20 min	(Bloom et al., 2014)
Milk protein concentrates	β-LG, α-LA, Caseins	Caseins remained same. β-LG showed increased allergenicity between 65 and 85 °C for 25 minutes, but decreased allergenicity between 85 and 90 °C for the same time period. α-LA, on the other hand, showed a decrease in allergenicity.	(Zhong et al., 2016)

2.3.2. Effects of Homogenization

Homogenization is a common method in the modern milk processing industry. For many years, the dairy sector utilized pressures ranging from 20 to 100 MPa to ensure product quality and stability. Homogenization is a mechanical procedure that reduces the size of fat globules in milk, resulting in a more homogenous and stable product (Gonçalves, 2022). Homogenization has the ability to affect milk allergenicity by changing the structure of allergenic proteins. When milk is homogenized, the surface area of the milk fat increases dramatically, providing more opportunity for allergic milk proteins to stick to it. Many of these antigenic proteins are encased within casein micelles in raw, untreated milk. The amount of exposed antigenic proteins in homogenized milk may increase. (Miciński et al., 2013). However, some studies have suggested that homogenization increases the potential of milk to trigger anaphylactic reactions, while others have found no significant differences in allergenicity between homogenized and non-homogenized milk. Poulsen et al. (1987) shown in a mouse model that homogenized milk and pasteurized milk caused anaphylactic reactions, whereas raw milk did not. Pelto et al. (2000), on the other hand, discovered no significant variations in allergenicity between groups treated with and without homogenized milk in a randomized, double-blind, cross-over research on humans. Overall, the evidence is insufficient to anticipate the effect of homogenization on the allergenic potential of milk proteins with any certainty.

2.3.3. Effects of Fermentation

Fermentation is a well-known food industry process that can impact milk allergenicity. Fermented foods, such as yoghurt, promote human health by hydrolyzing important dietary proteins enzymatically, resulting in the production of bioactive peptides. During fermentation, LAB breakdown milk proteins into peptides, amino acids, bioactive peptides and exopolysaccharides (Wang et al., 2021). This method has the potential to reduce the allergenic properties of milk proteins. Bacterial proteolytic systems include peptidases, proteinases, and transport systems, which are all required for their growth in milk and dairy products. These enzymes are capable of cleaving relevant epitopes. Substances such as lactic acid have the ability to influence secondary and tertiary protein structures, resulting in alterations in epitopes and, as a result, a reduction in allergenicity (Mu et al., 2021).

Unlike heat treatment, LAB fermentation has the potential to degrade casein. For instance, specific strains of Enterococcus faecium, which are commonly found in fermented milk and cheese, can create enzymes such as metalloproteases and cell envelope proteinase which are essential for breaking down casein in milk. This proteolytic activity of LAB helps to make dairy products less allergenic and more appropriate for people who are allergic to milk proteins (Wang et al., 2021). Moreover, a study conducted by Mu et al. in 2021 demonstrated that Lactobacillus fermentum IF3956 could effectively hydrolyze milk proteins, specifically targeting α-casein and β-LG. It's worth noting that the specific effects of fermentation on milk allergens can vary depending on factors such as the bacterial species utilized, the duration and conditions of the fermentation process, and the particular milk protein under consideration (Abd El-Salam & El-Shibiny, 2019). Furthermore, the dietary consumption of probiotics and fermented foods can mitigate some symptoms of atopy and reduce the development of allergies through the mechanism of immune regulation. Fermented foods containing LAB can also enhance the expression and secretion of both Type I and Type II interferons at the systemic level (Bu et al., 2010). Table 5 summarizes the effect of fermentation on antigenicity and allergenicity of bovine milk proteins for different bacterial strains.

Table 3. Fermentation effect on the milk allergenicity (a)

Bacterial Strain	Milk Protein	Findings	Reference
Lb. bulgaricus, Lb. helveticus, S. thermophilus (single; mixed 1:1)	α-LA, β-LG	Combination of Lb. helveticus, S. thermophilus resulted in the most significant reduction in immunoreactivity, with 87% for α-LA and 95% for β-LG	(Bu et al., 2010)
Lb. delkbreukii subsp bulgaricus CRL656	β-LG	After 8 hours, the immunoreactivity of β-LG decreased by 32% due to the degradation of the three primary epitopes of β-LG.	(Pescuma et al., 2011)
Lb. paracasei 1 -N-10	β-casein	The breakdown of two significant linear epitopes associated with the allergenicity of β-casein.	(Hadji Sfaxi et al., 2012)

Table 4. Fermentation effect on the milk allergenicity (b)

Lb. helveticus A75	β-casein, αS1-Casein	Both proteins had a 60% reduction in their IgE binding	(AHMADOVA et al., 2012)
Lb.Rhamnosus GG	α-LA, β-LG, α-Casein, β-Casein	After 12 hours of fermentation and 0.5 days of storage, the antigenicity was significantly reduced, resulting in a decrease in IgE binding from 6.5% to 70%	(Yao et al., 2013)
Lb.casei	α-LA, β-LG, α-casein, β-casein	IgE binding reduced from 15 to 90%	(Shi et al., 2014)
Lb.casei LcY	α-LA, β-LG, Caseins	Fermentation with or without simulated digestion: decreased allergenicity (higher with digestion)	(Wróblewska et al., 2016)
L. plantarum 7-2	α-casein, β-LG	significantly reduced the antigenicity of α-CN and β-LG.	(Mu et al., 2021).

2.3.4. Effect of Enzymatic Hydrolysis

Enzymatic hydrolysis is a well-established, safe, and effective processing technique for reducing the allergenic properties of milk proteins. This approach uses digestive enzymes to modify allergenic proteins. During enzymatic hydrolysis, specific peptide or disulfide bonds are altered, causing the disruption of conformational or linear epitopes and this can lead to the reduction of allergenicity. Moreover, the use of enzymatic hydrolysis has recently become popular for producing high-quality, hypoallergenic protein hydrolysates. Several investigations have indicated that hydrolysis with Alcalase can diminish β-LG and α-LA antigenicity (Liang et al., 2020).

Enzymatic hydrolysis breaks down the milk proteins into smaller peptide molecules and amino acids. Allergic epitopes are often located in the core of the protein structure and by breaking down the protein into smaller pieces, the allergenic epitopes may be destroyed or become less accessible, resulting in a reduced allergenicity of the protein (Bu et al., 2013). Typically, small peptides and free amino acids are considered non-allergenic. However, extensively hydrolyzed milk proteins are associated with undesirable characteristics such as poor flavor, a bitter taste, limited lipid

emulsification properties, high osmolality, and high costs. These drawbacks restrict their applicability in standard infant formula preparations. Therefore, research has been directed towards screening the type of enzyme, hydrolysis conditions, and use of complementary treatments to obtain partially hydrolyzed milk protein of maximum reduction in allergenicity and the least changes in the sensory and functional properties of the product (Abd El-Salam & El-Shibiny, 2019).

In the process of hydrolysis, several critical factors influence the outcomes, including the choice of enzymes, the hydrolysis model, and the extent of hydrolysis. These factors result in variations in the composition of peptides, the remaining antigenicity in the hydrolysate, and even the taste of the final product (Bu et al., 2013). Moreover, combining enzymes in the hydrolysis process was found more effective in reducing the allergenicity of whey protein compared to using a single enzyme. Another approach, known as the 'two-step' hydrolysis process using alcalase and papain, was found to be highly effective in decreasing the immune reactivity of cows' milk whey protein. However, it's important to note that even with this approach, some allergenic epitopes remained present in the hydrolysate (Wroblewska et al., 2004). Table 6 summarizes the impact of enzymatic hydrolysis on the antigenicity of milk proteins.

Table 5. Effect of enzymatic hydrolysis on milk allergenicity (a)

Enzyme	Milk allergen	Findings	Reference
Alcalase, Papain	β-LG, α-LA	The antigenicity of milk proteins was decreased, and the two-step hydrolysis process proved to be more effective in reducing antigenicity. However, it was not entirely successful in eliminating allergenic epitopes.	(Wroblewska et al., 2004)
Protease of Bacillus licheniformis	Whey protein concentrate	A hydrolysate with an average peptide chain length of around 4 amino acids was obtained. The antigenic whey protein in the product was reduced 99.97%,	(Guadix et al., 2006)
Trypsin, chymotrypsin	Whey protein isolate	Enzymatic hydrolysis combined with glycation significantly reduced the IgE binding capacity of whey protein isolate	(Xu et al., 2020)

Table 6. Effect of enzymatic hydrolysis on milk allergenicity (b)

Papain	β-LG	The hydrolysates demonstrated a complete elimination of allergenicity	(López-Expósito et al., 2012)
Alcalase, Protamex	Casein and β-LG	Efficiently reduced the antigenicity of milk allergens	(Liang et al., 2020)
Alcalase, Protamex, and Flavourzyme	Caseins, β-LG, and α-LA	The IgE-binding and IgG-binding ability in hydrolyzed were significantly reduced.	(Liang et al., 2021)

2.3.5. Effect of Combined treatment

Combining various processing methods can be more effective in reducing milk allergenicity compared to using a single approach. This is because each method has its own advantages and drawbacks. For instance, heat treatment can alter protein structures, potentially reducing allergenicity, but it might also create new allergens. Enzymatic hydrolysis breaks proteins into smaller, potentially less allergenic pieces, but it can also create new allergenic spots or introduce bitter flavors. Lactic acid fermentation can change protein properties, reducing allergenicity, but it might also introduce neo allergens. By combining different techniques, it is possible to mitigate the limitations of each method and enhance their combined impact on reducing milk allergenicity (Villa et al., 2017).

Some researchers have demonstrated that when enzymatic hydrolysis is combined with a preceding heat treatment, it can significantly boost the proteolytic breakdown of key milk allergens like α-LA and β-LG. The heat treatment plays a crucial role as it thermally denatures the proteins, potentially revealing more cleavage sites. This makes the proteins more susceptible to enzymatic proteolysis, ultimately reducing their allergenic properties (Bu et al., 2013). For example, in a study by Mecherfi et al. (2011), they studied the effects of combining microwaving and enzymatic hydrolysis on β-LG and bovine whey proteins. They found that microwaving enhanced the breakdown of these proteins compared to traditional heating methods. Importantly, this combined approach significantly reduced the proteins' ability to trigger an allergic response, indicating a decrease in their allergenicity. Additionally, it has shown that using microwaves to assist enzymatic

breakdown can improve the solubility and digestibility of milk proteins while reducing their allergenicity (Izquierdo et al., 2008).

In a recent study by Ye et al. in 2023, the research explored the combination between enzymatic hydrolysis and LAB fermentation. They specifically noted the effectiveness of an alkaline protease when used in tandem with Lactobacillus helveticus treatment, offering a practical method for eliminating allergenicity in milk processing. Additionally, their findings highlighted the possibility of achieving dairy products with excellent functional attributes and a well-balanced sensory experience. This approach opens promising avenues for addressing milk allergies while maintaining product quality. Table 7 provides an overview of the combined methodologies and their impacts on milk allergens.

Table 7. Effect of combined method on milk allergenicity (a)

Treatment	Matrix	Allergen	Findings	Reference
Heat and enzymatic hydrolysis	Purified proteins	α-Casein, β-LG	Heat treatment decreased β-LG by altering its structure and making it more vulnerable to enzymatic digestion, affecting B cell epitopes, while it had no impact on the allergenicity of α-casein.	(Morisawa et al., 2009)
Heat and enzymatic hydrolysis	Whey protein concentrate (WPC)	Whey protein	Heated WPC had lower antigenicity compared to native WPC when treated with various enzymes, and the most significant reduction in antigenicity occurred when using a high E/S ratio of pepsin followed by trypsin.	(Kim et al., 2007)

Table 8. Effect of combined method on milk allergenicity (b)

Heat treatment and enzymatic hydrolysis	Purified whey	α-LA, β-LG	Enzymatic hydrolysis with heat treatment significantly improved the breakdown α-LA and β-LG by tryptic and peptic enzymes, leading to a reduction in the allergenicity of milk.	(Bertrand-Harb et al., 2002; Peyron et al., 2006)
microwaves combined with enzymatic hydrolysis	Whey protein isolate	β-LG	Microwave treatment at 200 W not only improved the breakdown of β-lactoglobulin by pepsin within just 3 minutes but also substantially reduced its immunoreactivity.	(El Mecherfi et al., 2011)
Microwave irradiation Enzymatic treatments	WPC	Whey proteins	Microwave treatment significantly heightened the extent of hydrolysis across all enzymes. Pronase exhibited the most profound proteolysis when subjected to microwave irradiation, followed by Papain and Alcalase, resulting in remarkably low immunoreactivity.	(Izquierdo et al., 2008)
Enzymatic hydrolysis and LAB fermentation	Cow's milk	Whey and casein proteins	Allergenicity reduction of cow milk treated by alkaline protease combined with Lactobacillus Plantarum and Lactobacillus helveticus based on epitopes	(Ye et al., 2023)

2.4.Bioactive Peptides

Bioactive peptides are compounds with the potential for various biological functions, including antimicrobial, antihypertensive, antioxidant, anticancer, immunomodulatory and anti-inflammatory. Some evidence suggests that these bioactive peptides exhibit functional activities, including ACE inhibition and antithrombotic effects. Typically, these peptides consist of short amino acid chains, comprising 2 to 20 amino acid residues. They exist within animal and plant proteins in an inactive state. However, when released through processes like enzymatic hydrolysis or microbial fermentation, bioactive peptides can have significant positive effects on health (Cruz-Casas et al., 2021). Additionally, these peptides play a role in addressing food allergies by inducing tolerance and preventing allergic reactions. Notably, it has been found that bioactive peptide has been associated with a protective effect against atopic dermatitis and allergic diseases. A study demonstrated that regular yogurt consumption in infancy may prevent the development of food allergies (Shoda et al., 2017). Clinical studies further highlight yogurt's ability to induce tolerance in children with cow's milk allergy. Moreover, yogurt consumption is on the rise across different stages of our lifespan (Huang et al., 2023).

2.4.1. Antioxidant Peptides

Within the food sector, there has been an increasing emphasis on antioxidant peptides in the field of bioactive chemicals. These distinct protein fragments have antioxidant characteristics. Their function is to mitigate the consequences of oxidative stress and lipid peroxidation caused by the presence of free radicals during both biological and food-related oxidation processes. In essence, antioxidant peptides protect us against the harmful effects of oxidation on both our bodies and the foods we eat. The primary mechanisms by which antioxidant peptides work include inactivating reactive oxygen species, scavenging free radicals, and chelating metal ions that contribute to oxidative damage in the body. These processes may indirectly help individuals with milk allergies by reducing the overall oxidative stress in the body (López-García et al., 2022).

2.4.2. Angiotensin-I-Converting Enzyme

Angiotensin-I-converting enzyme (ACE) plays a crucial role in regulating blood pressure. Targeting ACE is effective in reducing blood pressure. ACE-inhibitory (ACE-I) peptides, derived from fermented milk, offer a natural and safe approach to treating hypertension. While milk proteins serve as a precursor to bioactive peptides, most peptides in milk are inactive and require

hydrolysis for release. Fermentation of milk with LAB, found in yogurt and cheese, hydrolyzes proteins, releasing bioactive peptides. LAB's proteolytic system makes them potential producers of antihypertensive peptides in fermented foods. Proteolytic activity varies among LAB species and strains, emphasizing the need to identify those with high proteolytic activity for effective antihypertensive peptide production. Notable lactic acid bacteria reported for this purpose include Lactobacillus delbrueckii ssp. bulgaricus, and. L. helveticus is widely utilized in dairy products for generating ACE-I peptides (Rubak et al., 2020).

3. MATERIALS AND METHODS

3.1. Procurement of Materials

In our experiment, we used ultra-high temperature (UHT) and extended self-life (ESL) milk. These milk products were brought from SPAR supermarket located in Budapest. The dairy products were transported to Laboratory 16, which is located in Building A of the Department of Food Engineering.

3.2. Preparation of Yogurt

Yogurt preparation requires two important processes. For starters, milk proteins were sequentially hydrolyzed with papain in 500 mL glass beakers. These beakers were placed carefully in an incubator (HACH, Düsseldorf, Germany) at a constant temperature of 50°C, allowing for a controlled protein hydrolysis reaction. Various quantities of papain (HIMEDIA, India) were used in this reaction, specifically 0.008 g/L and 0.012 g/L. These are represented by UHT-P1, UHT-P2, ESL-P1 and ESL-P2. Subsequently, when the milk temperatures reached 50°C consistently, precise amounts of a papain solution with a concentration of 0.009 $g \cdot mL^{-1}$were added into the milk samples. This introduction was made possible by a sterile polyethersulfone (PES) syringe filter with a pore size of 0.2 m manufactured by VWR International in Radnor, PA, USA. The milk samples were then subjected to a 10-minute papain hydrolysis process in the same 50°C incubator. The glass beakers were then transported to a thermostat, where the papain activity was deactivated at a higher temperature of 70°C. This deactivation procedure required a 30-minute incubation period in a water bath. (Nath et al., 2020). Following papain deactivation, all milk types were carefully cooled to 45°C within a controlled laminar flow chamber. Our research focused on the effect of glucose on the lactic acid fermentation process. To investigate this, we injected 40 µL of sterile β-galactosidase into each 90 mL portion of milk individually, ensuring aseptic conditions. For the microbial hydrolysis of the protein concentrate, we used LAB (Thermophilic YoFlex® Mild 1.0, sourced from Chr. Hansen in Nienburg, Germany). Each sample received an inoculum of 900 µL from the stock culture. Following inoculation, the levels of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in the fermentation medium were measured at 5.5×10^6 $CFU \cdot mL^{-1}$ and 1.5×10^7 $CFU \cdot mL^{-1}$, respectively, using Breed's method (Beshkova et al., 1998). Fermentation

was performed at a temperature of 45 °C for 6 h in an incubator (HACH, Düsseldorf, Germany) (Li et al., 2019). Fermented samples were represented by UHT-P1-Y, UHT-P2-Y, UHT-P1-Y-NL, UHT-P2-Y-NL, ESL-P1-Y, ESL-P2-Y, ESL-P1-Y-NL and ESL-P2-Y-NL. All types of yogurt samples were stored in refrigerator after microbial fermentation with 6 h.

3.3.Analytical Method

3.3.1. Understanding the Molecular Weight Distribution of Proteins and Peptides

To assess the molecular weights of proteins and peptides in various milk and yogurt samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli method (LAEMMLI, 1970). The running gel and stacking gel were generated in the vertical electrophoresis equipment (Bio-Rad Mini Protean 3 Cell and the power PAC1000) at 15% and 6% concentrations, respectively. Proteins and peptides were separated electrophoretically using a constant voltage of 200 V, a current of 54 mA, and a power of 11W. Laemmli sample buffer (2X) and 10% 2-mercaptoethanol were used to dilute the sample. Each well in the stacking gel received 10 L of the correctly diluted sample, and the electrophoresis was run at room temperature for 60 minutes. The molecular weights of proteins and peptides were determined using a standard protein marker from Bio-Rad (Hercules, CA, USA). A solution of 0.2% Coomassie Brilliant Blue R250 in 9% acetic acid (96%) and 45% ethanol was applied to the gel for 30 minutes at room temperature to stain it. The gel was then de-stained at room temperature with a solution of 50% methanol-water and 10% acetic acid (both volume-based). To acquire photos of the gel, the Gel Doc System 2000 (Bio-Rad CA, USA) was used.

3.3.2. Immunoblotting

The proteins and peptides from SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-blot semi-dry transfer cell (Bio-Rad in California, USA). To assess the antigenicity of different milk and yogurt samples, a rabbit polyclonal antibody against casein obtained from cow's milk was employed. Secondary antibodies, including peroxidase-labeled goat anti-human Immunoglobulin E (IgE) and peroxidase-labeled goat anti-rabbit (Rb) Immunoglobulin G (IgG), were provided by Sigma-Aldrich in Stockdorf, Germany. The transfer

of proteins and peptides onto a PVDF membrane with a pore size of 0.45 µm (Merck Millipore, MA, USA) was carried out following the manufacturer's instructions. The Trans-blot semi-dry transfer cell from Bio-Rad was used, operating at 0.25 V and 0.8 mA/cm² for 1 hour. The binding patterns of the antibodies were visualized using a substrate solution containing 4-chloronaphtol, hydrogen peroxide, and ethanol in a 16 mM phosphate-buffered saline solution at pH 7.2. The Gel Doc 2000 system (Bio-Rad in California, USA) was utilized for image analysis of the blot (Hajós et al., 2004).

3.3.3. Determination of Protein Concentration

All milk and yogurt samples were centrifuged in a temperature-controlled laboratory centrifuge (Z206A; Wehingen, Germany) prior to testing. Centrifugation was carried out at 10,000 rpm for 20 minutes at 4 °C, and the content of protein in supernatants was determined using the Bradford assay (Bradford, 1976). 100 L of appropriately diluted supernatant and 3 mL of Bradford reagent (Sigma-Aldrich, Schnelldorf, Germany) were vortexed. A blank sample produced with 100 L of DI water and 3 mL of Bradford reagent was used for colorimetric determination. Following that, the mixture was incubated at room temperature for 30 minutes in an incubator (HACH, Düsseldorf, Germany). Spectrophotometric analysis was performed with wavelength 580 nm in a UV-Vis spectrophotometer (EvolutionTM 300; Thermo ScientificTM, Waltham, MA, USA) at RT. Bovine serum albumin (Sigma-Aldrich, Schnelldorf, Germany) was used as a standard in assay.

3.3.4. Determination of the Antioxidant Capacity

3.3.4.1. Ferric Reducing Ability of Plasma (FRAP) Assay

All the samples were centrifuged in a laboratory centrifuge (Z206A, Wehingen, Germany). The centrifugation operational parameters remained constant with the previously indicated settings. Ferric-reducing ability of plasma (FRAP) was used to assess the antioxidant capacity of the supernatants.A part of 100 L of properly diluted supernatant was mixed with a reagent solution containing 5 mL of 20 mM ferric chloride, 5 mL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (Sigma-Aldrich in Schnelldorf, Germany) was used with 40 mM hydrochloric acid, and 50 mL of 300 mM sodium acetate buffer with a pH of 3.6. This combination was then incubated in an incubator (Düsseldorf, Germany (HACH)) for 30 minutes at 35 °C. Under low pH conditions and in the presence of antioxidant peptides or proteins, the Fe3+-TPTZ (ferric-tripyridyltriazine) complex is reduced to Fe2+-TPTZ, resulting in the development of a blue color. At room temperature, the

intensity of this blue color was measured using a UV-Vis spectrophotometer at a wavelength of 593 nm. The antioxidant capacity of the proteins and peptides was determined using ascorbic acid (99.7%, Merck, Darmstadt, Germany) as a reference. A blank sample for spectrophotometric analysis was also created by combining 100 L of deionized water and 2.9 mL of the reagent (Benzie & Strain, 1996).

3.3.4.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical-Scavenging Assay

Following the previously indicated operational conditions, supernatants from all samples were obtained using centrifugation. The goal was to assess the antioxidant capability of these supernatants using the DPPH radical-scavenging assay. 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich in Schnelldorf, Germany) was used for this experiment. In the experiment, 100L of supernatant was combined with 3.9 mL of a 610-5 M DPPH-methanol solution. 100 L of methanol was mixed with 3.9 mL of the same 610-5 M DPPH-methanol solution to make a control sample. These mixes were vortexed and then incubated for 30 minutes at room temperature in the dark. After incubation, a UV-Vis spectrophotometer was used to measure the absorbance at a wavelength of 517 nm. The percentage change in absorbance compared to the control sample was calculated to determine the DPPH radical-scavenging activity. This assay provides insights into the ability of the samples to neutralize the DPPH radicals, indicating their antioxidant activity (Arfaoui, 2020).

3.3.5. Estimation of Angiotensin-Converting Enzyme Inhibitory Activity

In this experiment, as previously done, samples were subjected to centrifugation to obtain supernatants. The purpose was to measure the angiotensin-converting enzyme inhibitory activity in these supernatants, following a method described earlier with slight modifications. Recombinant angiotensin-converting enzyme sourced from the Division of Clinical Physiology at the Institute of Cardiology, University of Debrecen, Hungary, was used. In each well, the enzymatic reaction mixture contained 100 mM TRIS HCl (pH 7), 50 mM sodium chloride, 10 mM zinc chloride, 15 mM substrate Abz-FRK(Dnp)-P (Sigma-Aldrich in Schnelldorf, Germany), and the recombinant angiotensin-converting enzyme. The concentration of recombinant angiotensin-converting enzyme was adjusted to yield around ten times the activity found in human serum. The IC50 value of the samples was determined using supernatants from various milk and yogurt samples that had been diluted 10-fold to 106-fold. The activity of the enzyme in the absence of sample supernatants served

as the reference point for unrestricted activity. The addition of the substrate started the process. In each plate, the level of inhibition was determined as a percentage of uninhibited activity. At 37°C, measurements were taken in a fluorescent plate reader (BMG Novostar, BMG Labtech, Germany), with variations in optical density recorded over at least 20 cycles at excitation wavelength 340 nm and emission wavelength 405 nm. This assay was used to assess the ability of the materials to inhibit the angiotensin-converting enzyme (Fagyas et al., 2014).

3.3.6. Texture Analysis of Yoghurt

Texture profile analysis (TPA) was performed on a TA-XTplus machine (Stable Micro Systems, UK), which was outfitted with a 5 kg load cell. A 20 mm diameter cylinder probe (P/20) was utilized in the study to deliver a steady 5 mm compression to a 50 ml cup-set yogurt. The process included a pre-test phase at 1 mm s^{-1} speed, followed by a compression and relaxation phase at 0.1 mm s^{-1} speed. All measurements were taken at a constant temperature of 25±1.0 °C, with three repetitions to ensure precision. Key textural parameters such as hardness, adhesiveness, and springiness were measured using the TPA curves to evaluate the texture of the yogurt.

4. RESULT AND DISCUSSION

4.1. Texture Analysis of the Prepared Yogurt Samples

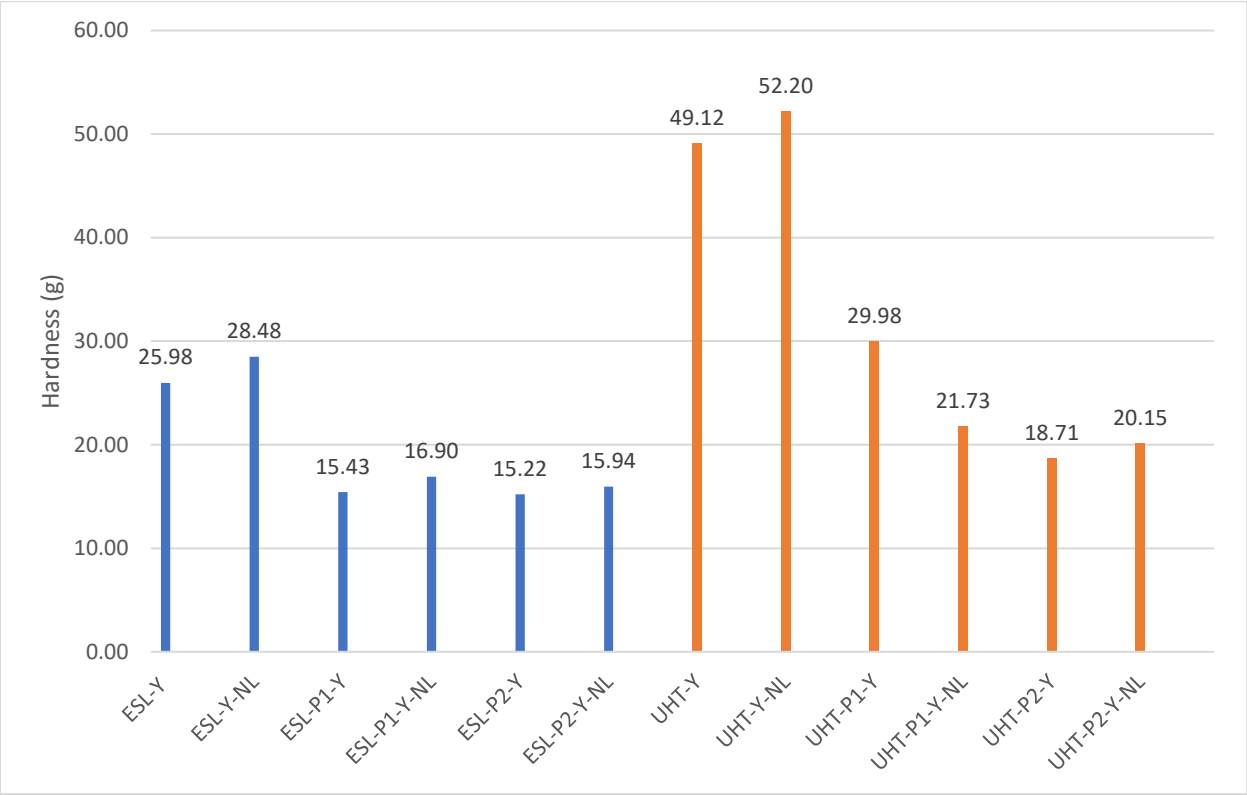

Figure 1. The value of Hardness in g of ESL-Y, ESL-Y-NL, ESL-P1-Y, ESL-P1-Y-NL, ESL-P2-Y, ESL-P2-Y-NL, UHT-Y, UHT-Y-NL, UHT-P1-Y, UHT-P1-Y-NL, UHT-P2-Y and UHT-P2-Y-NL. Results are represented with a mean value of three triplicate experimental results.

In Figure 1, the firmness of yogurt samples from ESL and UHT milk is depicted. When comparing yogurt samples from ESL-Y and UHT-Y that underwent only LAB fermentation, a noticeable difference in hardness is evident. UHT-Y shows significant firmness, likely attributed to reduced protein breakdown by LAB in UHT milk compared to ESL milk. The assumption is that the more intense heating of UHT milk makes it more prone to lactosylation (Lu et al., 2023). Interestingly, bacteria can more easily break down lactosylated molecules than proteins. This enzymatic degradation process subsequently leads to the production of exopolysaccharides. Exopolysaccharides is a complex carbohydrate produced by LAB which modifies rheological

properties including increasesd viscosity, and a denser yogurt structure. Moreover, when glucose is added it may promote the microbial growth and formation of microbial metabolites, such as nitrogenous compounds, exopolysaccharides and lactate (Purohit et al., 2009; Yang et al., 2014). This is evident when we compare the samples of ESL-Y-NL and UHT-Y-NL with β-galactosidase added, which promotes lactose breakdown into galactose and glucose and increases the exopolysaccharide, leading in firmer yogurt (Nath et al., 2020)

Upon further observation, it is apparent that the firmness of UHT-P1-Y, UHT-P1-Y-NL, UHT-P2-Y, and UHT-P2-Y-NL is noticeably reduced and resembles the texture of ESL-P1-Y, ESL-P1-Y-NL, ESL-P2-Y, and ESL-P2-Y-NL samples. Moreover, when the concentration of papain is increased the hardness of the sample reduces. This is because as the concentration increases, the hydrolysis of proteins breaking into lower molecular weights increases, which even causes the denaturation and coagulation of proteins during papain and microbial fermentation, leading to an alteration in the overall protein network of yogurt and impacting its texture and hardness (Csighy et al., 2020). It has been noted that yogurt samples derived from UHT milk, following both papain and microbial hydrolysis, consistently exhibit a slightly firmer texture compared to samples from ESL milk. This difference is attributed to the higher lactosylation of UHT milk, which effectively reduces the proteolytic activity of papain. The increased lactosylation introduces steric hindrance, acting as an inhibitory factor for papain's catalytic function. As a result, the yogurt samples from UHT milk maintain a degree of hardness even after undergoing hydrolysis processes (Nath et al., 2021; Lu et al., 2023).

4.2.Hydrolysis of Proteins in ESL and UHT Milk

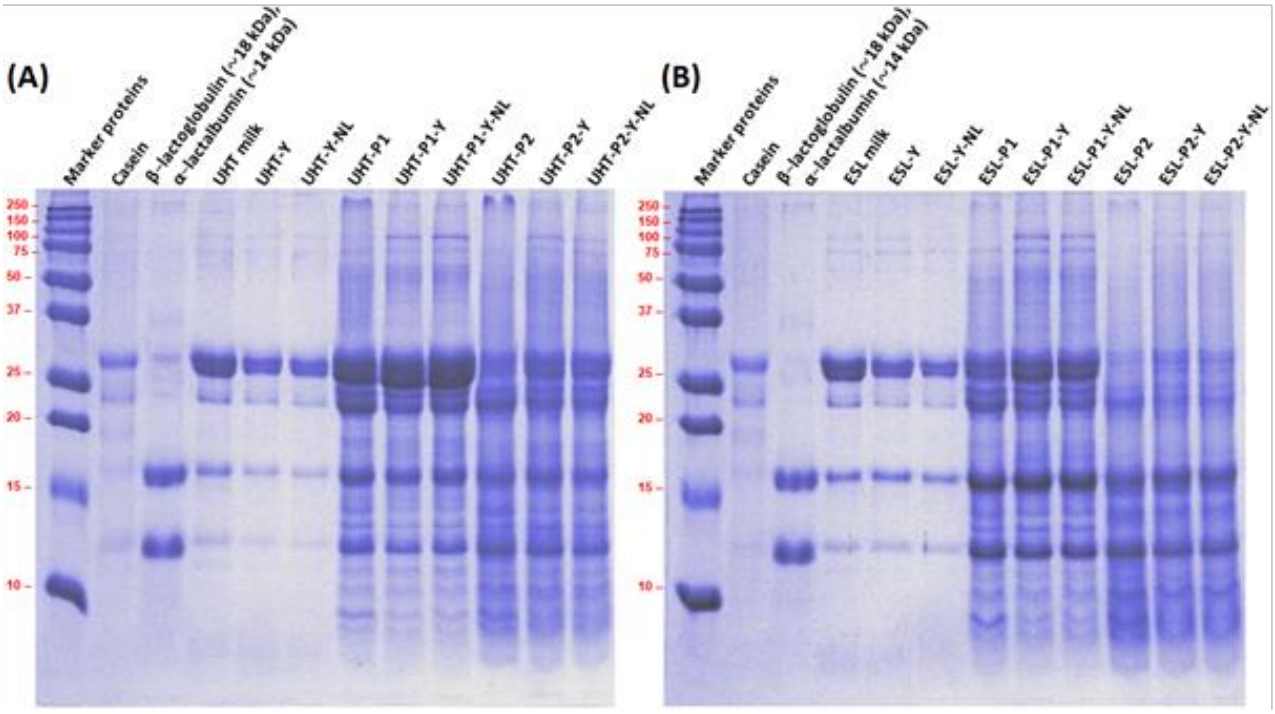

Figure 2: SDS-PAGE image of proteins and peptides; (A) lane 1: marker protein, lane 2: standard casein, lane 3: standard α-lactalbumin and β-lactoglobulin, lane 4: UHT milk, lane 5:UHT-Y, lane 6: UHT-Y-NL, 7: UHT-P1, lane 8: UHT-P1-Y, lane 9: UHT-P1-Y-NL, lane 10: UHT-P2, lane 11: UHT-P2-Y, lane 12:UHT-P2-Y-NL; (B)) lane 1: marker protein, lane 2: standard casein, lane 3: standard α-lactalbumin and β-lactoglobulin, lane 4: ESL milk, lane 5:ESL-Y, lane 6: ESL-Y-NL, 7: ESL-P1, lane 8: ESL-P1-Y, lane 9: ESL-P1-Y-NL, lane 10: ESL-P2, lane 11: ESL-P2-Y, lane 12: ESL-P2-Y-NL

From Figure 2A and B, both UHT milk and ESL milk have αs1-casein (~24kDa), αs2-casein (~26 kDa), β-casein (~24kDa), β-LG (~21kDa) and α-LA (~12kDa). From the SDS-PAGE image the UHT-Y and UHT-Y-NL have undergone bacterial fermentation and the molecular weight of αs1-casein, αs2-casein, β-casein, β-LG, and α-LA seem to be less apparent mostly due to the uptake of the proteins and protein coagulation. UHT-P1, UHT-P1-Y, and UHT-P1-Y-NL exhibit a wide range of protein and peptide molecular weights as papain enzymatic treatment likely induces partial hydrolysis of proteins, leading to various peptide molecular weights. Moreover, the combination of papain treatment and LAB fermentation introduces additional enzymatic and microbial

activities, leading to a further breakdown of proteins and peptides with varied sizes. This is evident because UHT-P2, UHT-P2-Y, and UHT-P2-Y-NL have a higher papain concentration leading to more profound protein hydrolysis. As a result, proteins are further degraded and conjugated. We can also see that UHT-P2, UHT-P2-Y, and UHT-P2-Y-NL have more apparent lower peptide molecular weights (Nath et al., 2020).

The alteration in sizes could likely be a consequence of changes in the sizes of milk proteins compared to their typical dimensions. When milk undergoes temperatures surpassing 80 °C, the tertiary structure of whey proteins unfolds, causing denaturation and potentially partial degradation of casein micelles through dephosphorylation. Subsequently, the denatured whey proteins either bind among themselves or associate with dephosphorylated casein molecules, potentially increasing the size of casein micelles. Additionally, intermolecular conjugation of whey proteins may increase the size of whey proteins, resulting in a larger range of lower molecular weight proteins (Galani & Apenten*, 1999; Morr, 1969).

Likewise, in Figure 2B, we observe the effects of papain and microbial hydrolysis on ESL milk. ESL-Y and ESL-Y-NL undergo exclusive fermentation, resulting in a narrower range of caseins. ESL-P1, the addition of papain causes partial hydrolysis and ESL-P1-Y, and ESL-P1-Y-NL due to papain and microbial hydrolysis they broaden the spectrum of lower molecular weight peptides. Similarly, ESL-P2, ESL-P2-Y, and ESL-P2-Y-NL, with a higher papain concetration caused a more significant protein hydrolysis which led to a smaller peptide molecular weight. Moreover, caseins show a dramatic hydrolysis as higher concentrations of papain provide more enzyme molecules to interact with casein substrates. This elevated enzyme-substrate interaction leads to increased hydrolysis as more casein molecules are exposed to the active sites of papain (Li et al., 2022).

It's noteworthy that yogurt produced from ESL milk displays a broader spectrum of lower molecular weight proteins when compared to yogurt from UHT milk. This discrepancy is attributed to the lower lactosylation of ESL milk compared to UHT milk. As a result, proteins in ESL milk are more susceptible to breakdown by both papain and microbial hydrolysis. In contrast, the lactosylation of UHT milk presents steric hindrance, and the proteins are more conjugated, making papain hydrolysis less effective, resulting in fewer proteins being broken down (Nath et al., 2020).

4.3. Antigenicity

As illustrated in Figure 3A, UHT milk exhibits antigenicity for casein. In contrast, LF, lactoperoxidase, BSA, and α-LA show no antigenicity. However, UHT-P1, UHT-P1-Y, and UHT-P1-Y-NL demonstrate antigenicity for these proteins, possibly due to cross-reactivity. UHT-P2, UHT-P2-Y, and UHT-P2-Y-NL only display antigenicity for BSA, LF, and lactoperoxidase. The antigenicity of casein is reduced in UHT-Y and UHT-Y-NL, possibly owing to the breakdown of proteins by LAB. This process, as indicated by Shi et al. in 2014, involves the cleavage of epitopes through proteolysis, leading to a reduction in antigenicity. However, microbial hydrolysis alone couldn't remove the antigenicity of casein which signifies that they are resistant to proteolytic enzymes produced by LAB during fermentation. In comparison to UHT-Y and UHT-Y-NL, the antigenicity of casein is further diminished in UHT-P1, UHT-P1-Y, and UHT-P1-Y-NL. This reduction can be attributed to the sequential application of papain and microbial hydrolysis on milk proteins, effectively eliminating antigenic epitopes in casein. Notably, the antigenicity of casein is nearly absent in UHT-P2, UHT-P2-Y, and UHT-P2-Y-N. The augmentation of papain concentration in milk is likely to promote extensive hydrolysis of milk proteins, leading to the breakdown of allergenic proteins into smaller peptides and a subsequent reduction in their size, as elucidated by Liang et al. in 2020b.

Similarly, Like the yogurt samples from UHT milk, we can observe from Figure 3B, there is a slight reduction in the antigenicity of casein in ESL-Y and ESL-Y-NL. However, microbial hydrolysis couldn't completely remove the antigenicity of casein. Furthermore, ESL-P1, ESL-P1-Y, and ESL-P1-Y-NL show a pronounced reduction in the antigenicity of casein and with an increase in the concentration of papain, the antigenicity of casein is nearly absent in ESL-P2, ESL-P2-Y, and ESL-P2-Y-N. However, a minor presence of antigenicity observed for α-LA at ~13kDa is observed which could be due to cross reactivity. From Figure 3A, B we can see that the inclusion of glucose showed some impact on reducing antigenicity across all instances of the samples, the addition of glucose stimulates microbial growth, leading to the breakdown of external proteins and their conversion into lactic acid and nitrogenous metabolites. In summary, yoghurt samples derived from ESL milk exhibit lower antigenicity compared to those from UHT milk. This distinction can be linked to the increased hydrolysis in ESL milk, a consequence of its non-lactosylated nature and the absence of significant steric hindrance (Nath et al., 2020).

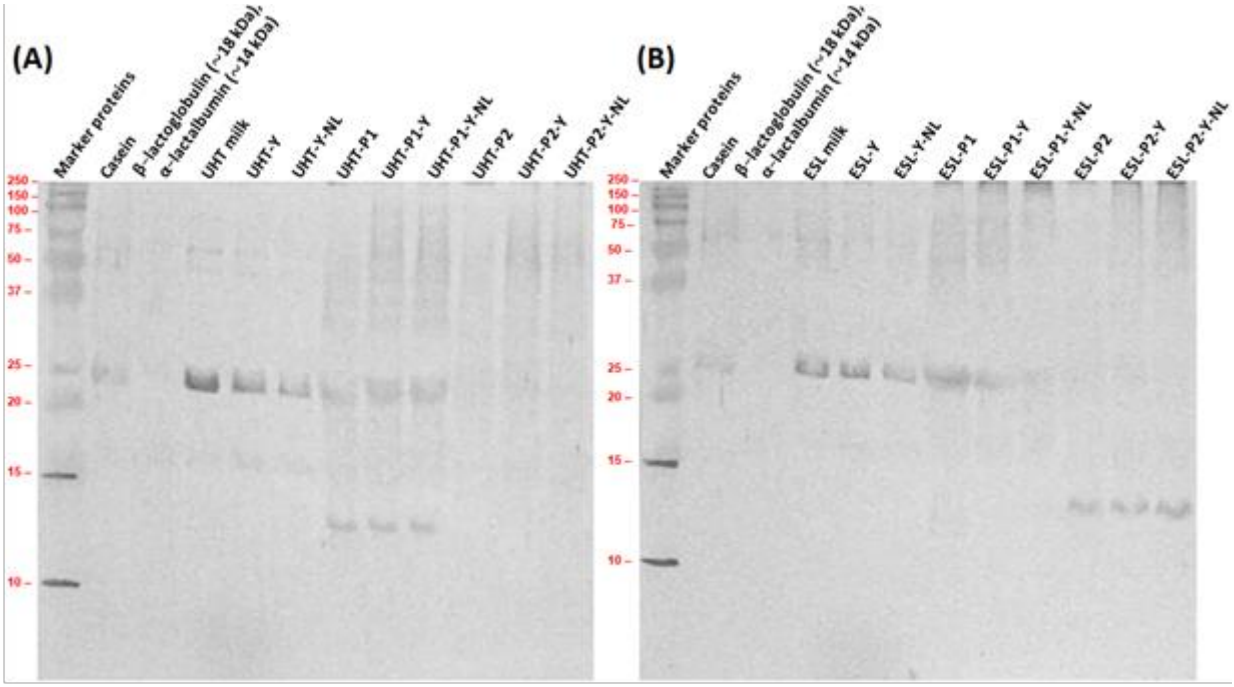

Figure 3: SDS-PAGE image of proteins and peptides. Antigenicity of proteins and peptides in UHT milk and yogurt from UHT milk (A), ESL milk and yogurt from ESL milk (B)

4.4.Angiotensin-Converting Enzyme Inhibitory Activity

The IC_{50} value is a measure of inhibitory concentration, and significantly decreases in a concentration-dependent manner following papain and LAB hydrolysis. This reduction is due to the treatment with papain and microbial hydrolysis, which unfolds the natural milk protein, exposing binding sites for the angiotensin-converting enzyme. Furthermore, because of the reduced steric hindrance, the resulting smaller molecular weight peptides have a better possibility of interacting with the enzyme (Asoodeh et al., 2016). Peptides contain hydrophobic amino acids like phenylalanine, proline, tryptophan, and tyrosine at the C-terminal position that have the capacity to bind with the angiotensin-converting enzyme, which helps in facilitating the inhibitory activity. Furthermore, we can observe the reduction in the IC_{50} value when the concentration of papain with LAB hydrolysis is increased. This is a result of the proteolytic system of LAB releasing angiotensin-converting enzyme-inhibitory peptides. In Figure 4, it is evident that the IC_{50} values in yogurt derived from ESL milk samples are lower compared to those from UHT milk samples. This difference arises because ESL milk undergoes a more extensive hydrolysis process, resulting in

smaller molecular weight peptides. These smaller peptides exhibit a higher likelihood of interacting with enzymes, attributed to reduced steric hindrance (Nath et al., 2021). Furthermore, the introduction of glucose into the samples leads to even lower IC_{50} values. The rationale behind this observation lies in the fact that glucose presence in the fermentation medium fosters microbial growth, subsequently influencing the proteolysis of milk proteins (Ramchandran & Shah, 2010).

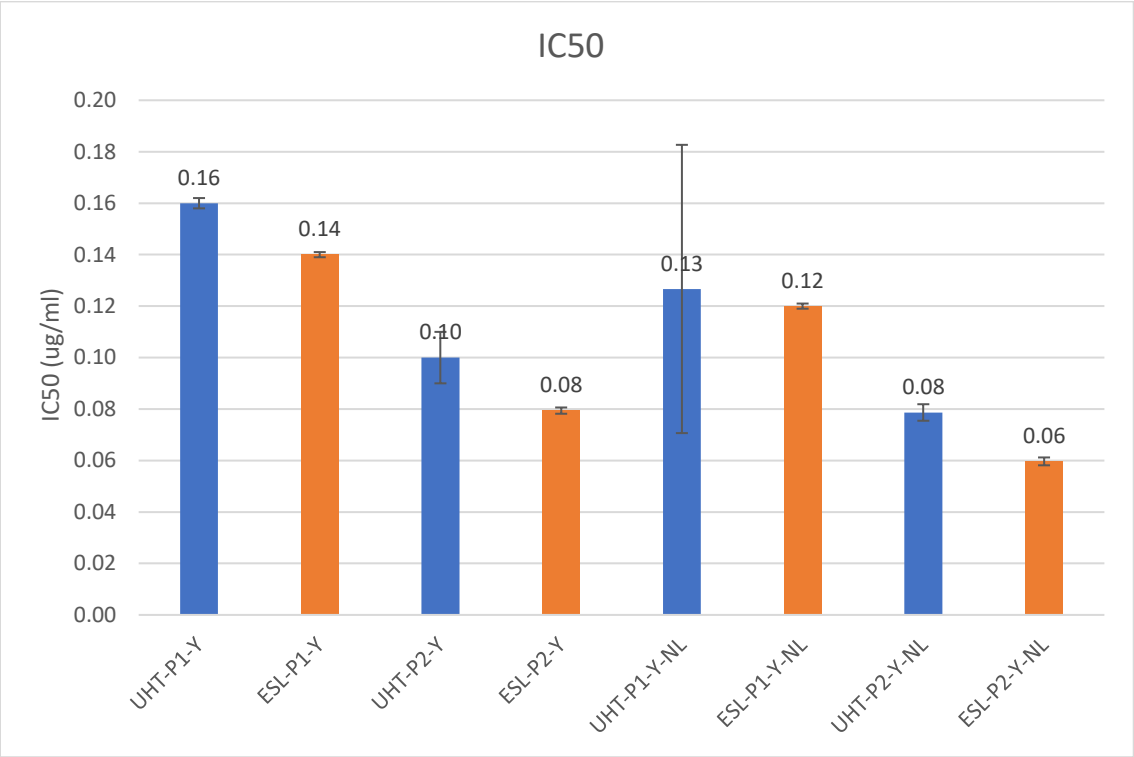

Figure 4. The value of IC_{50} of UHT-P1-Y, ESL-P1-Y, UHT-P2-Y, ESL-P2-Y, UHT-P1-Y-NL, ESL-P1-Y-NL, UHT-P2-Y-NL, and ESL-P2-Y-NL. Results are represented with a mean value and standard deviation (± values) of three triplicate experimental results.

4.5. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

DPPH is a stable free radical with its peak absorbance observed at a wavelength of 517 nm in a methanol solution. When DPPH encounters a substance capable of donating a proton, like an antioxidant, it undergoes scavenging, leading to a reduction in absorbance at 517 nm. In Figure 4, the DPPH antioxidant activity percentage of UHT-P1-Y, ESL-P1-Y, UHT-P2-Y, ESL-P2-Y, UHT-P1-Y-NL, ESL-P1-Y-NL, UHT-P2-Y-NL, and ESL-P2-Y-NL is depicted. We can see that the

DDPH antioxidant capacity in yogurts from ESL samples is higher compared to UHT samples. Furthermore, the antioxidant capacity increases with a higher concentration of papain. This indicates an elevated antioxidant capacity in papain-treated milk with an increased dose level. Peptides with lower molecular weight and higher charge density exhibit enhanced scavenging activity against free radicals, attributed to their low steric hindrance. The higher concentration of papain leads to increased hydrolysis of peptide linkages in milk, exposing hydrophobic amino acids. These hydrolyzed peptides from milk protein act as electron donors, effectively converting DPPH free radicals into more stable molecules. The hydrophobic amino acid at the N-terminus position of the peptide contributes to radical scavenging activity (Bamdad et al., 2017).

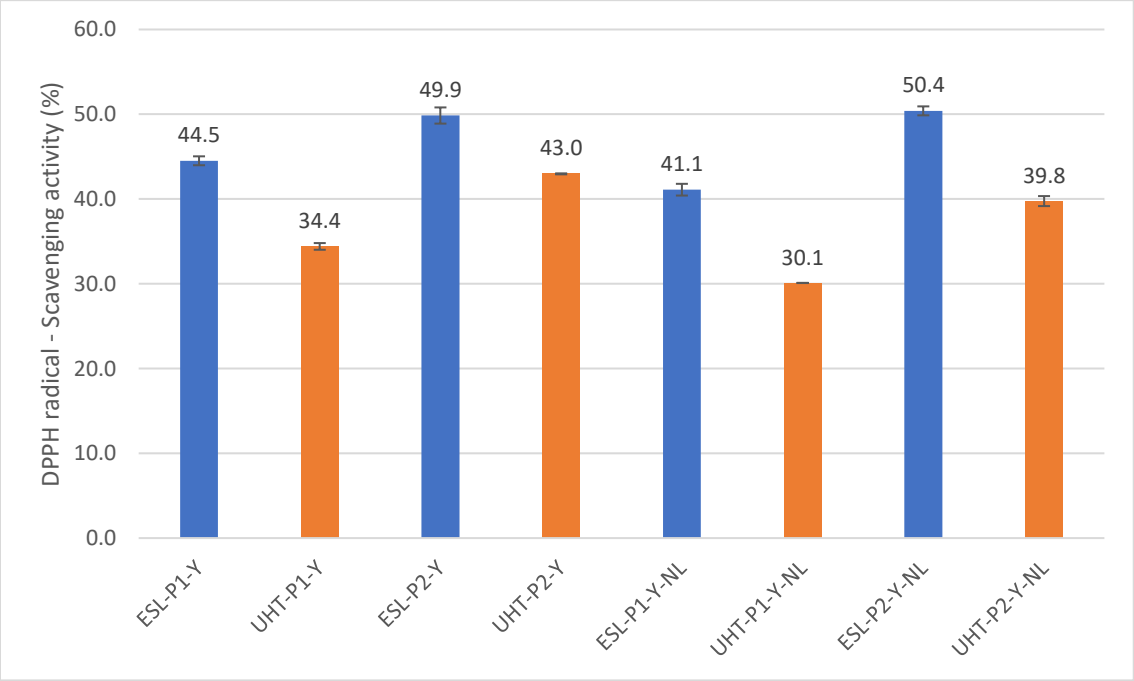

Figure 5. The DPPH antioxidant capacity of ESL-P1-Y, UHT-P1-Y, ESL-P2-Y, UHT-P2-Y, ESL-P1-Y-NL, UHT-P1-Y-NL, ESL-P2-Y-NL, and UHT-P2-Y-NL. Results are represented with a mean value and standard deviation (± values) of three triplicate experimental results.

4.6.Ferric Reducing Ability of Plasma (FRAP) Assay

The antioxidant potential of yogurt samples from ESL and UHT milk was assessed using the ferric reducing antioxidant power (FRAP) assay, depicted in Figure 11. In this assay, hydrophobic amino acids or aromatic groups within antioxidant peptides play a role in reducing ferric ions (Fe3+) to ferrous ions (Fe2+). Upon closer examination of Figure 6, it becomes evident that the antioxidant capacity of all samples increased with higher concentrations of papain. Papain, with a preference for cleaving peptide bonds at hydrophobic amino acids (Arg, Lys, Glu, His, Gly, or Tyr) in the amino acid sequence, demonstrates reducing activity towards ferric ions, contributing to the reduction of Fe3+–TPTZ complex. It is noteworthy that peptides with a lower molecular weight exhibit superior reducing activity towards Fe3+, attributed to their higher charge-to-mass ratio and the exposure of more electron-rich side chains (Nath, Kailo, et al., 2020). Furthermore, the yogurt samples from ESL milk exhibited a greater antioxidant capacity compared to those from UHT milk. This discrepancy is attributed to the more profound hydrolysis undergone by ESL milk, leading to the breakdown of proteins into even smaller peptides (Nath et al., 2020).

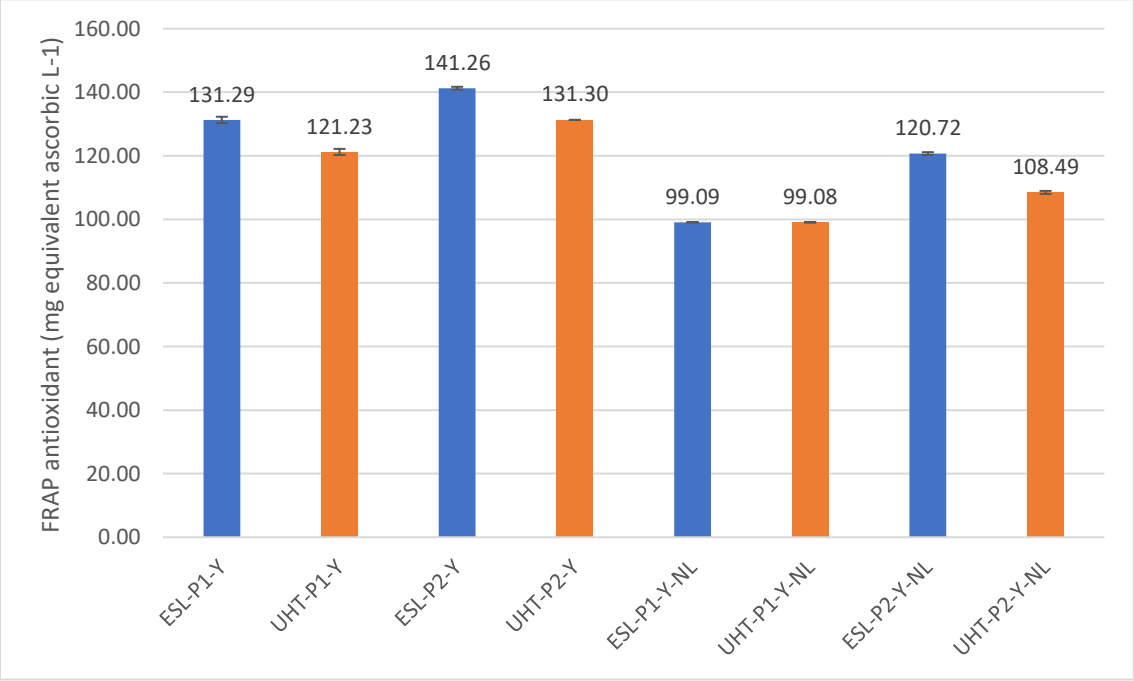

Figure 6. FRAP Assay antioxidant capacity of ESL-P1-Y, UHT-P1-Y, ESL-P2-Y, UHT-P2-Y, ESL-P1-Y-NL, UHT-P1-Y-NL, ESL-P2-Y-NL, and UHT-P2-Y-NL. Results are represented with a mean value and standard deviation (± values) of three triplicate experimental results.

5. CONCLUSION

In the examination of yogurt samples derived from ESL and UHT milk, it is evident that hardness diminishes with both papain and microbial hydrolysis. Furthermore, an increase in papain concentration correlates with a reduction in hardness, indicative of intensified hydrolysis. Notably, UHT milk yields slightly firmer textures, attributed to higher lactosylation impeding papain activity. The SDS-PAGE gel electrophoresis showed varied molecular weights in proteins and peptides. Samples from ESL milk exhibited broader lower molecular weight peptides compared to samples from UHT milk due to lower lactosylation and rendering proteins more susceptible to hydrolysis which also indicates lower antigenicity by eliminating antigenic epitopes. Moreover, the IC_{50} values showed ESL milk samples to have lower IC_{50} than UHT milk samples and the antioxidant capacity, assessed by FRAP and DPPH assays, was higher in ESL samples, attributed to more profound hydrolysis leading to smaller peptides with enhanced scavenging activity. In conclusion, the study demonstrates the efficacy of enzymatic and microbial hydrolysis in reducing allergenicity, with ESL milk presenting as a more favorable substrate for extensive hydrolysis and improved functional properties.

6. SUMMARY

Cow's milk is a crucial component of the diets of infants and children worldwide, offering a range of nutrients that contribute to the development of a robust immune system and overall health. However, as we all know, cow's milk is a leading cause of food allergies in children, generating abnormal immunological reactions. While complete milk avoidance is a common approach for preventing milk allergy, these proteins are included in a variety of processed foods and avoiding them may result in nutritional deficits. Heating, enzymatic, and microbial hydrolysis are all food processing procedures that can modify protein structures by removing or degrading epitopes.

In our study, we used papain enzymatic hydrolysis and LAB fermentation. Fermented foods not only improve nutritional value but also include bioactive peptides produced by bacteria during the fermentation process. Yogurt bioactive peptides have functional features such as ACE inhibition and antithrombotic action. This has piqued the food industry's interest in developing novel food supplements and functional goods based on these bioactive peptides.

We examined the texture, molecular weight, antigenicity, ACE inhibitory concentration, and antioxidant capacity of yogurt samples derived from UHT and ESL milk. This investigation involved the application of papain enzymatic hydrolysis and LAB fermentation. Two concentrations of papain, namely 0.008 g/L (UHT-P1, ESL-P1) and 0.012 g/L (UHT-P2, ESL-P2), were prepared. Additionally, we investigated the impact of glucose on lactic acid fermentation by introducing β-galactosidase into individual milk portions. For microbial hydrolysis, LAB strains Streptococcus thermophilus and Lactobacillus bulgaricus were utilized, and fermentation at 45 °C for 6 hours occurred. The resulting fermented samples were designated as UHT-P1-Y, UHT-P2-Y, UHT-P1-Y-NL, UHT-P2-Y-NL, ESL-P1-Y, ESL-P2-Y, ESL-P1-Y-NL, and ESL-P2-Y-NL

The texture analysis of yogurt samples revealed that the hardness of both UHT and ESL milk-derived yogurts decreased with papain and microbial hydrolysis, particularly at higher papain concentrations. Interestingly, UHT milk-based yogurts exhibited a slightly firmer texture, attributed to increased lactosylation inhibiting papain's proteolytic activity.

To understand the molecular weight and distribution of proteins and peptides, SDS-PAGE gel electrophoresis was conducted. The results showed that higher enzyme-to-substrate ratios led to the conversion of large molecular weight protein subunits into low molecular weight peptides.

UHT-P1, UHT-P1-Y, and UHT-P1-Y-NL exhibited a wide range of molecular weights due to partial hydrolysis induced by papain treatment. The combination of papain and LAB hydrolysis further broke down proteins into peptides, with UHT-P2, UHT-P2-Y, and UHT-P2-Y-NL showing more profound protein hydrolysis and lower peptide molecular weights. Notably, yogurt from ESL milk displayed a broader spectrum of lower molecular weight proteins compared to UHT milk, attributed to lower lactosylation in ESL milk, making proteins more susceptible to papain and microbial hydrolysis.

The antigenicity of yogurt samples from UHT and ESL milk using a rabbit polyclonal antibody against cow's milk casein was studied. Papain hydrolysis demonstrated a dose-dependent reduction in allergenicity, with UHT-Y, ESL-Y, UHT-Y-NL, and ESL-Y-NL showing decreased casein antigenicity, likely due to LAB proteolysis. However, microbial hydrolysis alone was insufficient in eliminating casein antigenicity. The sequential application of papain and microbial hydrolysis in UHT-P1, ESL-P1, UHT-P1-Y, ESL-P1-Y, UHT-P1-Y-NL, and ESL-P1-Y-NL further reduced casein antigenicity. Remarkably, UHT-P2, ESL-P2, UHT-P2-Y, ESL-P2-Y, UHT-P2-Y-NL, and ESL-P2-Y-NL nearly eliminated casein antigenicity, attributed to increased papain concentration promoting extensive hydrolysis. Moreover, samples from ESL milk showed greater reduction in antigenicity.

The IC_{50} values, measuring ACE inhibitory concentration, significantly decreased in a concentration-dependent manner following papain and LAB hydrolysis. The lower molecular weight peptides resulting from reduced steric hindrance had a higher likelihood of interacting with enzymes. Higher papain concentration with LAB hydrolysis further reduced IC_{50} values, indicating the release of ACE-inhibitory peptides by LAB. Yogurt from ESL milk exhibited lower IC_{50} values than UHT milk, attributed to ESL milk undergoing more extensive hydrolysis, producing smaller peptides with enhanced enzyme interaction. The addition of glucose further decreased IC_{50} values, as it facilitated microbial growth and influenced milk protein proteolysis.

The antioxidant capacity was measured by using FRAP and DPPH tests, which revealed dose-dependent antioxidant activity. Because of reduced steric hindrance, peptides with lower molecular weight and higher charge density displayed improved scavenging efficacy against free radicals. Yogurt samples from ESL milk had better antioxidant capacity than UHT milk, which was

attributed to the more extensive hydrolysis in ESL milk, which broke down proteins into smaller peptides.

In conclusion, dual hydrolysis with papain and LAB lowered allergenicity in yogurt. Increased papain concentration increased proteolysis while decreasing allergenicity. ESL milk-derived yogurts regularly outperformed, with lesser antigenicity and increased antioxidant capacity due to their non-lactosylated nature, which allows for more thorough hydrolysis. The findings offer suggestions for improving yogurt production in terms of texture, molecular properties, and health-related aspects. This study advances our understanding of enzymatic and microbial hydrolysis in the development of sensitive dairy products.

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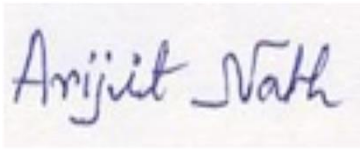

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