ANTI-MICROBIAL PROPERTIES AND BIOCHEMICAL CHARACTERIZATION OF MAILLARD REACTION PRODUCTS FROM LEGUME PROTEIN HYDROLYSATES AND D-GLUCOSE

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ABSTRACT

ANTI-MICROBIAL PROPERTIES AND BIOCHEMICAL CHARACTERIZATION OF MAILLARD REACTION PRODUCTS FROM LEGUME PROTEIN HYDROLYSATES AND D-GLUCOSE

This thesis investigates the antimicrobial properties and biochemical characterization of Maillard Reaction Products (MRPs) derived from chickpea, faba bean, common bean, and soybean protein hydrolysates reacted with D-glucose at various thermal conditions (175°C to 275°C).

The study assessed the antimicrobial activity of these MRPs *against Escherichia coli, Staphylococcus aureus, Lactococcus lactis, Candida albicans, Saccharomyces var. boulardii*, and *Saccharomyces cerevisiae*. Results demonstrated that chickpea MRPs exhibited the highest antimicrobial activity, followed by faba bean MRPs, with significant reductions in bacterial and yeast counts—the antimicrobial efficacy varied with the processing temperature, indicating optimal activity at specific conditions. Biochemical characterization using UV-Vis spectroscopy, FTIR, HPLC, and NMR analyses revealed differences in melanoidin content and structural properties based on the legume source and thermal treatment.

Colorimetric analysis and browning index determination showed that higher temperatures led to darker MRPs with increased browning indices. These findings suggest that legume-based MRPs have potential as natural antimicrobial agents in food preservation, contributing to developing novel food products with enhanced health benefits and safety.

ÖZET

BAKLAGİL PROTEİN HİDROLİZATLARI VE D-GLUKOZDAN ELDE EDİLEN MAİLLARD REAKSİYON ÜRÜNLERİNİN ANTİ-MİKROBİYAL ÖZELLİKLERİ VE BİYOKİMYASAL KARAKTERİZASYONU

Bu tez, çeşitli termal koşullarda (175°C ila 275°C) D-glukoz ile reaksiyona sokulan nohut, bakla, adi fasulye ve soya fasulyesi protein hidrolizatlarından elde edilen Maillard Reaksiyon Ürünlerinin (MRP'ler) antimikrobiyal özelliklerini ve biyokimyasal karakterizasyonunu araştırmaktadır.

Çalışmada bu MRP'lerin *Escherichia coli, Staphylococcus aureus, Lactococcus lactis, Candida albicans, Saccharomyces var. boulardii* ve *Saccharomyces cerevisiae*'ye karşı antimikrobiyal aktivitesi değerlendirilmiştir. Sonuçlar nohut MRP'lerinin en yüksek antimikrobiyal aktiviteyi sergilediğini, bunu bakla MRP'lerinin izlediğini, bakteri ve maya sayılarında önemli azalmalar olduğunu göstermiştir; antimikrobiyal etkinlik işleme sıcaklığına göre değişiklik göstermiş ve belirli koşullarda optimum aktiviteye işaret etmiştir. UV-Vis spektroskopisi, FTIR, HPLC ve NMR analizleri kullanılarak yapılan biyokimyasal karakterizasyon, melanoidin içeriğinde ve yapısal özelliklerinde baklagil kaynağına ve ısıl işleme bağlı olarak farklılıklar olduğunu ortaya koymuştur.

Kolorimetrik analiz ve esmerleşme indeksi tayini, daha yüksek sıcaklıkların daha koyu MRP'lere yol açtığını ve esmerleşme indekslerinin arttığını göstermiştir. Bu bulgular, baklagil bazlı MRP'lerin gıda muhafazasında doğal antimikrobiyal ajanlar olarak potansiyele sahip olduğunu ve gelişmiş sağlık yararları ve güvenliği ile yeni gıda ürünlerinin geliştirilmesine katkıda bulunduğunu göstermektedir.

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CHAPTER 1

INTRODUCTION

1.1. Protein Isolates

1.1.1. Legumes

Legumes' nutritional and functional benefits have led to their recommendation for use in new food preparations, and their impact on food quality is substantial (Carbonaro et al., 2021). Legumes are particularly esteemed for their abundant protein content, which surpasses that of cereals, and therefore serve as a vital supply of amino acids for human nutrition. The consumption of legume-based foods provides a significant source of alternative protein, contributing to a balanced diet (Rebello et al., 2014).

Soybean (*Glycine max*) and several varieties of beans (*Phaseolus*) are extensively cultivated in tropical and subtropical regions. Legumes are well-known for their vital contribution to sustainable agriculture by improving soil quality through nitrogen fixation (Bechthold et al., 2019). Grain legumes, sometimes known as pulses, are fundamental components of traditional meals in numerous countries. In industrialized nations, dietary recommendations strongly encourage the consumption of these foods due to their numerous health benefits (Conti et al., 2021).

Although legumes are typically low in lipids, except soybean, peanut, and lupin, which contain 30–35% lipids, they are an excellent source of proteins and important minerals, including iron, zinc, and calcium (Diplock et al., 1999). Their composition includes a high carbohydrate content (up to 60%), mostly in the form of starch, and essential vitamins, including thiamine, niacin, biotin, riboflavin, and folic acid. Furthermore, legumes are a substantial provider of dietary fiber, accounting for up to 37%

of their composition. This characteristic classifies them as low-glycemic foods, as stated by Rajnincová et al. (2019).

A body of research suggests a positive association between regular consumption of legumes and enhanced well-being. A synthesis of long-term prospective observational studies indicates a reduced probability of developing coronary heart disease in subjects who consume legumes (Bechthold et al., 2019; Conti et al., 2021). As defined in (Diplock et al., 1999), a nutraceutical refers to a food or component offering health advantages, including illness prevention and therapy.

The non-nutritious components of legumes, including isoflavones, alkaloids, phytates, saponins, and diverse proteins, are believed to possess nutraceutical benefits. These components include protease and amylase enzyme inhibitors, lectins, storage proteins, and peptides. Although previously regarded as antinutrients due to their negative impact on nutrient metabolism, further research has revealed the health advantages of these components, emphasizing the intricate function of legumes in human nutrition (Carbonaro et al., 2021). Table 1 shows the composition of 4 legumes used in this thesis.

Table 1. Composition of the Legumes.

Components (%)	Chickpea[a]	Faba Bean[b]	Common Bean[c]	Soybean[d]
Protein	23.6	26.6	21.3	36.9
Carbohydrate	62.3	35.4	47.8	6.1
Fiber	3.8	31.3	18.4	20.9
Lipid	6.4	1.8	1.6	18.1
Ash	3.7	4.1	4	4.7
References	Alajaji et al., 2006	Edwards et al., 1993	Muzquiz et al., 2007	Muzquiz et al., 2012

1.1.1.1. Amino Acid Profiles of Legumes

The amino acid profiles of protein isolates derived from legumes serve as vital markers of the nutritional value and functional characteristics of these products. The profiles offer a comprehensive analysis of the essential and non-essential amino acid composition, which is crucial for the evaluation of legume protein quality (Table 1). The essential amino acids are of significant importance to humans, as the body lacks the ability to synthesize them internally, necessitating their acquisition from dietary sources. Conversely, the body itself may produce non-essential amino acids. Furthermore, an analysis of the composition and abundance of these amino acids offers useful information on the nutritional effectiveness of legume proteins. This was demonstrated by Keskin et al. (2022).

Protein characteristics in food systems are greatly influenced by amino acids. Glutamic acid (Glu) and aspartic acid (Asp) are nonessential amino acids that are usually found in the highest quantities in most legumes, except chickpeas. However, the nutritional value of the protein is limited by the presence of these limiting amino acids, which are found in minimal quantities and thus restrict the protein's nutritional value. The presence and quantity of limiting amino acids in specific legume species varies. Understanding these characteristics enables the optimization of legume protein utilization in food formulations, thereby enhancing their nutritional value and efficacy (Berrazaga et al., 2019).

Glutamic acid and aspartic acid are the primary contributors to the umami taste, which is characterized by its savory flavor. They can affect proteins' solubility and emulsifying characteristics, which are crucial for their use in different food items. The variation in the essential amino acids that are limited in various bean species highlights the significance of consuming a broad diet to guarantee sufficient intake of all necessary amino acids. The diversity mentioned here emphasizes the possibility of using complementary protein techniques, which include mixing several protein sources to obtain a well-balanced amino acid profile (Trevino et al., 2007).

1.1.1.2. Functional and Physicochemical Properties of Legume Protein Isolates

Protein isolates (PIs) derived from legumes are suitable for use in the food processing and nutritional formulation industries, provided that their physicochemical and functional characteristics are optimal. The stability of PIs at elevated temperatures and their processing behavior are two of the most important characteristics to consider. The thermal denaturation temperature (Td) of protein isolates provides an indication of their stability. To provide further illustration, the thermal decomposition temperature (Td) of chickpea protein isolates with a purity of 70% is 205°C, while faba bean isolates with a purity of 88% have a Td of 183°C. Bean isolates with 55% and 75% purities exhibited thermal decomposition (Td) values of 211,5°C and 193.8°C, respectively. Conversely, lentil isolates with purities of 45% and 75% have Td values of 199,5°C and 183.4°C, respectively, according to Ricci et al. (2018). These findings demonstrate that the thermal stability of pea protein isolates (PIs) is positively correlated with their purity level. The denaturation enthalpies of different bean PIs exhibit a range of values between 90°C and 152°C, with a mean of 118,8°C and a standard deviation of 14.3°C. Their values range from 32.9 to 134 J/g, as reported by Gundogan and Karaca in 2020.

The water-holding capacity (WHC) and oil-holding capacity (OHC) of legume protein isolates (PIs) are noteworthy. The reported ranges are 1.8 to 6.8 g/g for WHC and 3.5 to 6.8 g/g for OHC. They demonstrate exceptional foaming capacity, emulsion capacity, solubility, and emulsifying activity index while retaining these attributes even under severely acidic (pH 2.0) and alkaline (pH 10.0) conditions (Gundogan & Karaca, 2020; Lafarga et al., 2020). The solubility of soybean isolates in their natural state is highest at pH values of 3.0, 5.0, 7.0, and 8.0, whereas the solubility of adzuki isolates is the lowest. Barać et al. (2015) found that naturally occurring soy proteins could generate stable foams.

In addition to their use in culinary applications, legume proteins are widely employed in encapsulation technology. The protein isolates derived from peas and chickpeas are utilized to enclose various nutrients, including vitamin B9 (folate), α-tocopherol, ascorbic acid, and phytase, with a high degree of efficiency in encapsulation, ranging from 62% to 100% (Ariyarathna & Karunaratne, 2015). Combining phytase and

pea PI leads to slow release rates and great bioaccessibility when exposed to simulated stomach and intestinal fluids (Gharibzahedi & Smith, 2021). Furthermore, vegetable oils can be fortified with lentil and red kidney bean PIs to improve their stability and bioavailability, as demonstrated by Joshi et al. (2012). The survival and stability of probiotic bacteria are enhanced when they are encapsulated with soy and pea protein isolates, particularly under gastrointestinal circumstances (Gharibzahedi & Smith, 2021). Furthermore, legume protein concentrates are used to produce edible films that have excellent mechanical and barrier qualities. These films are ideal for packaging food items that are sensitive to light (Hopkins et al., 2015).

The functionality of legume proteins can be enhanced by emerging technologies, including enzymatic hydrolysis, high hydrostatic pressure (HP), and ultrasound. Treatment with HP has been shown to enhance the solubility and emulsifying activities of kidney bean and lentil protein isolates. Enzymatic hydrolysis has been demonstrated to decrease the thixotropic properties of kidney bean PIs, resulting in their ability to exhibit Newtonian fluid behavior at greater shear rates (Ahmed et al., 2018). The application of high-intensity ultrasonic treatment has been demonstrated to enhance the solubility, emulsifying, foaming, and gelling properties of chickpea protein isolates (PIs) when subjected to heat (Wang et al., 2020). Furthermore, enzyme hydrolysis using Alcalase and bromelain has enhanced the antioxidant and anti-inflammatory activities of pigeon peas, lentils, and chickpea PIs (Xu et al., 2021). The aforementioned alterations have rendered legume proteins suitable for a multitude of food and nutraceutical applications, offering enhanced nutritional and functional attributes (Al-Ruwaih et al., 2019; Wang et al., 2020; Xu et al., 2021).

1.2. Extraction Methods

1.2.1. Wet Extraction Methods

The functional and physico-chemical qualities of protein isolates (PIs) are important in determining their suitability for different dietary applications. Legumes are

often extracted using wet extraction procedures, which involve the use of aqueous solvents or chemicals such as alkali, acid, or water. The typical method of this process involves an additional stage in which proteins are precipitated or recovered to increase the amount obtained. In order to enhance the yield of protein, solvents are occasionally combined with other techniques, thereby increasing the efficiency and efficacy of the extraction procedure (Deleu et al., 2019; Kumar et al., 2021; Zhang et al., 2019).

The choice of starting material for extraction may vary and could include flaked, milled, or air-classified fine protein fractions. Each of these presents distinct advantages and difficulties in the subsequent extraction procedure (Guo et al., 2021). An integral part of the process is the drying of the extracted protein, which is necessary to enhance the protein isolates' shelf life and overall quality throughout storage and transit. It is essential to ensure thorough drying to maintain the functional qualities of protein isolates, as this allows them to be used effectively in food items (Byanju et al., 2020).

1.2.2. Alkaline Extraction of Legume Proteins

Alkaline extraction is a well-established and frequently used approach for separating proteins extracted from plant matter, including legumes. The process involves adjusting the pH of the extraction solution by adding alkaline solvents such as sodium hydroxide and potassium hydroxide, which raise the pH to a basic range of 8–11 or higher (Cui et al., 2020). The pH levels mentioned below facilitate the solubilization of proteins by creating a basic environment that disrupts the disulfide bonds present in the proteins, thereby improving the proteins' release, recovery, and yield.

In order to precipitate the proteins, the pH is typically lowered to a range of 4.0–4.8 using an acid such as HCl. This procedure commences with the solubilization of the proteins by reducing the pH to an alkaline condition. Proteins reach their isoelectric point at a specific pH, where their solubility is at its lowest, forming protein precipitates (Boye et al., 2010). The supernatant obtained after precipitation is primarily composed of starch and fibers. It is separated from the precipitated protein using techniques such as centrifucentrifugation, filtering, or screening techniques the recovered proteins, an additional washing step with either an acid solution or water can be employed, followed

by resolubilization at a neutral pH (Guo et al., 2021). In order to guarantee the microbiological safety of the separated proteins, it may be necessary to implement heating phases (D'Agostina et al., 2006).

The final stage of the alkaline extraction process is the removal of moisture from the protein isolate, which generates a low-moisture product that may be stored for an extended period without spoilage. Freeze drying, followed by milling, is commonly employed in laboratory settings. In contrast, spray-drying is frequently utilized at the commercial level to produce isolates that can be conveniently packed (Burger & Zhang, 2019).

Although widely used, the alkaline extraction process has some disadvantages. According to reports, the recovery rate is around 50% and there is a decrease in the functioning of the isolated proteins (Karki et al., 2010). The poor extraction efficiency is frequently attributed to the development of intricate interactions between the solvent and polysaccharides inside the cell matrix, which impede protein release (Rahman & Lamsal, 2021). In addition, the excessive use of alkali in the process of increasing protein release might have adverse effects, including the racemization of amino acids, reduced protein digestibility, and the destruction of important amino acids such as lysine and cysteine.

It is possible to overcome the limitations of alkaline extraction in terms of the quantity of protein obtained by optimizing numerous variables, including the ratio of sample to solvent, concentration of alkali, extraction duration, and temperature. By implementing this optimization, it is possible to achieve the maximum yield of protein while minimizing costs (Zhang et al., 2014). A significant advantage of the alkaline extraction process is that the resulting proteins have a relatively high bioavailability and digestibility (Deleu et al., 2019). Yoshie-Stark, Wada, and Wäsch (2008) demonstrated the effectiveness of isolating rapeseed protein using alkaline conditions and then precipitating it at an isoelectric point of 5.8, resulting in a crude protein content of 708.4 g/kg. In a study conducted by Salgado et al. (2012), an optimization study on the alkaline extraction of proteins from sunflower meal was performed. The results indicated that the protein recovery was 70.4% based on the dry weight of the sample.

1.2.3. Acid-Assisted Extraction of Legume Proteins

Extracting proteins from legumes may present considerable difficulties due to the insolubility of some pulse proteins and their loss of function when extracted using traditional denaturants. Acid-assisted extraction is a potential technology that helps overcome these obstacles by preserving the proteins in their inactive states, preventing denaturation. Acidic solutions, such as butanol, pentanol, hexane, and acetone, are frequently employed for this objective. The solvents mentioned in the study by Cui et al. (2020) can dissolving non-polar side chains, lipid-binding proteins, polar side chains, and amino acids containing aromatic rings. This procedure entails reducing the pH of the protein solution below the protein's isoelectric point, which is especially effective for legume proteins because of their overall positive charge, hence improving solubility at lower pH levels (Andreou et al., 2022).

Although it functions at a lower pH, the acid extraction method is analogous to the alkaline extraction process in its operation. The mixture separates into three distinct components after adding an acidic solution to a protein-containing solution, followed by the introduction of ammonium sulfate. Proteins segregate into an intermediate layer between the upper organic and lower aqueous phases, contingent upon the quantity of ammonium sulfate used. Research has demonstrated that this technique effectively disables lipoxygenase, an enzyme that causes the unpleasant beany flavor in legume-derived protein products. Consequently, the sensory characteristics of the products are much improved compared to the use of alkaline extraction methods (De Angelis et al., 2020). Although less frequently employed, this technique has been utilized to extract proteins from pulses and legumes, including pea and faba bean protein isolates (De Angelis et al., 2020).

Nevertheless, there are significant constraints associated with this methodology. Neucere and Ory (1968) observed that proteins obtained from peanut seeds using acetone and hexane exhibited increased insolubility following acid treatment. More precisely, the two primary reserve proteins, arachin and conarachin, exhibited decreased solubility and modified ion exchange characteristics after being extracted. Alterations in protein structure can result in reduced solubility and alterations in ionic properties, which can impact the total production and usefulness of the extracted proteins. According to Hrynets

et al. (2011), acid-aided extraction is not extremely effective as it can only recover up to 20% of yellow pea protein. Therefore, acid-assisted extraction is not considered a favorable method and is not commonly used for protein separation from legumes.

1.2.4. Legume Protein Extraction Using Enzymes

Enzyme-assisted methods for extracting legume proteins have become a popular alternative to classic alkaline and acid-assisted extraction procedures. Enzyme-assisted extraction effectively tackles several issues related to traditional procedures, such as environmental risks and adverse impacts. Enzymes facilitate the release of proteins by degrading the polymers present in cell walls, thereby promoting protein solubility (Sari et al., 2013). This approach is more ecologically friendly and has fewer negative impacts than alkaline and acid extractions.

Enzymes that are frequently utilized in this process include proteases, such alcalase, and carbohydrases, including cellulase and pectinases. Enzymes function as a first step that promotes the use of alkaline extraction instead of acid extraction, with proteases being especially efficient. Proteases enhance protein synthesis by liberating proteins from their polysaccharide matrix and transforming larger proteins into more soluble forms, hence improving extraction efficiency. In addition, they safeguard against protein denaturation by functioning within optimal pH settings and preventing the formation of complexes between liberated proteins and cellular constituents such as polysaccharides (Rahman & Lamsal, 2021).

Rommi (2016) showed that cellulolytic, pectinolytic, and xylanolytic enzymes were effective in obtaining protein yields of 74% and 56% from dehulled and hulled rapeseed press cakes, respectively. The proteins that were retrieved demonstrated resilience to substances that cause oxidation and had low levels of thickness, which makes them well-suited for use in food-related contexts. Enzymatic protein extraction, while advantageous, has some limitations, such as operational complexity, high prices, scalability issues, inconsistent recovery, and high energy needs (Kumar et al., 2021). Nevertheless, the advantages of this approach are deemed favorable because of its little

ecological footprint and its favorable influence on protein functioning and recovery (Perovic et al., 2022).

Perovic et al. (2022) found that using enzymes to help in alkaline protein extraction from chickpeas led to a 30-fold increase in the amount of extracted protein, resulting in a 93% recovery of the protein. This approach also enhanced the functional characteristics of the protein, including its solubility, capacity to hold water, ability to absorb oil, capacity to form emulsions, and ability to create foam. These improvements were measured at 14%, 130%, 22%, and 150% respectively. The improvements mentioned make enzyme-assisted extraction a highly promising method for generating legume protein isolates of exceptional quality.

1.2.5. Method of Salt In / Salt Out Extraction

Leveraging the solubility of legume and pulse proteins in salt solutions, the salt in/salt out extraction method is a widely used approach for protein extraction. This technique utilizes the varying ionic strengths of different salts to extract proteins in the form of micelles or aggregates (Muranyi et al., 2016). Salts often used in this procedure include ammonium sulfate, sodium chloride, and calcium chloride. The extraction process consists of two primary stages: salting-in and salting-out.

During the salting-in phase, the addition of salts with low ionic strength (less than 0.15 M) is employed to charge the surfaces of proteins positively. This process enhances their solubility and prevents denaturation. Subsequently, the salting-out phase occurs, wherein a salt with a significantly greater ionic concentration (about 5 M) is added. This results in the clustering of dissolved proteins and their subsequent precipitation (Jiang et al., 2021). The proteins precipitate due to the dilution effect caused by the change in ionic strength during salt-in extraction. After the extraction process, carbs and insoluble fibers are eliminated, and the protein extracts are then diluted with cold water (Lam, Can Karaca, Tyler, & Nickerson, 2018). Then, protein concentration and desalting are carried out, followed by centrifugation, filtration, resolubilization, and spray-drying of the protein. Alternatively, one can utilize ultrafiltration/diafiltration or isoelectric precipitation.

Ultrafiltration (UF) is a method that employs semipermeable membranes to segregate solubilized proteins. The protein extract is filtered using ultrafiltration (UF) membranes, which selectively retain proteins while allowing smaller soluble components, such as carbohydrates, to pass through (Hadidi et al., 2020). The protein isolates can be subjected to either spray-drying or freeze-drying processes comparable to those used for isoelectrically precipitated isolates. The University of Florida (UF) provides several benefits compared to the isoelectric point precipitation methods utilized in alkali and acid extraction operations. One of the advantages of this method is that it allows for the retention of more complete protein components, such as albumins. In contrast, alkali and acid procedures tend to recover globulins more selectively (Boye et al., 2010).

In addition, UF does not necessitate very acidic or alkaline pH conditions, hence enabling the proteins to maintain their original structures. Not including a neutralization phase leads to isolates with reduced levels of ash and salt but increased protein recovery (Alonso-Miravalles et al., 2019). In their study, Boye et al. (2010) found that protein concentrates derived from peas, chickpeas, and lentils utilizing ultrafiltration (UF) and isoelectric precipitation procedures consistently exhibited greater protein levels compared to those obtained by alkali and acid extraction methods. The combination of isoelectric point and UF/DF processing procedures led to isolates with a protein content that was four times higher, ranging from 63.9% to 88.6% (w/w).

Fuhrmeister and Mesuer (2003) discovered that wrinkled pea concentrates produced using ultrafiltration (UF) had a greater protein content (70-80%) and a lower fat level (2.3%) compared to isolates obtained through isoelectric precipitation (68% protein content and 3.8% fat). Nevertheless, Muranyi et al. (2016) noted that the salt-in technique decreased productivity and diminished functionality compared to the alkali extraction approach. Similarly, employing the salt-in technique, Tanger, Engel, and Kulozik (2020) observed alterations in the structure of proteins isolated from pea flour, resulting in decreased concentration and yield. While the solubility of salt-extracted proteins was greater at 96.6%, they demonstrated worse retention of their natural structure.

1.2.6. Process For The Extraction of Pulse / Legume Protein by Air Classification

Air classification is a method of separating particles in an air stream based on their size and density. The process entails suspending finely ground flour in a chamber with the help of air movement, resulting in the separation of particles based on their size and density (Sozer et al., 2017). Flour may be effectively separated into various fractions using regularly employed methods such as impact, jet milling, and rotor-type classifiers (Pelgrom et al., 2013).

The air classification approach has been effectively utilized to extract protein from legumes such as peas, faba beans, and lupin (Pelgrom et al., 2015). The process often starts with a dehulling phase, which eliminates antinutrient elements that contribute to bitterness and astringency. This step also enhances the color of the protein flour and marginally boosts its protein content (Do Carmo et al., 2020). Following removing the hulls, the finely ground flour is delivered onto a rotating classification wheel using a stream of air. Within the separating chamber, the flour undergoes centrifugal separation, which divides it into smaller protein-rich pieces measuring roughly 3 μm and bigger starch granules measuring 25-40 μm. This process yields a fraction or concentrates that is rich in protein.

The classifier wheel separates smaller particles to create the fine fraction, while bigger particles settle at the bottom of the chamber, generating the coarse starch fraction. The larger particles include a higher concentration of carbohydrates and fiber, whereas the smaller particles have a higher protein content. As an additional step, the coarse fraction can be re-milled and passed through the air classifier. This process aims to enhance the purity and yield of each fraction (Schutyser & Van Der Goot, 2011).

The crucial elements in the air classification technique encompass the velocity of milling and the velocity of the air classifier. Optimal milling velocity is essential to achieve thorough cell disintegration, hence allowing the separation of starch and protein components. Although the air classification approach has benefits, it has the drawback of yielding less protein than wet extraction methods. In their study, Schutyser et al. (2015) found that protein concentrates obtained from the air classification of several pulses had protein content ranging from 49% to 70%.

Nevertheless, the air classification process possesses significant benefits compared to wet fractionation. The absence of a drying stage and the employment of chemicals in the process results in lower energy and water requirements while also preserving the natural structure of proteins (Vogelsang-O'dwyer et al., 2020). Air categorization offers a more sustainable and eco-friendly alternative for extracting protein from legumes.

1.3. Protein Hydrolysates

The economic feasibility and environmental benefits of protein hydrolysates have made them a prominent subject of current study (Harnedy & FitzGerald, 2012). The hydrolysates are generated by enzymatic hydrolysis of proteins, resulting in the formation of smaller peptides that possess bioactive characteristics. Legume protein hydrolysates are essential for obtaining bioactive peptides. These peptides have shown promise in preventing and treating several diseases, such as cancer, immunological disorders, infections, and cardiovascular diseases (Kamran & Reddy, 2018). The addition of bioactive peptides from legumes to regularly consumed meals might augment their nutritional quality and functioning, hence enhancing the total dietary value (Malaguti et al., 2014).

It is crucial to encourage the use of legumes and provide cost-effective products for disadvantaged people in order to alleviate poverty and combat malnutrition. Approximately 170 million preschoolers and nursing mothers in poor African and Asian nations experience protein-energy malnutrition, a serious nutritional disorder (Nedumaran et al., 2020). Researchers are increasingly prioritizing utilizing naturally occurring, wild, and underutilized legumes that have been previously ignored or are particular to certain regions (Bhat & Karim, 2009). This method focuses on resolving nutritional inadequacies and highlights the potential of these legumes as excellent sources for future functional meals. Legume proteins have a crucial role in different meal preparations by supplying energy, amino acids, and substantial nutritional content. They also have a considerable impact on the physical and chemical properties of food (Etemadian et al., 2021).

Out of the roughly 1,000 legume species, only around 20 are widely grown and farmed (Singh et al., 2022). The genetic resources of these neglected crops are quickly decreasing in their original habitats, resulting in their growing rarity on a global scale. In order to minimize neglect and increase exploitation of these species, it is imperative to undergo a paradigm change in how we produce, exploit, and consume them (Paul et al., 2019). Studies have shown that hydrolyzed plant proteins have somewhat superior functional and physiological properties compared to crude proteins (Ashaolu et al., 2020). The identification of novel and cost-effective sources of protein from abundant plant resources has become essential to satisfy the increasing need for protein (Prakash et al., 2001).

Protein hydrolysates are frequently employed as supplements in manufacturing food and feed because of their capacity to alter different protein characteristics (Ward, 2011). The food sector has shown significant interest in protein hydrolysates due to their advantageous effects on food items and human health (Ahlström, 2022). Hydrolysis is the process by which proteins are decomposed into peptides of different sizes, usually composed of 2 to 20 amino acid units (Shahidi et al., 2019). Legumes, being the most abundant sources of plant protein, meet around 10% of the global protein intake needs (Azman et al., 2023). Nevertheless, only a limited number of legumes have been thoroughly utilized for their full potential (Wikandari et al., 2021). Legume-derived hydrolysates and peptides have many biological effects, such as anticancer, antihypertensive, anti-inflammatory, hypolipidemic, antioxidant, and immunomodulatory activities (Matemu et al., 2021).

1.3.1. Enzymatic Hydrolysis

Enzymatic hydrolysis is a commonly employed method for generating protein hydrolysates and peptides with favorable biological characteristics. This technique entails the enzymatic breakdown of protein substrates in a controlled laboratory environment utilizing particular external proteolytic enzymes. Enzymatic proteolysis has various advantages over chemical treatments. These include the use of softer processing conditions, with a pH range of 6.0-8.0 and a temperature range of 40-60°C. Additionally,

enzymatic proteolysis allows for better control over the hydrolysis process, as stated by Kristinsson et al. in 2000. The amino acid makeup of enzymatic protein hydrolysates roughly mirrors that of the original protein substrate, with minor alterations contingent upon the enzymes used. Furthermore, enzymatic digestion does not need the use of organic solvents or harmful chemicals, which makes it appropriate for applications in the food and pharmaceutical industries (Wijesekara et al., 2011).

The enzymatic approach yields protein hydrolysates with diverse peptide profiles, which differ from those produced during microbial fermentation due to the use of various proteases and enzyme/substrate ratios. This selectivity enables the creation of consistent and replicable bioactive protein hydrolysates (Van der Ven et al., 2002). Various methods, including as chemical synthesis, enzymatic synthesis, and recombinant DNA technologies, can be employed to create bioactive peptides. Nevertheless, enzymatic hydrolysis continues to be favored because of its selectivity and gentle operating conditions.

Selecting protein substrates is crucial in generating protein hydrolysates with the intended biological characteristics. The potential of proteins derived from plants or animals, such as milk, eggs, wheat, and marine sources, to produce bioactive peptides has been investigated (Bhat et al., 2015). The composition and primary amino acid sequence of the protein substrate are crucial determinants. Peptides containing Trp, Tyr, Phe, Pro, or hydrophobic amino acids at the C-terminal have been found to be efficient in inhibiting ACE activity, as reported by Gobbetti et al. in 2000.

Thermal treatment and sonication are two types of pretreatments that can improve enzymatic hydrolysis. These pretreatments work by promoting interactions between enzymes and proteins, which is facilitated by the unfolding of proteins. In their study, Jia et al. (2010) showed that using ultrasonic pretreatment on wheat germ protein enhances the production of ACE-inhibitory peptides during enzymatic hydrolysis.

The degree of specificity shown by the proteolysis enzyme considerably influences the biological activity of the resultant hydrolysates. Proteases, such as trypsin, chymotrypsin, and pepsin, break down proteins at particular locations, resulting in peptides of different lengths and amino acid contents (Simpson et al., 1998). As an illustration, Lassoued et al. (2015) generated hydrolysates from thornback ray gelatin by employing several enzymes, leading to various levels of hydrolysis and antioxidant properties.

The degree of hydrolysis (DH) and the bioactivities of protein hydrolysates are significantly influenced by essential parameters such as hydrolysis conditions, which include time, temperature, pH, and enzyme/substrate ratio. Balti et al. (2010) found that the ACE-inhibitory activity of cuttlefish protein hydrolysate rose as the degree of hydrolysis (DH) increased, with the highest activity seen at a DH of 16%.

Enzymatic hydrolysis can be performed in either batch reactors or continuous enzymatic membrane reactors. Utilizing immobilized proteases provides benefits such as enhanced enzyme stability and the capacity to recycle the enzyme, resulting in decreased manufacturing expenses. Enzymes that are immobilized may be used to produce hydrolysates continuously in a regulated manner without the presence of proteolysis enzymes. This makes them well-suited for use in the food and pharmaceutical sectors (Pedroche et al., 2007).

To achieve protein hydrolysates with certain functional qualities, it is important to carefully choose proteolytic enzymes that are suitable for the task. To achieve consistent and desirable bioactivity of hydrolysates, it is crucial to meticulously regulate factors such as enzyme specificity, hydrolysis conditions, and pretreatment of protein substrates (Nasri et al., 2014).

1.3.2. Degree of Hydrolysis

The degree of hydrolysis (DH) is crucial in the enzymatic hydrolysis of proteins since it measures the amount of peptide bond breaking that occurs during hydrolysis. The term "cleavage efficiency" refers to the ratio of broken peptide bonds, which are expressed as a fraction of the total number of peptide bonds. This ratio may be determined using the following formula:

$$DH(\%) = (h/htot) \times 100 \qquad (1.1.)$$

where *h* represents the number of hydrolyzed peptide bonds, and *htot* is the total number of peptide bonds present (Adler-Nissen, 1979).

The absence of a universally applicable approach for determining DH has resulted in the creation and utilization of several analytical methodologies. Some often used techniques for this purpose are pH-stat, osmometric, soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), 2,4,6-trinitrobenzene sulfonic acid (TNBS), o-phthaldialdehyde (OPA), amino acid nitrogen, and formol titration methods. Each of these approaches possesses its own set of principles, benefits, and constraints.

pH-Stat Method: This method ensures a consistent pH level throughout hydrolysis by constantly introducing a titrant (either an acid or a base). The quantity of titrant supplied is directly correlated with the number of peptide bonds hydrolyzed, which makes this technique appropriate for continuous monitoring of DH (Adler-Nissen, 1979).

Osmometric Method: This technique quantifies the rise in osmotic pressure resulting from the formation of smaller peptides and free amino acids during the process of hydrolysis. The alteration in osmotic pressure approximates the DH (Kristinsson & Rasco, 2000).

Soluble Nitrogen After Trichloroacetic Acid Precipitation (SN-TCA): In this method, the hydrolyzed material is subjected to trichloroacetic acid (TCA) treatment in order to cause the precipitation of substantial peptides and proteins. The nitrogen concentration in the supernatant, which includes soluble peptides and free amino acids, is then quantified to ascertain the DH (Church et al., 1983).

2,4,6-Trinitrobenzenesulfonic Acid (TNBS) Method: TNBS chemically combines with unbound amino groups present in peptides and amino acids, resulting in the formation of a chromophore that can be quantitatively quantified by spectrophotometry. The rise in absorbance is directly related to the DH (Spies, 1967).

O-Phthaldialdehyde (OPA) Method: OPA undergoes a chemical reaction with primary amines, resulting in the formation of a substance that emits light and may be detected via fluorescence. The fluorescence intensity is linearly related to the concentration of unbound amino groups, enabling the measurement of DH (Church et al., 1985).

Amino Acid Nitrogen: This technique quantifies the nitrogen concentration of amino acids liberated during the process of hydrolysis. The nitrogen concentration is used to directly estimate the extent of hydrolysis (Moore & Stein, 1963).

Formol Titration Method: Formaldehyde undergoes a chemical reaction with amino groups, resulting in a decrease in the pH of the solution. The amount of base needed to neutralize the solution is directly proportional to the DH (Taylor et al., 1980).

The choice of an appropriate technique for detecting DH relies on several aspects, including the inherent characteristics of the protein substrate, the specificity of the protease, and the planned use of the hydrolysates. Each technique provides distinct perspectives on hydrolysis, allowing researchers to customize their methodology in order to attain the specific level of hydrolysis and functional characteristics in protein hydrolysates that they seek.

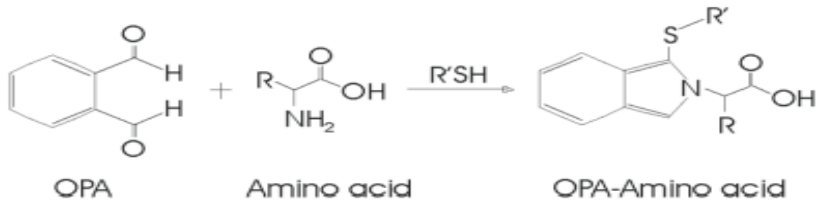

Figure 1. O-phthaldialdehyde (OPA) reacts with amino acids (Source: Rutherford et al., 2010).

1.4. Maillard Reaction and It's Products

Food is composed of an intricate combination of substances, including proteins, amino acids, carbs, fats, vitamins, and minerals. Throughout the ages, several culinary techniques have been created to maintain food quality. Heat treatment is particularly important in facilitating non-enzymatic processes such as the Maillard reaction, caramelization, chemical oxidation of phenols, and maderization (Manzocco et al., 2000). Out of them, the Maillard reaction has particular importance since it plays a crucial role in the creation of different chemicals, some of which have the potential to be harmful.

The Maillard reaction, initially documented by French chemist L.C. Maillard in 1912, elucidates the chemical phenomenon that transpires when glucose and glycine undergo heating, resulting in the creation of a melanoidin, a dark-hued pigment. Hodge clarified this reaction in 1953, outlining the route in the Journal of Agriculture and Food Chemistry. The significance and physiological impacts of the response were emphasized

during the inaugural Maillard conference that took place in Sweden in 1979 (Gerrard, 2006).

The Maillard reaction takes place during the preparation and storage of different food items, such as fruits, vegetables, cereals, milk, and meat, and has a substantial impact on the overall quality of the finished product. This process entails the interaction between electrophilic carbonyl groups of reducing sugars and nucleophilic amino groups of proteins, peptides, or amino acids. The process consists of three distinct stages: initiation, propagation, and the advanced stage. At first, glucose, which is a type of sugar that may be reduced, combines with a molecule that has a free amino group. This combination forms a glycosylamine, which then undergoes a rearrangement to generate Amadori rearrangement products (ARPs) and 1-amino-1-deoxy-2-ketose. During the second stage, sugar molecules undergo dehydration and fragmentation, resulting in the breakdown of amino acids and the production of 5-hydroxymethylfurfural (HMF) and other byproducts of fission. The advanced stage encompasses a sequence of intricate events, such as cyclizations, dehydrations, retro-aldolisations, rearrangements, isomerizations, and further condensations, which ultimately result in the creation of melanoidins (Tamanna & Mahmood, 2015).

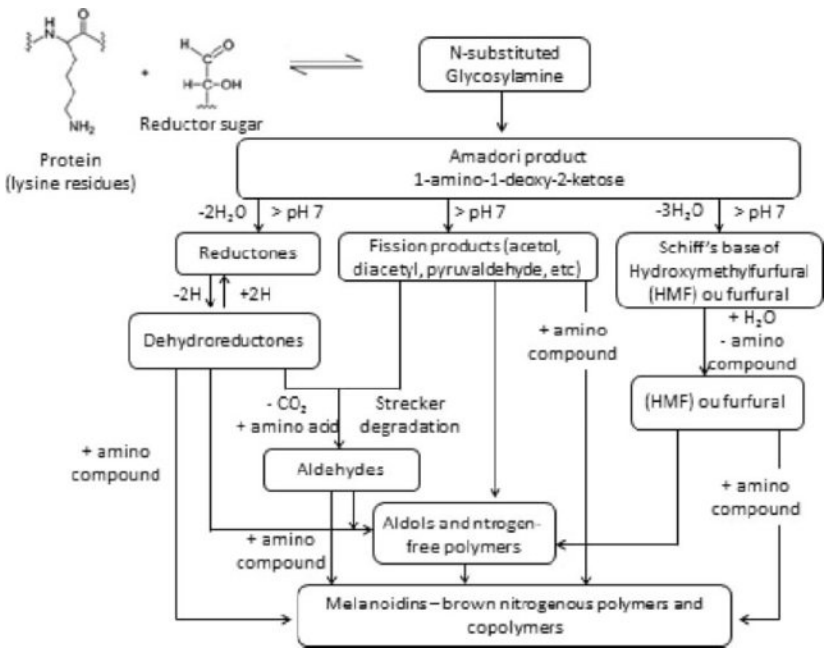

Figure 2. Maillard Reaction mechanism scheme (Source: Hodge, 1953).

The degree of the Maillard reaction is influenced by several parameters, such as the processing technique, type of sugar, amino acid, temperature, pH, and water activity. In creating various items such as bread, cakes, meat, fish, potato-based products, cocoa, and coffee, it is advantageous to use this ingredient as it enhances the visual appearance, texture, and scent. However, in goods such as milk and fruit juices, this reaction is considered undesirable since it causes a brown coloration. Furthermore, Maillard reaction products (MRPs) can have both advantageous and harmful impacts on health. Melanoidins have antioxidant and antibacterial characteristics due to their capacity to form complexes with metals (Bastos et al., 2012). On the other hand, some substances such as HMF, furosine, acrylamide, and heterocyclic amines have been proven to be dangerous and cancer-causing. Therefore, it is important to identify and reduce the presence of these substances in food items (ALjahdali & Carbonero, 2019).

The Maillard reaction also enhances the production of reactive carbonyl compounds, which can lead to carbonyl stress and contribute to the creation of advanced glycation end-products (AGEs). Elevated use of these substances is associated with heightened susceptibility to diabetes, cancer, chronic heart failure, Parkinson's, and Alzheimer's illnesses. Thus, it is crucial to implement strategies aimed at decreasing or averting the formation of these chemicals in foods that undergo heat processing (Wu et al., 2015).

1.5. Factors That Affect The Maillard Reaction

1.5.1. Reducing Sugar

The Maillard reaction (MR) is a chemical process that occurs without the involvement of enzymes. It occurs when an amino group (such as amino acids) reacts with a carbonyl group (such as reducing sugars). The reaction described is of utmost importance in the process of altering proteins with reducing sugars, which has a considerable effect on the flavor, color, and general quality of food items (Manzocco et al., 2000). Reducing sugars play a crucial role in the Maillard reaction (MR), and their

quantity and composition greatly impact the creation and properties of Maillard reaction products (MRPs).

The concentration of reducing sugars in food matrices can significantly impact sensory characteristics such as scent, taste, and color. The presence of reduced sugars in coffee beans can have a significant effect on the fragrance, flavor, and color of the beans when they are being roasted (Borrelli et al., 2002). Similarly, steak that is cooked at high temperatures, resulting in lower amounts of sugars but greater levels of Maillard reaction products (MRPs), has a better overall taste and roasted meat quality compared to steak cooked in an oven.

Although similar discoveries have been made, several research has concentrated on the impact of the overall amount of reducing sugars on taste generation without determining the individual reducing sugars with significant functions. Yang et al. (2018) showed that glucose and ribose may undergo the Maillard reaction with α-amino acids, leading to the production of aroma molecules seen in cooked meat. Furan, an essential intermediary in certain chemical systems, is formed from pentose and is produced by the 1,2-enolization of the Amadori intermediate. According to Yang et al. (2018), the reaction involving xylose produces greater amounts of furfural and volatile taste compounds associated with meat than ribose and glucose.

In addition, introducing reducing sugars can cause protein glycation and improve the solubilization of myosin, perhaps leading to an improvement in the meat's ability to retain water (Yang et al., 2018). This phenomenon implies that the presence of reducing sugars has a role in the creation of flavors and enhances the texture of food items.

1.5.2. Protein Source

The Maillard reaction (MR) is a chemical process that involves the interaction between amino groups, which come from proteins or peptides, and carbonyl groups, which come from reducing sugars. This reaction is a type of non-enzymatic browning process. The choice of protein source substantially impacts the taste and characteristics of Maillard reaction products (MRPs). Current research has placed a growing emphasis

on using vegetable proteins or peptides to create taste enhancers through MR, hence expanding the variety of flavors in food items.

Soybean peptides have been widely employed to create diverse tastes, such as umami, sweet, salty, and meaty characteristics. A study showed that when xylose and soybean peptides were reacted at temperatures ranging from 100 to 140°C and pH 7.6 for a duration of 2 hours, the resulting MRPs had unique tastes. According to Chen et al. (2019), the presence of cysteine decreased the perception of bitterness at a temperature of 140°C, while simultaneously increasing the perception of umami and salty at a temperature of 100°C. The ability of soybean peptides to adapt and be used in many ways makes them a highly important component in creating flavors.

Additionally, proteins produced from other plants have a considerable impact on the flavor characteristics of MR. Flaxseed protein hydrolysates capture and consistency of umami soups. Hydrolysates or extracts derived from meats have been utilized to infuse food items with tastes like those of meat. Nevertheless, these investigations frequently lack precision in discerning the distinct flavors that emerge, such as distinguishing between chicken, beef, or fish flavors (Oh et al., 2018).

Scientists have investigated the combination of meat hydrolysates with certain sugars and amino acids to enhance the specificity of flavor. Kang et al. (2020) found that adding xylose and L-cysteine to beef hydrolysate increased its kokumi, meaty, umami, and kokumi-enhancing abilities. Enzymatic hydrolysis and microbial reduction (MR) can transform animal bone extracts into acceptable taste constituents, improving flavor profile and diminishing unpleasant sensations. Ribose, when subjected to enzymatic hydrolysis and heat treatment with beef bone hydrolysates, generates specific meat taste compounds such as 2-methyl pyrazine, dimethyl disulfide, and dimethyl trisulphide. Simultaneously, this process suppresses the formation of bitter compounds such as 2-furan methanol (Song et al., 2017).

Other animal protein sources have also been subjected to similar methodologies. The fragrance components were enhanced, and the overall flavor was improved by the enzymatic hydrolysis of swine lard and goat by-product protein hydrolysates. The use of Flavourzyme significantly intensified the taste of chicken bone extracts by the augmentation of pyrazine and sulfur compounds. The use of sequential hydrolysis using Protamex® and Flavourzyme® resulted in a notable decrease in bitterness and an enhancement in the acceptance of taste, as demonstrated by Zhao et al. in 2018. Using

thermal treatment on chicken proteins with xylose produces meat tastes that enhance the richness and freshness of the final solution (Kim et al., 2021).

In addition, combining low-valued fish hydrolysates with meat hydrolysates and sugars such as xylose can improve the taste of sauces by intensifying meat aromas and reducing unpleasant fishy smells. For example, when shrimp hydrolysate is reacted with xylose, it produces MRPs (Maillard Reaction Products) that have a strong seafood flavor and freshness (Wang et al., 2020). Using fermented tilapia fish head hydrolysate may be extended to create a concentrated taste, showcasing the possibility of obtaining a wide range of flavor characteristics from different protein sources (Kan et al., 2021).

1.5.3. Reaction Conditions of Maillard Reaction

The Maillard reaction (MR) is an intricate process that is greatly impacted by reaction circumstances, including pH and temperature. These factors substantially impact the creation and characteristics of Maillard reaction products (MRPs).

First and foremost, pH has a crucial impact on MR by affecting the amounts of sugars in their open-chain state and the active forms of amino reactants. Alkaline circumstances enhance the rearrangement of sugars at a molecular level and facilitate nucleophilic addition processes. Amadori compounds, which serve as early intermediates in the Maillard reaction (MR), exhibit a higher propensity for 1,2-enolization at pH 8 and 2,3-enolization at pH 9.7 (Lotfy et al., 2021). The variation in enolization routes impacts the nature and amounts of MRPs produced. Moreover, raising the initial reaction pH can augment caramelization, albeit its influence is confined to the pH range of 6.7 to 8.0 (Liu et al., 2021).

During the process of microbial respiration (MR), amino groups, sugars, free amino acids (FAAs), and peptides are metabolized and broken down, resulting in the production of acidic chemicals. For instance, the introduction of cysteine into a soybean peptide and D-xylose system leads to a reduction in the final pH. This is because cysteine speeds up the production of formic and acetic acids (Lotfy et al., 2021). A research study utilizing enzymatically hydrolyzed quinoa protein as a precursor to produce thermal process flavorings discovered that altering the pH from 5 to 9 resulted in a shift in the

sensory characteristics of the flavors produced, transitioning from a caramel-like taste to that of burned coffee. The generation of furan was linked to low pH, whereas pyrazine creation was promoted by high pH (Lotfy et al., 2021). Nevertheless, the impact of pH on MRPs varies in different systems. Li and Liu (2021) found that the volatile profiles of heat-treated pork hydrolysate remained unchanged despite fluctuations in pH. Regardless of pH changes, identical quantities of volatile chemicals, such as furfural and furans, were formed.

Temperature is a significant variable that has a notable impact on MR. The reaction rate substantially increases as the temperature rises, with each 10°C increment leading to a 3-5 times acceleration of the process. Below a temperature of 110°C, the polypeptide and xylose system is primarily characterized by cross-linking processes. At temperatures over 110°C, the production of tiny molecular molecules increases, facilitating the conversion into volatile chemicals such as pyrazine, sulfur-containing compounds, and pyrrole (Deng et al., 2023). For instance, the level of furans, which are chemicals generated from sugar, escalates when the temperature elevates from 80 to 140°C in the soybean peptide-xylose system (Deng et al., 2023). Heating xylose and chicken hydrolytic peptides at temperatures between 80-100°C for 60-90 minutes increases the creation of umami and thick-flavor compounds. However, higher temperatures (100-140°C) promote the development of barbecue and meat tastes (Wang et al., 2020).

Nevertheless, temperatures that are too elevated might have adverse consequences. They can make malodorous compounds like thiazole, disrupt the binding sites of enzymes in polypeptides, and form cellulose analogs that are resistant to decomposition and absorption by the human body, hence diminishing the nutritious content of meals. In addition, temperatures over 110°C can result in the production of hazardous compounds such as acrylamide and advanced glycation end-products (AGEs), which can have adverse effects on health (Singh et al., 2021).

1.6. Antimicrobial Activity of MRP and Melanoidins

Certain antimicrobials have been recorded in the literature as being employed as food preservatives to prolong the shelf life of processed foods by regulating bacterial growth in the end products. Antimicrobials like potassium sorbate and sodium benzoate can hinder the growth of several bacteria, including Vibrio parahaemolyticus, Bacillus mucoides, Bacillus subtilis, and Staphylococcus aureus. Various minimum inhibitory concentration (MIC) values have been documented for Pseudomonas aeruginosa, Escherichia coli, Aspergillus flavus, Candida albicans, Fusarium oxysporum, Trichoderma harsianum, and Penicillium italicum (Beuchat LR. 1993). Protamine is an antimicrobial agent that hinders the development of bacteria (Potter R et al., 2005). Furthermore, nisin is a peptide with a low molecular weight that is synthesized by Lactococcus lactis subsp. It is commonly employed as a bacteriocin (Punyauppa-Path S et al., 2015). Nisin is commonly employed to manage Listeria monocytogenes by exploiting its strong sensitivity to the antimicrobial effects of nisin, particularly against Gram-positive bacteria (Delves-Broughton J et al., 1996).

Nevertheless, several customers voice apprehension over preservatives, highlighting potential adverse health consequences (Zhong et al., 2021). When sodium benzoate is present in drinks that include ascorbic acid, it can undergo a chemical reaction in the solution and produce a carcinogenic compound called benzene (Gardner & Lawrence, 1993). In addition, sorbic acid, which is used in soft drinks, bread, and cheese, is metabolized in a similar way to certain fatty acids. This reduces the chances of generating other negative consequences (Silva et al., 2016). However, sorbic acid has been shown to cause allergic responses, such as urticaria (Dendooven et al., 2021). Hence, there is an ongoing requirement to create innovative antibacterial substances obtained from natural materials and/or food items.

Ongoing research is being conducted to create novel antimicrobial agents, with a specific focus on the antibacterial properties of melanoidins. These compounds are anticipated to serve as alternative antimicrobial agents derived from dietary sources. For instance, studies have shown that melanoidins derived from roasted coffee could impede the development of germs, such as *S. aureus* (Rufián-Henares & de la Cueva, 2009; Rurián-Henares & Morales, 2008). Furthermore, researchers have shown that

melanoidins derived from crops like sunflowers exhibit antibacterial properties against *S. aureus* and *Escherichia coli* O157:H7 (Habinshuti et al., 2019). Furthermore, the Maillard reaction products derived from squid skin compounds exhibited antibacterial properties against *E. coli*, methicillin-resistant *S. aureus*, and *Vibrio harveyi*, as reported by Ji et al. in 2020.

Staphylococcus aureus is a kind of bacteria that is classified as gram-positive, catalase-positive, and coagulase-positive. It is characterized by its clustered spherical shape. These bacteria can induce several inflammatory illnesses, such as skin infections, pneumonia, endocarditis, septic arthritis, osteomyelitis, and abscesses. In addition, *S. aureus* can induce toxic shock syndrome (TSST-1), scalded skin syndrome (exfoliative toxin), and food poisoning (enterotoxin) (Sizar O et al., 2023). *S. aureus* causes food poisoning by synthesizing toxins in food (Kobayashi T. et al., 2015). The incubation period ranges from one to six hours, while the duration of the sickness ranges from 30 minutes to three days. The reference citation is Nakatsuji T. et al., 2016. Effective preventive techniques to mitigate the transmission of the disease encompass the complete washing of hands with soap and water prior to food preparation. The Centers for Disease Control and Prevention advises refraining from consuming food when experiencing illness and suggests the use of gloves during food preparation in the event of any open wounds on the hands or wrists. It is advisable to keep food at a temperature between 4.4 °C (40 °F) and 60 °C (140 °F) if it is stored for more than two hours.

Infectious infections are predicted to be the second leading cause of death worldwide. The escalating threat of drug-resistant bacteria is a significant worldwide public health issue (Salam et al., 2023). *Staphylococcus aureus*, a well-known bacterium in medical and community settings, is renowned for its resistance to penicillin and other antimicrobial drugs (Romero et al., 2021). The resistance is attributed to the synthesis of β-lactamase enzymes, with the initial documentation of a penicillin-resistant strain of *Staphylococcus aureus* dating back to 1945 (Bush 2018). The bacteria was initially discovered by Friedrich Julius Rosenbach in 1884. However, the utilization of enzyme testing to identify a staphylococcal infection caused by coagulase production by this microbe did not occur until the 1930s. Later, doctors started identifying and treating *Staphylococcus aureus* infections with penicillin. Before 1940, the mortality rate for those infected with *Staphylococcus aureus* was 75%. Nevertheless, by the late 1940s, a strain that was resistant to penicillin had emerged, causing typical penicillin to lose its effectiveness in treating the illness (Tălăpan et al., 2023).

It is important to recognize, nonetheless, that only a tiny percentage of antibiotics with new chemical categories have been launched in the last 30 years. This section will provide illustrations of medication categories utilized in treating *Staphylococcus aureus* infections, along with their respective action methods. Vancomycin, a glycopeptide antibiotic, is commonly employed for the treatment of serious infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains in patients who are admitted to the hospital. It interacts with the dipeptide D-Ala4-D-Ala5 of lipid II, inhibiting the transglycosylation and transpeptidation reactions catalyzed by PBP2 (penicillin-binding protein 2) and PBP2a (penicillin-binding protein 2). These proteins are crucial for bacterial cell wall synthesis, and their inhibition can impede peptidoglycan remodeling (Salah et al., 2022).

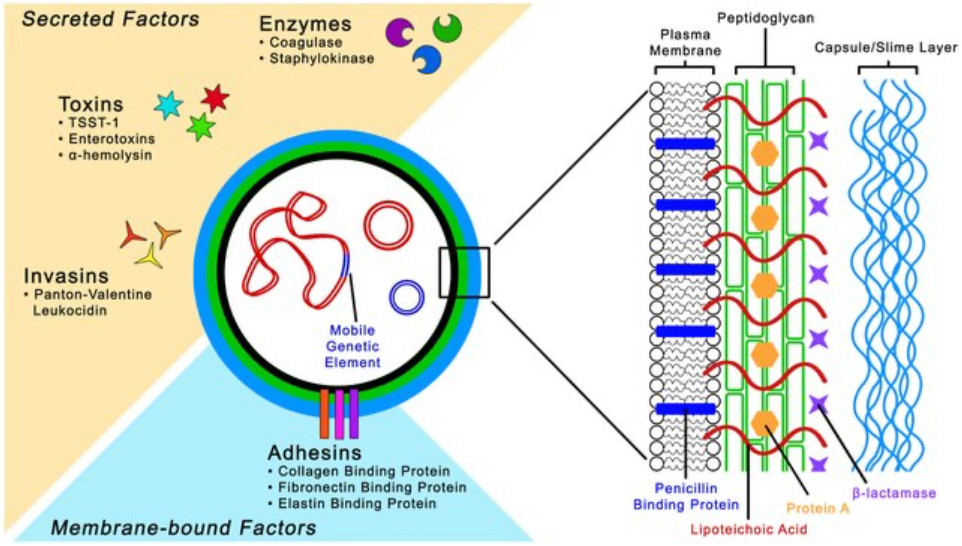

Figure 3. The cell wall structure of *S.aureus* (Source: E. Kong et al.,2016).

According to Su et al. (2021), a minimum of six gene alterations is necessary to decrease the effectiveness of vancomycin. Linezolid, an oxazolidinone medication, received approval in the year 2000 to treat difficult-to-treat HA-MRSA infections. Linezolid is the sole antibiotic that is entirely synthesized and targets the ribosome. The binding site is in the peptidyl transferase center (PTC) within the 50S ribosome subunit. It hinders the amino-acyl moiety of aa-tRNA, hence blocking peptidyl transferase and the creation of peptide bonds (Toh et al., 2007). Erythromycin is a kind of macrolide

antibiotic that blocks the movement of polypeptides near the peptidyl transferase center (PTC). At present, macrolides are not frequently employed for the treatment of staphylococcal infections. Nevertheless, they play a crucial part in managing *Staphylococcus aureus* infections. Semisynthetic macrolides, such as clarithromycin and azithromycin, are utilized for therapeutic purposes in the treatment of bacterial infections caused by microorganisms that are not part of the *Staphylococcus* genus. Consequently, the commensal staphylococci are frequently subjected to macrolides, which might explain the prevalent occurrence of erythromycin resistance in clinical samples (Urban-Chmiel et al., 2022).

Escherichia coli (E. coli) is a significant bacterium in the field of food microbiology and safety. Food-borne diseases have become a major public health issue due to the presence of many microorganisms, such as bacteria, viruses, fungus, parasites, and chemical pollutants, in food and water. These disorders cause more than 2,500 diseases worldwide, impacting almost 600 million people and leading to 420,000 deaths per year (Elbehiry et al., 2023). *E. coli* is a diverse genus of gram-negative bacteria consisting of several innocuous strains. However, some pathogenic strains, such as *E. coli* O157 (STEC), are well-known for generating significant food poisoning outbreaks (Ramos et al., 2020).

E. coli is a coliform bacterium belonging to the Enterobacteriaceae family, which includes other species including *E. adecaroxylate, E. blattae, E. fergusonii, E. hermannii*, and *E. vulneris*. This bacterium is a gram-negative, non-spore-forming, facultatively anaerobic, rod-shaped microorganism that can survive at temperatures between 7°C and 45°C. Coliform bacteria, such as *Citrobacter*, *Enterobacter*, and *Klebsiella*, are markers of fecal contamination and unsanitary conditions in food. The presence of *E. coli* in food, particularly raw milk, fruits, and vegetables, indicates poor hygiene standards, making it an important pathogen to monitor to ensure food and water safety (Lim et al., 2010).

The relevance of *E. coli* in food microbiology lies in its function as an indication of fecal contamination and its ability to cause serious infections as a pathogenic organism. *Escherichia coli* is commonly found in feces and the surrounding environment, forming a significant component of the intestinal microbiota. Pathogenic strains of *E. coli* can generate toxins, resulting in poisoning and causing gastroenteritis. They can also lead to severe illnesses such as newborn meningitis, which has significant rates of illness and death globally. Intestinal pathogenic *E. coli* is divided into different pathotypes, such as enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC),

diffusely-adherent (DAEC), enteroinvasive (EIEC), and enterohemorrhagic (EHEC) strains (Luna-Guevara et al., 2019), (Castro-Rosas et al., 2012).

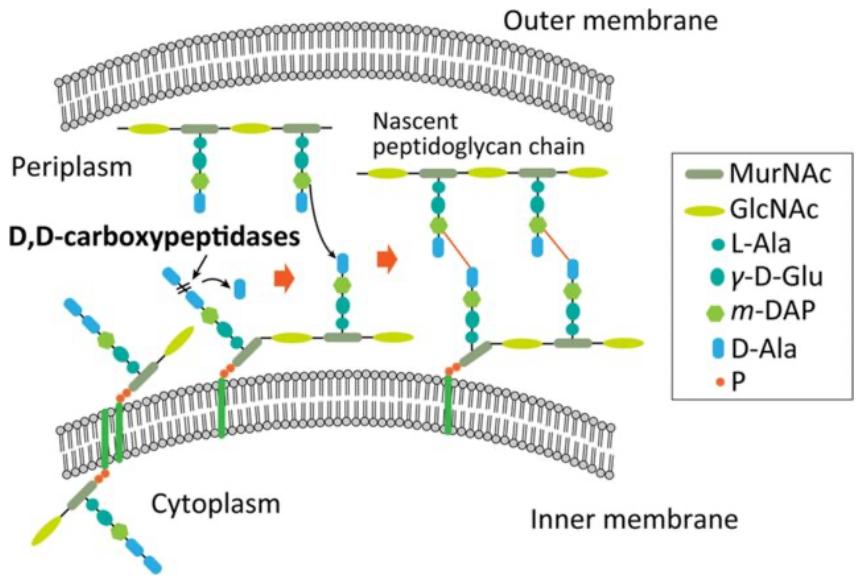

Figure 4. The cell wall structure of *E.coli* (Source: Y. Haiquan et al.,2019).

Antibiotics hinder the growth of *E. coli* by interfering with crucial bacterial activities in various ways. For example, beta-lactam antibiotics such as penicillin disrupt the process of cell wall formation, destroying the cell. Tetracyclines hinder protein synthesis by attaching to the bacterial ribosome, whereas fluoroquinolones interfere with DNA replication by specifically targeting DNA gyrase and topoisomerase IV. Sulfonamides and trimethoprim hinder the production of folic acid, which is an essential process for the synthesis of nucleotides. The efficacy of these antibiotics is contingent upon the strain and its resistance mechanisms, underscoring the importance of meticulous selection and monitoring in clinical and food safety contexts (Kapoor et al., 2017).

Lactococcus lactis is a major species of lactic acid bacteria (LAB) that is extensively employed in the dairy sector to produce fermented goods. Lactic acid bacteria (LAB) are a kind of bacteria that produce lactic acid as the main result of fermentation. This contributes to the distinct aromas and textures found in fermented foods. The primary genera of lactic acid bacteria (LAB) consist of *Lactobacillus, Leuconostoc,*

Pediococcus, and *Streptococcus*. *Lactococcus lactis* is crucial in dairy fermentation processes (Mathur et al., 2005).

Lactococcus lactis plays a vital role in food microbiology since it is widely employed in the fermentation process of dairy products, including cheese, buttermilk, and sour cream. The production of lactic acid by this organism reduces the pH of the food, which prevents the growth of harmful microorganisms and extends the shelf life of the product, so improving food safety. *Lactococcus lactis* plays a crucial role in enhancing the flavor and texture of fermented dairy products, making it an essential component in the dairy sector (Clementi et al., 2011).

Antibiotic inhibition of *Lactococcus lactis*, like other bacteria, involves disrupting essential cellular processes. Commonly used antibiotics include:

Beta-lactams (e.g., penicillins): These antibiotics hinder the production of cell walls by specifically attacking penicillin-binding proteins, resulting in the rupture and demise of the cells.

Tetracyclines: These substances hinder the process of protein synthesis by attaching to the 30S ribosomal subunit, which stops the incorporation of amino acids into the developing peptide chain.

Macrolides (e.g., erythromycin): These substances hinder the process of protein synthesis by attaching themselves to the 50S ribosomal subunit. This blocks the exit tunnel of the ribosome, causing the elongation of proteins to come to a stop.

Glycopeptides (e.g., vancomycin): They hinder the formation of cell walls by attaching to the D-Ala-D-Ala end of cell wall building blocks, therefore blocking their integration into the cell wall.

Candida albicans is a prominent microbe in the field of food microbiology because it serves as both a commensal organism and an opportunistic pathogen. This yeast often resides on the human skin, genitals, and gastrointestinal systems without causing any harm in normal circumstances. Nevertheless, alterations in the host's immune system or microbiota can result in significant colonization of the mucosal surfaces and the occurrence of infections, both locally and systemically (Li et al., 2022). The incidence of *Candida* bloodstream infections has increased in parallel with developments in medical procedures. Among patient samples, *C. albicans* is the most often isolated yeast, which has been associated with higher fatality rates (Bac et al., 2019).

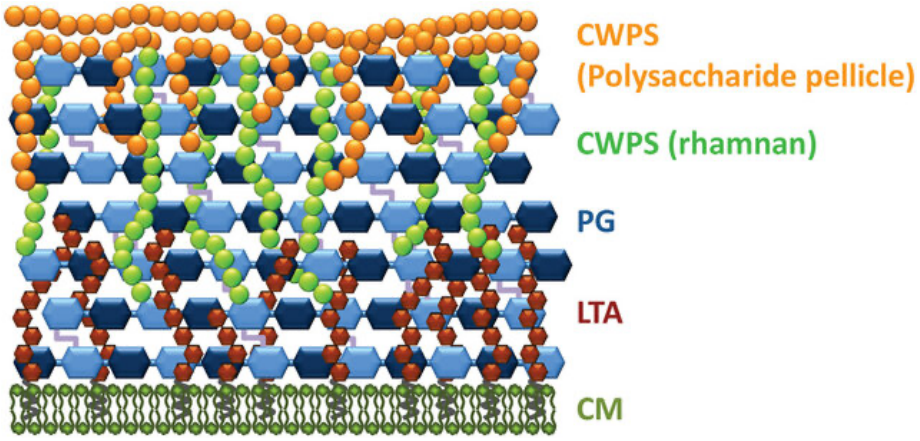

Figure 5. The cell wall structure of *L. lactis* (PG: Peptidoglycan, LTA: Lipoteichoic acids, CM: Cytoplasmic membrane) (Source: Martinez et al., 2020).

Opportunities for C. albicans infections to occur in intensive care units (ICUs) arise from factors such as colonization of skin and mucous membranes, wounds, operations, and the presence of indwelling intravascular catheters (Costa-de-Oliveira et al., 2020). A major obstacle in the treatment of Candida albicans infections is the issue of diagnosing them accurately. This is because the clinical signs are not specific, and discovery is sometimes delayed, leading to a delay in starting antifungal medication (Fang et al., 2023).

Antifungal agents used against *C. albicans* can be classified into several categories, each with distinct mechanisms of action:

Polyenes: Amphotericin B and nystatin are also included in this class. Polyenes specifically act on ergosterol within the fungal cell membrane, creating openings that interfere with the integrity of the membrane and ultimately result in the cell's demise (Brian et al., 2008). Amphotericin B is considered the most effective treatment for severe fungal infections, although its usage is limited due to its nephrotoxicity (Cavassin et al., 2021).

Pyrimidine Analogues: 5-Flucytosine is the main medication in this group. Within fungal cells, it converts into 5-fluorouracil, which disrupts the synthesis of DNA and RNA, hence limiting cellular activity and division. 5-flucytosine is commonly used in combination with other antifungals due to its tendency to rapidly build resistance (Delma et al., 2021).

Triazoles: Fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole are all part of this extensive class. Triazoles function as inhibitors of the enzyme lanosterol 14α-demethylase, which leads to the disruption of ergosterol production and the formation of faulty cell membranes (de Oliveira Santos et al., 2018). Fluconazole is commonly utilized due to its high level of safety and cost-effectiveness. Nevertheless, the presence of resistance mechanisms, such as the increased production of efflux pumps and genetic alterations in the ERG11 gene, presents significant obstacles (Lee et al., 2021).

Echinocandins: Echinocandins, such as caspofungin, micafungin, and anidulafungin, block the activity of β-(1,3)-glucan synthase, which is necessary to produce fungal cell walls. These medications exhibit fungicidal properties and provide a highly favorable safety profile, rendering them the preferred first treatment for invasive Candida infections (Szymański et al., 2022).

Candida albicans has several resistance mechanisms, which complicate the process of therapy. Polyene resistance to amphotericin B is uncommon but can arise from mutations in the ERG3 gene or heightened catalase activity (Bohner et al., 2022). Resistance to triazoles frequently occurs through the increased production of efflux pumps (such as CDR1 and CDR2) and genetic mutations in the ERG11 gene. The main cause of echinocandin resistance is mutations in the FKS1 gene, which codes for β-(1,3)-glucan synthase, decreasing drug sensitivity (Scorzoni et al., 2021).

Saccharomyces cerevisiae var. *boulardii*, a specific kind of *Saccharomyces* yeast, has attracted considerable interest in the field of food microbiology because of its beneficial effects as a probiotic. In contrast to *Saccharomyces cerevisiae*, the baker's yeast that does not possess significant health advantages, S. var. boulardii is highly efficient in the treatment of acute gastrointestinal diseases such as diarrhea and chronic illnesses such inflammatory bowel disease (IBD) (Abid et al., 2022). *S.boulardii* CNCM I-745, manufactured by Laboratoires Biocodex, is the sole yeast probiotic that is backed by strong scientific data, as demonstrated in more than 80 randomized clinical studies. It is important to emphasize that the effectiveness demonstrated in this strain cannot be applied to other strains, such as *S.* var. *boulardii* CNCM 1079 (Moré et al., 2018).

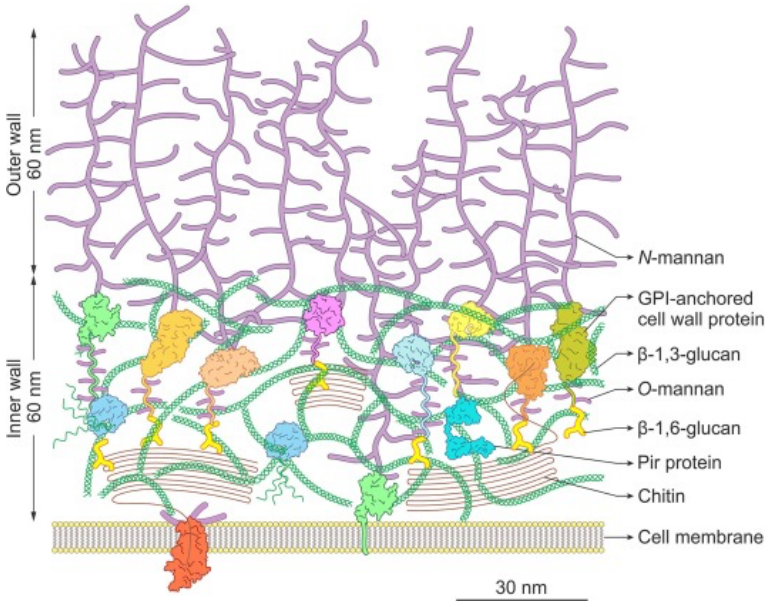

Figure 6. The cell wall structure of *Candida albicans* (Source: Lenardon et al., 2020).

The significance of *S. boulardii* in food microbiology stems from its distinct function as a probiotic yeast. It enhances gut health through several pathways, such as antitoxin effects, physiological protection, control of the microbiota, regulation of metabolism, modulation of the immune system, competition with pathogens, and interactions with the brain-gut axis (Pais et al., 2020). The diverse range of effects exhibited by *S.boulardii* makes it highly beneficial in dietary supplements and functional foods for enhancing gastrointestinal health and general well-being.

Saccharomyces boulardii is genetically identical to *Saccharomyces cerevisiae*, with a comparable karyotype. Molecular typing techniques, such as pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), are used. Restriction fragment length polymorphisms (RFLP) analysis has provided evidence that *S. boulardii* strains form clusters within the *S. cerevisiae* species. This suggests that they are variants of the same species rather than separate species (Pais et al., 2020). Phylogenetic investigations and experiments using DNA/RNA hybridization provide more evidence for this, showing that *S.* var. *boulardii* has specifically lost some Ty elements, which distinguishes it from *S. cerevisiae* (Edwards-Ingram et al., 2007).

Saccharomyces boulardii is added to many food items in the food business to improve gastrointestinal health. The use of probiotics is especially beneficial in the

management of gastrointestinal problems. Nevertheless, guaranteeing the stability and sustainability of *S. boulardii* in food items presents difficulties. To be efficacious, the yeast must endure preparation, storage, and transit via the gastrointestinal system. To ensure the probiotic's effectiveness, it is crucial to employ advanced formulation processes and rigorous quality control procedures.

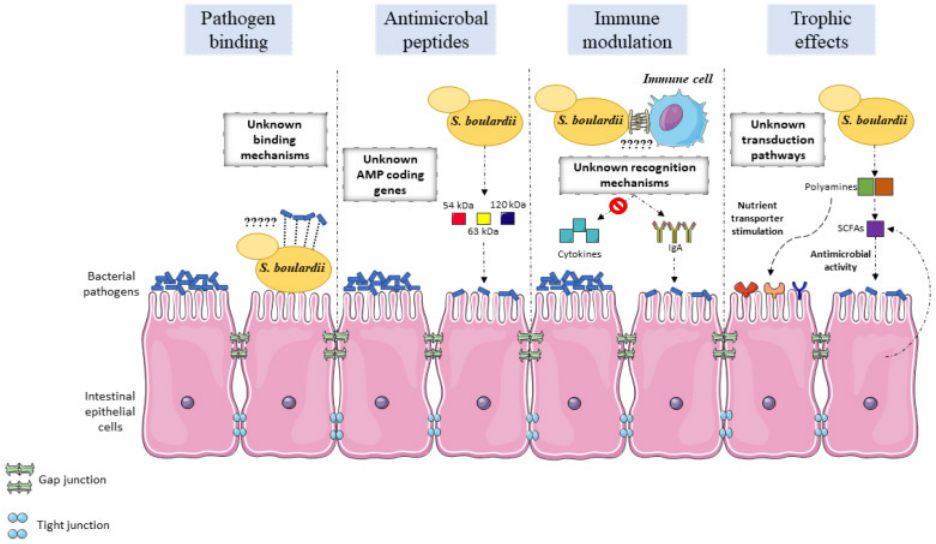

Figure 7. Review the key mechanisms that support *S. boulardii* probiotic activity in the intestinal epithelium (Source: Pais et al., 2020).

Saccharomyces cerevisiae, sometimes known as baker's yeast, plays a crucial role in the field of food microbiology and is widely used in other industrial applications. The widespread utilization of yeast in bread making and the creation of fermented drinks highlights its economic and cultural importance. Moreover, *S. cerevisiae* plays a fundamental role in scientific investigations, serving as a model organism to investigate a wide range of biological phenomena such as metabolism, aging, apoptosis, gene expression regulation, the cell cycle, and signal transduction (Parapouli et al., 2020).

The significance of *S. cerevisiae* in food microbiology arises from its adaptable use in culinary and health-related settings. As a dietary supplement, it is acknowledged for its abundant nutritional composition, including vital minerals such as zinc, selenium, phosphorus, magnesium, chromium, B vitamins, amino acids, and proteins. These supplements are commonly suggested for those who are suffering weakness, exhaustion,

or problems relating to their skin, hair, and nails. They are also indicated for those who are recovering from sickness or surgery (Perli et al., 2020).

Although *S. cerevisiae* has useful applications, it is becoming more frequently found in other parts of the human body, such as the gastrointestinal, respiratory, and vaginal tracts. There are also new reports of diseases caused by this microorganism. Although severe infections like fungemia are not common, they are a major worry, especially for susceptible groups such as preterm newborns, elderly adults, and patients with weakened immune systems (Górzyńska et al., 2024). Infections can vary in severity, ranging from localized fungal infections to more widespread illnesses such as fungemia, endocarditis, liver abscesses, and pneumonia. The risk factors including intestinal translocation, organ spread, and infections connected to catheters (Barros et al., 2023).

The treatment of *S. cerevisiae* infections depends on antifungal medications, often utilizing fluconazole and amphotericin B as the primary options. Nevertheless, there is limited documentation on the susceptibility profile of *S. cerevisiae* to antifungal medications. Reports suggest that fluconazole and itraconazole have high minimum inhibitory concentration (MIC) values for this organism. Caspofungin has demonstrated favorable in vitro efficacy and is recommended as a viable alternative for therapy (Górzyńska et al., 2024).

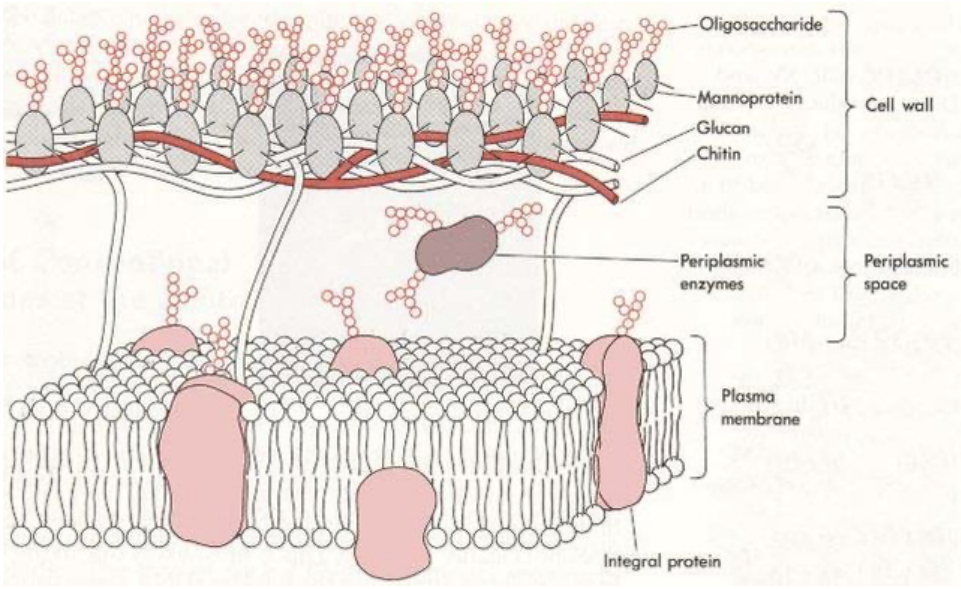

Figure 8. The cell wall structure of *S. cerevisiae* (Source: I. Anwar et al., 2017).

Recent research has investigated the vulnerability of *S. cerevisiae* to several antifungal medications, including a new compound called manogepix. Manogepix acts as an inhibitor of the fungal Gwt1 enzyme, which leads to the disruption of cell wall integrity, biofilm development, and germ tube production. As a result, it effectively suppresses the growth of fungi (Kapoor et al., 2020). According to Almajid et al. (2024), it has a wide range of effectiveness against many types of fungi, including those that are resistant to azole and echinocandin. This makes it a potentially effective antifungal treatment.

1.7. Aim of the Thesis

The primary aim of this thesis is to investigate the antimicrobial properties and biochemical characteristics of Maillard Reaction Products (MRPs) derived from various legume protein hydrolysates, specific chickpea, faba bean, common bean, and soybean when reacted with D-glucose under different thermal conditions (175°C to 275°C). This study seeks to assess the efficacy of these MRPs against a range of bacterial and fungal strains, including *Escherichia coli, Staphylococcus aureus, Lactococcus lactis, Candida albicans, Saccharomyces var. boulardii*, and *Saccharomyces cerevisiae*. Through detailed biochemical characterization using techniques such as UV-Vis spectroscopy, FTIR, HPLC, and NMR, the research aims to elucidate the structural and functional properties of melanoidins formed in these reactions. Additionally, the study will explore the colorimetric changes and browning indices of the MRPs, providing insights into their potential applications in food preservation and developing novel food products with enhanced safety and health benefits.

CHAPTER 2

MATERIAL AND METHOD

2.1 Alkali Extraction - Acid Precipitation Method For Legumes

The alkaline extraction-acid precipitation procedure, as described by Ferreira et al. (2018), was used to prepare all four legumes.

Initially, the beans were pulverized in a blender till they transformed into a fine powder. Subsequently, they were filtered using a sieve with a mesh aperture of 150 microns.

The lipid extraction process involved combining the sample with n-hexane in equal proportions (1:1 ratio) and allowing it to sit in a fume hood for 24 hours to eliminate the alcohol. Subsequently, they were diluted with distilled water at a ratio of 1:10, pH was adjusted to 9 using 1 Molar NaOH, and agitated for 1 hour. The sample was subjected to centrifugation using a Refrigerated Centrifuge, model L535R, at a speed of 11,000 times the force of gravity (11,000xg) at a temperature of 4 C^o for a duration of 20 minutes.

The liquid portion was extracted, and the acidity was adjusted to 4.5 using a solution of 1 Molar Hydrochloric Acid and agitated for a duration of 1 hour. The suspensions underwent a second round of centrifugation at a speed of 11,000 times the force of gravity at a temperature of 4 C^o, for a duration of 20 minutes.

The resulting solid masses were collected. The pellets that were collected were reconstituted in distilled water. The pH was then adjusted to 7. The solution was frozen at a temperature of -80 C^o and subsequently dried using the Labconco Benchtop Freezer for a duration of 24 hours.

2.2. Composition Analysis of Protein Isolates

2.2.1. Kjeldahl For Protein Determination

Exactly 1 gram of each sample (Chickpea seed, Chickpea Protein Isolate, Faba bean seed, Faba bean Isolate, Common bean seed, Common Bean Isolate, Soybean seed, and Soybean Protein Isolate) was put in a digestion flask. Each flask received an addition of 12-15 mL of sulfuric acid, followed by 7 grams of a catalyst combination consisting of potassium sulfate and copper sulfate. The specimens were subjected to heating on a heating block within the temperature range of 370°C to 400°C until the occurrence of white fumes, followed by an additional heating period of 60-90 minutes. After the process of digestion was finished, the samples were chilled and carefully mixed with 250 mL of distilled water.

To carry out distillation, a precise measurement of 15 mL of an acid standard solution was combined with 70 mL of water, and then 3-4 drops of methyl red indicator were added. The sample mixture was rendered very alkaline by adding 80 mL of sodium hydroxide solution. Subsequently, the flask was attached to distillation equipment, and the resulting liquid was gathered until a volume of 150 mL was achieved.

The ammonia, when dissolved in the acid-trapping solution, reacted with and neutralized a portion of the hydrochloric acid (HCl). The residual acid was titrated using a standardized sodium hydroxide (NaOH) solution. The quantity of ammonia that was distilled was utilized to ascertain the nitrogen concentration present in the sample.

The amount of nitrogen was calculated based on the moles of ammonia trapped. Using the following equations (2.1.), the mass of nitrogen, the percentage of nitrogen, and the crude protein content were determined: (F: 6.25 coefficient factor).

$$\text{Mass of nitrogen= (Moles of Ammonia) (Moles of N/moles of NH3) (14.01 g N/moles of N)}$$

$$\text{Nitrogen (\%)} = \text{Mass of N in sample/Mass of analyzed sample} \times 100$$

$$\text{Crude protein (CP\%)} = \%\,\text{N} \times \text{F} \qquad (2.1.)$$

2.2.2. Soxhlet For Lipid Determination

The Soxhlet extraction technique was used to quantify the lipid content in different seed and protein isolate samples. More precisely, a quantity of 1 gram from each sample, including Chickpea seed, Chickpea Protein Isolate, Faba bean seed, Faba bean Isolate, Common bean seed, Common Bean Isolate, Soybean seed, and Soybean Protein Isolate, was measured and put in individual cellulose or glass fiber thimbles. Subsequently, every thimble was placed within the primary compartment of a Soxhlet extractor. Hexane, an appropriate solvent for extracting lipids, was introduced into a round-bottom flask that was linked to the extractor. The system was furnished with a condenser and heated via a heating mantle, enabling the solvent to undergo boiling, vaporization, and subsequent condensation into the Soxhlet chamber. The extraction procedure was prolonged for a duration of 6 hours to guarantee the occurrence of many cycles of solvent extraction. Subsequently, the solvent containing the lipids was collected and subjected to evaporation using a rotary evaporator to separate and get the lipid extracts. The lipid material that was obtained was measured to quantify the amount produced, and the resulting lipid extracts were kept in vials that were labeled for future study. This technique guarantees the extraction of lipids from different seed and protein isolate samples in a manner that is both efficient and comprehensive, so enabling the subsequent study of their lipid content.

2.2.3. Ash Content of Samples

The ash content of several samples, such as Chickpea seed, Chickpea Protein Isolate, Faba bean seed, Faba bean isolate, common bean seed, common bean isolation, soybean seed, and soybean protein isolate, was determined using the AOCS Official Method. Every individual sample, weighing 1 gram, was carefully put within a crucible that had been previously weighed and ensured to be clean and dry. The samples were enclosed in crucibles and subsequently sent to a muffle furnace, where they were heated to a temperature of 550°C. This temperature was sustained until all organic substances

were entirely incinerated, a process that normally lasted around 5-6 hours. Following the process of burning, the samples underwent a transformation into a pale gray residue, which signifies the achievement of total incineration. Subsequently, the crucibles were cautiously extracted from the furnace and cooled in a desiccator to avert moisture absorption. After being allowed to cool down to the temperature of the surrounding room, the crucibles were reweighed in order to ascertain the mass of the ash. The ash content was determined by subtracting the starting weight of the empty crucible from the final weight of the crucible with the ash, and then expressing it as a percentage of the beginning weight of the sample using the following formula (2.2.):

$$\text{Ash content (\%)} = (\text{Weight of ash} / \text{Initial sample weight}) \times 100 \qquad (2.2.)$$

2.3. Hydrolysis of Legume Proteins

Four samples of legumes were hydrolyzed with the Alcalase enzyme (Sigma Aldrich) according to the methodology described (Ghribi et al., 2015). This is achieved through the addition of 0.8% (v/v) alkalase (2.4 AU/g protein) to 8% (w/w) protein isolate dispersions that have been pre-equilibrated at pH 8.0 and 55 °C. The reaction was maintained at 55 °C and pH 8.0 with the continuous addition of 0.1 N NaOH for various times, including 30 min (H30), 60 min (H60), 90 min (H90), 120 min (H120), and 180 min (H180). After the required response time had passed, hydrolysis was terminated by inactivating the alkalase at 85 °C for 20 minutes. Controls were prepared in H0, which comprised a temperature-treated slurry with pH 8.0 and without alkalase.

2.4. Measurement of Protein Hydrolysis Degree

The extent of hydrolysis was assessed by quantifying the α-amino groups liberated during the hydrolysis process using the o-phthaldialdehyde (OPA) spectroscopic test, as outlined by Church et al. with certain adjustments. According to the procedure outlined

by Church et al. (1983), 200 µL of OPA reagent (composed of 97.5 mL of 100 mM sodium tetraborate with a pH of 9.9, 0.5 mL of 20% SDS, and 5 mg/mL of OPA dissolved in 1 mL of methanol) was introduced into Eppendorf tubes. The specimen (either leucine standards or hydrolysate) was combined with the reagent, then sealed with aluminum foil and subjected to incubation at a temperature of 25 °C for a duration of 5 minutes. Subsequently, the sample was placed in an incubator set at a temperature of 37 °C for a duration of 20 minutes. A 5 µL portion of the sample (either leucine standards or hydrolysate) was combined with 5 mg/mL dithiothreitol dissolved in 1 mL water. The mixture was then covered with aluminum foil and kept at a temperature of 25 °C for a duration of 5 minutes. Subsequently, the sample was placed in an incubator at a temperature of 37 °C for a duration of 20 minutes. Afterwards, the sample underwent analysis using UV-Vis spectroscopy at a specific wavelength of 340 nm. The extent of hydrolysis was determined using the following equation (2.3.):

$$\mathrm{DH}(\%): \frac{\mathrm{CSxDF1xDF2x100}}{\mathrm{mxHtot}} \qquad (2.3.)$$

CS denotes the sample concentration that is derived from the linear regression equation. DF1 refers to dilution factor 1, which specifies the extent to which the sample is diluted before to the OPA technique. DF2 refers to dilution factor 2, which specifically indicates the extent to which the sample is diluted during the OPA procedure. m indicates the protein concentration in grams per liter, whereas Htot represents the total number of peptide bonds in the protein substrate (which is 7.8 millimoles per gram).

2.5. Preparation of Maillard Reaction Products

2.5.1. Glucose – Glycine Maillard Reaction Model System

1 mol of D-glucose and 1 mol of Glycine were each dissolved separately in individual beakers for 1 hour. Subsequently, the two solutions were combined into a single beaker and stirred for an additional hour. The combined mixture was then frozen

at -80°C before undergoing lyophilization for 24 hours to remove water content. After lyophilization, the water-free samples were transferred to a clean beaker and subjected to thermal treatment in an oven at 120°C for 2 hours to induce the Maillard Reaction. This process resulted in the formation of melanoidins, which indicates the Maillard Reaction products between glucose and glycine. All 20 products were first dissolved in distilled water and filtered using Whatman filter paper, then 10 kDa was separated from the filter with the help of an ultrafiltration device, the retentate and filtrate parts were separated, and retentate part (larger than 10 kDa) was frozen at -80°C and kept in a lyophilized device for 24 hours to remove water.

2.5.2. Legume Protein Hydrolysates – Glucose Maillard Reaction System

Equal amounts of Legume Protein Hydrolysates and D-glucose were accurately weighed in grams. Maillard reaction was carried out based on the method in (Zhou et al., 2021). Each component was dissolved separately in phosphate buffer at pH 7 in individual beakers. Subsequently, the solutions were combined into a single beaker and stirred for 1 hour to ensure thorough mixing. After mixing, the combined solution was frozen at -80°C. The frozen samples were then subjected to lyophilization for 24 hours to remove all water content, resulting in dry samples.

The water-removed samples were then subjected to thermal treatment at five different temperatures: 175°C, 200°C, 225°C, 250°C, and 275°C. This thermal treatment induced the Maillard Reaction, leading to the formation of melanoidins. Products were first dissolved in distilled water and filtered using Whatman filter paper, then 10 kDa was separated from the filter with the help of an ultrafiltration device, the retentate and filtrate parts were separated, and the retentate part (larger than 10 kDa) were frozen at -80°C and kept in a lyophilized device for 24 hours to remove water.

2.6. Colour Measurements of MRPs

For the colorimetric analysis of the Maillard Reaction Products (MRPs), an equal number of samples was taken from each of the 20 different Maillard Reaction samples. The color measurements were performed using a Minolta CM 508d spectrophotometer (Minolta Co. Ltd., Osaka, Japan). The spectrophotometer was calibrated, and the measurements were taken on a white background to ensure accuracy and consistency.

The color parameters measured were L*, a*, and b*:

L*: This parameter represents the lightness of the sample, ranging from 0 (black) to 100 (white).

a*: This parameter represents the green-red spectrum, where negative values indicate green and positive values indicate red.

b*: This parameter represents the blue-yellow spectrum, where negative values indicate blue and positive values indicate yellow.

These measurements provided quantitative data on the color changes of the MRPs resulting from the Maillard Reaction under different thermal conditions. The colorimetric data are essential for understanding the visual properties of the MRPs and their potential applications in food products.

2.7. Browning Index Determination

The Browning Index (BI) (Palou et al., 1999) for each of the 20 Maillard Reaction Products (MRPs) was calculated using the color parameters (L*, a*, and b*) obtained from the colorimetric analysis. The formula used for calculating the BI is as follows:

$$BI = [100 * (x - 0.31)] / 0.172$$

$$x = (a * +1.75xL *)/(5.645xL * +a * -0.3012b *) \quad (2.3.)$$

The L*, a*, and b* values were plugged into this formula to determine the corresponding BI values for each MRP. These BI values provide a quantitative measure of each sample's browning intensity, which indicates the extent of the Maillard Reaction that has occurred under different thermal conditions. This analysis is crucial for understanding the visual and, potentially, the sensory properties of the MRPs, as the browning intensity can influence the appearance and acceptability of food products.

2.8. UV-Vis Spectroscopy

For the UV-Vis spectroscopy analysis, 10 mg samples from two different types of faba beans were dissolved in 1 mL of distilled water. These solutions were then diluted 100-fold to achieve appropriate concentrations for measurement. The samples were scanned across a wavelength range of 200 nm to 600 nm to capture the absorbance spectrum. Specific absorbance measurements were taken at 420 nm to determine the melanoidin content. The melanoidin content was calculated using a standard curve generated from glucose-glycine melanoidins, allowing for the quantification of these compounds in the samples. This method provides a detailed understanding of the melanoidin content and its relation to the Maillard reaction products formed in the legume protein hydrolysates.

2.9. FTIR Analyzes

2.9.1. Only MRP Samples

Lyophilized Maillard reaction products were analyzed using an ATR-FTIR spectrometer. The analysis was conducted by measuring the absorbance against the wavelength, which provided a detailed spectral profile of the samples. This technique allowed for the identification of functional groups and the assessment of the chemical

composition of the Maillard reaction products. The ATR-FTIR analysis offers valuable insights into the molecular changes occurring during the Maillard reaction, contributing to a better understanding of the formation and properties of the resulting melanoidins.

2.9.2. MRP – Microorganisms

The following procedure investigated the interaction between Maillard Reaction Products (MRPs) and microorganisms: 100 mg of Fava Bean MRP and 100 mg of Chickpea MRP were prepared and separately added into microplate wells. Into each well, either *Candida albicans* or *Lactococcus lactis* was introduced, with control wells containing only the microorganisms without MRPs.

The microplate wells were incubated for 24 hours under suitable conditions for microbial growth. After incubation, the samples were centrifuged at 5000 g for 10 minutes to separate the supernatant and pellet. The supernatant was removed, and the pellet was resuspended in peptone water, with this washing step repeated three times. The cleaned pellet was then transferred to Eppendorf tubes, frozen at -80°C, and lyophilized for 24 hours to remove moisture.

The lyophilized samples were then analyzed using Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR), recording spectra as absorbance against wavelength to profile chemical interactions and structural changes in the MRPs due to the presence of microorganisms.

2.10. HPLC - DAD Analysis

A modified technique was employed in the HPLC-DAD study to examine the melanoidin fractions. The melanoidin fractions were suspended in methanol at a concentration of 6 mg/mL. The solution was agitated using an orbital shaker for 1 hour and thereafter centrifuged at a speed of 10,000 rpm for a duration of 20 minutes. The liquid portion was gathered, and the liquid was removed by evaporation using a nitrogen

stream. The remaining substance was subsequently dissolved in a solution of methanol and water (5% v/v, 200 µL).

The solutions, each measuring 20 µL, were examined using a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA). The system was outfitted with a C18 Zorbax Eclipse column (250 × 4.6 mm, 5 µm particle size) and a Diode Array Detector (DAD). The experiment utilized a gradient elution technique, employing a mixture of acetonitrile and water at a flow rate of 1 mL/min. The gradient started with a concentration of 5% acetonitrile, progressively rose to 35% acetonitrile during a time span of 30 minutes, and then reached a concentration of 100% acetonitrile at the 42-minute mark. This technique enabled the efficient isolation and study of the melanoidin components, enabling a comprehensive investigation of their chemical characteristics.

2.11. Antimicrobial Analysis

2.11.1. Test Microorganisms

Fungal and bacterial strains obtained from the Izmir Institute of Technology Food Engineering Department and IYTE Integrated Research Center were used. The strains used were *Staphylococcus aureus* RSKK 1009, *Escherichia coli* ATCC 25922, *Candida albicans* DSMZ 5817, *Saccharomyces var. boulardii* CNCM-I745, *Lactococus lactis ssp. lactis* C24, *Saccharomyces cerevisiae*. *S. aureus* and *E.coli* bacteria were grown on TSA (Tryptic Soy Agar), *L. lactis* on MRS agar, and *S. var boulardii*, *S. cerevisiae,* and *C.albicans* yeasts on YPD (Yeast Peptone Dextrose) agar. *S. aureus* and *E. coli* bacteria were incubated for 24 hours at 37°C, and yeasts were incubated for 48 hours at 35°C, after which medium-sized colonies were transferred to liquid culture and incubated under the same conditions.

2.11.2. Antimicrobial Activity Test

Chickpea - D-glucose MRPs and Faba bean - D-glucose MRPs were made using 100 mg. 100 mg samples were added to a Well plate containing 24 wells (1 mL each well), and 990 µL of broth and 10 µL of microorganisms were added. The assay was performed with 10^4 CFU/mL bacteria for each bacterial species and 10^3 CFU/mL yeast for each yeast. S. aureus and E. coli bacteria were incubated for 24 hours at 37°C and yeasts were incubated for 48 hours at 35°C in a Thermo Scientific™ Varioskan™ Multimode Microplate containing both samples and microorganisms in a 24-well plate and wells containing only microorganisms for control. After incubation, they were diluted and plated on agar for colony counts.

2.11.3. Well Diffusion Antimicrobial Activity Test

Chickpea - D-glucose MRPs and Broad Bean - D-glucose MRPs were performed using 50 mg. Bacteria and yeasts (10^6 CFU/mL) were first smeared with a swab stick on each agar. Then, wells with a diameter of 2 cm were made in the agars, and 50 mg of Chickpea-D-glucose MRP and Broad Bean-D-glucose MRP were first added, and 800 µL of peptone water was added to ensure diffusion into the agar. S. aureus and E. coli bacteria were incubated at 37°C for 24 hours, and yeasts at 35°C for 48 hours. After incubation, zone formation was observed.

2.12. ^{1}H-NMR Analysis

The faba bean-MRP and chickpea-MRP samples underwent NMR analysis using the specified procedure. 10 mg of dried samples obtained from the previously mentioned preparation techniques were diluted in 0.5 mL of deuterated water (D2O). The solutions were subjected to ultrasonication for a duration of 15 minutes to guarantee thorough

dissolution and uniformity. Following ultrasonication, the samples underwent centrifugation with a force of 1000 times the acceleration due to gravity for a duration of 5 minutes to eliminate insoluble particles. The liquid portion obtained from this procedure was utilized for the NMR examination. The NMR spectra were acquired with an AV III400 M NMR spectrometer manufactured by Bruker in Germany. 1H-NMR spectra were obtained to get an in-depth understanding of the structural and compositional properties of the melanoidin products formed from faba bean and chickpea protein hydrolysates through their Maillard reaction with D-Glucose. This investigation proved crucial in comprehending the molecular interactions and chemical composition of the Maillard Reaction Products (MRPs) in these legume-based systems.

2.13. Statistical Analysis

Experiments were rigorously conducted simultaneously, and results were presented with standard deviations. Subsequent data analysis was performed using Minitab 18.0 software developed by Minitab Inc. and based in State College, PA, USA. To assess differences between samples, statistical analysis included applying the analysis of variance (ANOVA) and Tukey tests. Statistical analysis of the FTIR analyses was performed with MATLAB software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Chemical Composition Analysis of Legume Proteins

The protein content, determined using the Kjeldahl method, showed significant increases in the protein isolates compared to their respective pretreatment samples. Chickpea protein isolate exhibited a protein content of 90.297 ± 0.531%, significantly higher than its pretreatment sample at 20.497 ± 0.855%. Similarly, faba bean, common bean, and soybean isolates displayed protein contents of 91.621 ± 0.348%, 89.273 ± 0.327%, and 91.884 ± 0.259%, respectively, all significantly higher than their pretreatment counterparts.

Carbohydrate content was notably reduced in the protein isolates compared to the pretreatment samples. Chickpea protein isolate had a carbohydrate content of 5.308 ± 0.570%, compared to 45.510 ± 0.501% in the pretreatment sample. Faba bean, common bean, and soybean isolates also showed reduced carbohydrate contents of 5.367 ± 0.752%, 7.113 ± 0.844%, and 3.407 ± 0.429%, respectively.

The lipid content, determined using the Soxhlet extraction method, decreased significantly in the protein isolates. Chickpea protein isolate had a fat content of 2.730 ± 0.229%, while its pretreatment sample had 18.707 ± 0.741%. Faba bean, common bean, and soybean isolates had fat contents of 1.420 ± 0.437%, 1.517 ± 0.401%, and 3.427 ± 0.117%, respectively, all lower than their pretreatment states.

Ash content was measured following the AOCS Official Method, showing a decrease in the protein isolates. Chickpea protein isolate exhibited an ash content of 1.697 ± 0.215%, compared to 15.287 ± 0.475% in the pretreatment sample. Faba bean, common bean, and soybean isolates had ash contents of 1.593 ± 0.102%, 2.487 ± 0.199%, and 1.287 ± 0.191%, respectively.

These results demonstrate the efficiency of the alkaline extraction-acid precipitation method in producing high-protein isolates with reduced carbohydrate, fat, and ash contents. The significant increase in protein content and the reduction in other components highlight the potential application of these protein isolates in food formulations where high protein content is desirable.

Table 2. Chemical composition of different legumes pretreatment and after protein extraction (%).

		Protein (N x 6.25)	Carbohydrate	Lipid	Ash
Chickpea	Pretreatment	20.497 ± 0.855B	45.510 ± 0.501B	18.707 ± 0.741B	15.287 ± 0.475AB
	Protein isolate	90.297 ± 0.531b	5.308 ± 0.570b	2.730 ± 0.229a	1.697 ± 0.215b
Faba bean	Pretreatment	22.283 ± 0.384A	46.601 ± 0.209AB	16.183 ± 0.350C	14.933 ± 0.427B
	Protein isolate	91.621 ± 0.348a	5.367 ± 0.752ab	1.420 ± 0.437b	1.593 ± 0.102b
Common bean	Pretreatment	19.243 ± 0.437B	47.743 ± 0.836A	16.937 ± 0.502C	16.077 ± 0.450A
	Protein isolate	89.273 ± 0.327c	7.113 ± 0.844a	1.517 ± 0.401b	2.487 ± 0.199a
Soybean	Pretreatment	22.747 ± 0.372A	40.893 ± 0.713C	20.997 ± 0.435A	15.457 ± 0.189AB
	Protein isolate	91.884 ± 0.259a	3.407 ± 0.429c	3.427 ± 0.117a	1.287 ± 0.191b

Notes: Results are shown as means ± standard deviation, columns with different lowercase or uppercase letters differ statistically ($p \leq 0.05$).

3.2. Degree of Hydrolysis Determination

The results demonstrated a progressive increase in hydrolysis over time for all legume protein isolates. At the 30-minute mark, the DH values were relatively low, with chickpea exhibiting 7.24 ± 0.44%, faba bean 6.83 ± 0.29%, common bean 5.25 ± 0.35%, and soybean 5.27 ± 0.27%. As the hydrolysis duration increased, the DH values also increased significantly. At 60 minutes, chickpea showed a DH of 12.84 ± 0.43%, faba bean 11.60 ± 0.40%, common bean 8.90 ± 0.52%, and soybean 7.73 ± 0.33%.

Table 3. Degree of hydrolysis of legume proteins.

Degree of Hydrolysis (%) / Time	Chickpea	Faba Bean	Common Bean	Soybean
0 min	0	0	0	0
30 min	7.24 ± 0.44^{A}	6.83 ± 0.29^{A}	5.25 ± 0.35^{A}	5.27 ± 0.27^{A}
60 min	12.84 ± 0.43^{B}	11.60 ± 0.40^{B}	8.90 ± 0.52^{B}	7.73 ± 0.33^{B}
90 min	16.83 ± 0.56^{C}	16.02 ± 0.22^{C}	14.39 ± 0.63^{C}	13.75 ± 0.23^{C}
120 min	21.92 ± 0.29^{D}	19.33 ± 0.27^{D}	18.63 ± 0.18^{D}	18.21 ± 0.51^{D}
180 min	26.41 ± 0.47^{E}	22.50 ± 0.41^{E}	20.00 ± 0.47^{D}	19.62 ± 0.35^{D}

Notes: Results are shown as means ± standard deviation, columns with different uppercase letters differ statistically ($p \leq 0.05$).

Further hydrolysis led to even higher DH values, with chickpea reaching 16.83 ± 0.56%, faba bean 16.02 ± 0.22%, common bean 14.39 ± 0.63%, and soybean 13.75 ± 0.23% at 90 minutes. The trend continued at 120 minutes, where chickpea exhibited a DH of 21.92 ± 0.29%, faba bean 19.33 ± 0.27%, common bean 18.63 ± 0.18%, and soybean 18.21 ± 0.51%. Finally, at 180 minutes, chickpea showed the highest degree of hydrolysis at 26.41 ± 0.47%, followed by faba bean at 22.50 ± 0.41%, common bean at 20.00 ± 0.47%, and soybean at 19.62 ± 0.35%.

These results indicate that chickpea protein isolates undergo the most extensive hydrolysis among the legumes studied, followed by faba bean, common bean, and soybean. With time, the increasing degree of hydrolysis reflects the effective enzymatic breakdown of protein structures, resulting in the release of α-amino groups.

3.3. Color Measurements of Legume Protein Hydrolysates – D-Glucose

At 175°C, the common bean MRP showed an L* value of 6.715 ± 0.092, indicating a darker color compared to faba bean and chickpea MRPs, which had L* values of 7.390 ± 0.526 and 7.127 ± 0.392, respectively. Soybean MRP had the lowest L* value of 6.321 ± 0.171 at this temperature. The a* values indicated a more pronounced red hue in faba bean (6.511 ± 0.130) and chickpea (6.613 ± 0.174) MRPs, while common bean and soybean MRPs exhibited lower a* values of 3.277 ± 0.423 and 3.360 ± 0.075, respectively. The b* values were highest for faba bean (11.700 ± 0.340) and chickpea (11.017 ± 0.705) MRPs, reflecting a strong yellow hue, whereas common bean and soybean MRPs had lower b* values of 8.553 ± 0.270 and 8.813 ± 0.197, respectively.

At 200°C, L* values generally increased slightly for all MRPs, with common bean MRP reaching 7.733 ± 0.092, faba bean 7.347 ± 0.580, chickpea 7.503 ± 0.445, and soybean 7.453 ± 0.468. The a* values decreased for common bean (2.043 ± 0.468) and soybean (2.733 ± 0.081) MRPs, while chickpea MRP showed a higher a* value of 4.963 ± 0.141. The b* values remained relatively high, with chickpea MRP exhibiting the highest value at 10.663 ± 0.474.

Table 4. Color parameters of MRPs.

	L*	a*	b*
Common bean MRP (175°C)	6.715 ± 0.092^{EFGHI}	3.277 ± 0.423^{C}	8.553 ± 0.270^{D}
Faba bean MRP (175°C)	7.390 ± 0.526^{EFGH}	6.511 ± 0.130^{A}	11.700 ± 0.340^{A}
Chickpea MRP (175°C)	7.127 ± 0.392^{EFGHI}	6.613 ± 0.174^{A}	11.017 ± 0.705^{AB}
Soybean MRP (175°C)	6.321 ± 0.171^{HI}	3.360 ± 0.075^{C}	8.813 ± 0.197^{D}
Common bean MRP (200°C)	7.733 ± 0.092^{CDEF}	2.043 ± 0.468^{E}	8.3233 ± 0.131^{D}
Faba bean MRP (200°C)	7.347 ± 0.580^{EFGH}	2.817 ± 0.326^{CD}	8.777 ± 0.191^{D}
Chickpea MRP (200°C)	7.503 ± 0.445^{EFG}	4.963 ± 0.141^{B}	10.663 ± 0.474^{BC}
Soybean MRP (200°C)	7.453 ± 0.468^{EFG}	2.733 ± 0.081^{CD}	9.993 ± 0.603^{C}
Common bean MRP (225°C)	8.730 ± 0.191^{BC}	1.420 ± 0.187^{F}	8.3267 ± 0.106^{D}
Faba bean MRP (225°C)	7.3233 ± 0.081^{EFGH}	1.123 ± 0.145^{F}	7.196 ± 0.0945^{E}
Chickpea MRP (225°C)	6.093 ± 0.161^{I}	2.726 ± 0.069^{CD}	8.400 ± 0.0557^{D}
Soybean MRP (225°C)	9.843 ± 0.249^{A}	2.5367 ± 0.1358^{DE}	10.5367 ± 0.109^{BC}
Common bean MRP (250°C)	7.633 ± 0.482^{DEF}	-0.960 ± 0.180^{HIJ}	5.230 ± 0.060^{GH}
Faba bean MRP (250°C)	8.690 ± 0.075^{BCD}	-1.197 ± 0.174^{IJ}	4.583 ± 0.111^{H}

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Table 4 (cont.)

Chickpea MRP (250°C)	7.530 ± 0.1353^{EFG}	-0.367 ± 0.097^{GH}	6.433 ± 0.096^{EF}
Soybean MRP (250°C)	7.593 ± 0.336^{EF}	-0.2833 ± 0.075^{G}	6.133 ± 0.0961^{F}
Common bean MRP (275°C)	10.483 ± 0.196^{A}	-0.880 ± 0.102^{GHI}	4.793 ± 0.152^{H}
Faba bean MRP (275°C)	9.733 ± 0.052^{AB}	-1.513 ± 0.101^{J}	4.4533 ± 0.162^{H}
Chickpea MRP (275°C)	6.4567 ± 0.077^{GHI}	-0.651 ± 0.070^{GHI}	4.600 ± 0.040^{H}
Soybean MRP (275°C)	8.070 ± 0.506^{CDE}	-0.843 ± 0.072^{GHI}	5.797 ± 0.225^{FG}

Notes: Results are shown as means ± standard deviation, columns with different uppercase letters differ statistically ($p \leq 0.05$).

At 225°C, a significant increase in L* values were observed for soybean MRP (9.843 ± 0.249), indicating a lighter color, while common bean and faba bean MRPs showed L* values of 8.730 ± 0.191 and 7.323 ± 0.081, respectively. Chickpea MRP had a lower L* value of 6.093 ± 0.161. The a* values decreased across the board, with faba bean MRP showing the lowest value of 1.123 ± 0.145. The b* values for soybean MRP remained high at 10.537 ± 0.109, while common bean, faba bean, and chickpea MRPs had lower b* values of 8.327 ± 0.106, 7.196 ± 0.095, and 8.400 ± 0.056, respectively.

At higher temperatures of 250°C and 275°C, L* values increased further, with common bean MRP reaching 10.483 ± 0.196 at 275°C, indicating significant lightening. Faba bean MRP showed a high L* value of 9.733 ± 0.052 at 275°C, while chickpea and soybean MRPs had lower values. The a* values turned negative for several MRPs at these temperatures, indicating a shift towards the green spectrum, with faba bean MRP showing the lowest a* value of -1.513 ± 0.101 at 275°C. The b* values also decreased, particularly for faba bean and common bean MRPs, indicating reduced yellowness.

3.4. Browning Index Determination

Browning index values shown in Table 5. At 175°C, chickpea MRPs exhibited the highest browning intensity with a BI of 75.76 ± 2.78, closely followed by faba bean MRPs at 73.64 ± 2.77. Soybean and common bean MRPs showed lower BI values of 49.84 ± 1.45 and 45.05 ± 2.76, respectively. This indicates a more pronounced browning reaction in chickpea and faba bean MRPs at this temperature.

As the temperature increased to 200°C, the BI values generally decreased. Chickpea MRPs still maintained a relatively high BI of 58.98 ± 3.05, while faba bean and soybean MRPs showed moderate BI values of 39.08 ± 1.88 and 38.09 ± 2.01, respectively. Common bean MRPs exhibited the lowest BI at this temperature, with a value of 29.85 ± 4.15.

At 225°C, a further reduction in BI values was observed. Chickpea MRPs displayed a BI of 45.34 ± 0.58, significantly higher than the other legumes, indicating that chickpea MRPs continue to undergo significant browning. Faba bean and soybean MRPs had similar BI values of 21.12 ± 0.84 and 29.28 ± 0.17, respectively, while common bean MRPs had a BI of 21.44 ± 0.98.

Table 5. Browning index values.

MRP Products	**Browning Index Values**
Common Bean (175°C)	45.05 ± 2.76^{C}
Faba Bean (175°C)	73.64 ± 2.77^{A}
Chickpea (175°C)	75.76 ± 2.78^{A}
Soybean (175°C)	49.84 ± 1.45^{B}
Common Bean (200°C)	29.85 ± 4.15^{D}
Faba Bean (200°C)	39.08 ± 1.88^{C}
Chickpea (200°C)	58.98 ± 3.05^{B}
Soybean (200°C)	38.09 ± 2.01^{C}

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Table 5 (cont.)

Common Bean (225°C)	21.44 ± 0.98^{D}
Faba Bean (225°C)	21.12 ± 0.84^{D}
Chickpea (225°C)	45.34 ± 0.58^{C}
Soybean (225°C)	29.28 ± 0.17^{D}
Common Bean (250°C)	-2.57 ± 1.58^{G}
Faba Bean (250°C)	-5.03 ± 1.16^{H}
Chickpea (250°C)	5.08 ± 0.60^{E}
Soybean (250°C)	5.46 ± 0.58^{E}
Common Bean (275°C)	-1.65 ± 0.55^{G}
Faba Bean (275°C)	-7.10 ± 0.75^{I}
Chickpea (275°C)	-0.31 ± 0.73^{F}
Soybean (275)	-0.55 ± 0.59^{F}

Notes: Results are shown as means ± standard deviation, columns with different uppercase letters differ statistically ($p \leq 0.05$).

At higher temperatures of 250°C and 275°C, negative BI values were recorded for some MRPs, indicating a possible degradation or different browning dynamics at these elevated temperatures. Common bean and faba bean MRPs exhibited negative BI values at both temperatures, with common bean MRPs at -2.57 ± 1.58 (250°C) and -1.65 ± 0.55 (275°C), and faba bean MRPs at -5.03 ± 1.16 (250°C) and -7.10 ± 0.75 (275°C). Chickpea and soybean MRPs showed slightly positive or less negative BI values, with chickpea MRPs at 5.08 ± 0.60 (250°C) and -0.31 ± 0.73 (275°C), and soybean MRPs at 5.46 ± 0.58 (250°C) and -0.55 ± 0.59 (275°C).

These results highlight the significant variations in browning intensity among different legume MRPs, influenced by both the type of legume and the thermal conditions.

3.5. Antimicrobial Analysis

The results indicated varying degrees of antimicrobial activity for the different MRPs as shown in Table 6. For *Escherichia coli*, the chickpea MRP at 175°C achieved a log reduction of 0.98, reducing the final concentration from an initial 2.5 x 10^6 CFU/mL to 3.02 x 10^8 CFU/mL. Faba bean MRP at 175°C showed a slightly lower log reduction of 0.79. Against *Staphylococcus aureus*, chickpea MRP exhibited a log reduction of 0.84, while faba bean MRP showed a log reduction of 0.77.

Lactococcus lactis displayed the highest sensitivity to the MRPs, with chickpea MRP achieving a log reduction of 2.67 and faba bean MRP showing a log reduction of 2.43. For *Saccharomyces cerevisiae var. boulardii*, chickpea MRP resulted in a log reduction of 1.52, whereas faba bean MRP achieved a log reduction of 1.26. In the case of *Saccharomyces cerevisiae*, the chickpea MRP had a log reduction of 1.47, while the faba bean MRP showed a log reduction of 1.75.

Candida albicans was also effectively inhibited by the MRPs, with chickpea MRP achieving the highest log reduction of 2.92, reducing the final concentration from an initial 1.4 x 10^3 CFU/mL to 1.1 x 10^5 CFU/mL. Faba bean MRP showed a log reduction of 1.88 against C. albicans.

Table 6. Antimicrobial activity of Chickpea hydrolysate – glucose and Faba bean hydrolysate – glucose MRPs.

	Microorganisms	Initial Concentration	Final Concentration	Log reduction
Control	*Escherichia coli*	2.5 x 10^6 CFU/mL	2.95 x 10^9 CFU/mL	
Faba Bean 175°C		2.5 x 10^6 CFU/mL	4.73 x 10^8 CFU/mL	0.77 ± 0.035 ^{A}
Chickpea 175°C		2.5 x 10^6 CFU/mL	3.02 x 10^8 CFU/mL	0.84 ± 0.034 ^{B}

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Table 6 (cont.)

Control	*Staphylococcus aureus*	3.7 x 10^6 CFU/mL	1.97 x 10^8 CFU/mL	
Faba Bean 175°C		3.7 x 10^6 CFU/mL	3.34 x 10^7 CFU/mL	0.79 ± 0.095 ^{A}
Chickpea 175°C		3.7 x 10^6 CFU/mL	2.84 x 10^7 CFU/mL	0.98 ± 0.097 ^{C}
Control	*Lactococcus lactis*	2.5 x 10^4 CFU/mL	9.5 x 10^7 CFU/mL	
Faba Bean 175°C		2.5 x 10^4 CFU/mL	3.5 x 10^5 CFU/mL	2.43 ± 0.120 ^{D}
Chickpea 175°C		2.5 x 10^4 CFU/mL	2 x 10^5 CFU/mL	2.67 ± 0.130 ^{D}
Control	*Saccharomyces cerevisiae* var. *boulardii*	1.8 x 10^3 CFU/mL	4.78 x 10^7 CFU/mL	
Faba Bean 175°C		1.8 x 10^3 CFU/mL	2.6 x 10^6 CFU/mL	1.26 ± 0.130 ^{E}
Chickpea 175°C		1.8 x 10^3 CFU/mL	1.43 x 10^5 CFU/mL	1.52 ± 0.150 ^{E}
Control	*Saccharomyces cerevisiae*	1.2 x 10^3 CFU/mL	1.7 x 10^7 CFU/mL	
Faba Bean 175°C		1.2 x 10^3 CFU/mL	2.75 x 10^5 CFU/mL	1.75 ± 0.140 ^{F}
Chickpea 175°C		1.2 x 10^3 CFU/mL	5.7 x 10^5 CFU/mL	1.47 ± 0.130 ^{E}
Control	*Candida albicans*	1.4 x 10^3 CFU/mL	9.2 x 10^7 CFU/mL	
Faba Bean 175°C		1.4 x 10^3 CFU/mL	1.19 x 10^6 CFU/mL	1.88 ± 0.52 ^{F}
Chickpea 175°C		1.4 x 10^3 CFU/mL	1.1 x 10^5 CFU/mL	2.92 ± 0.48 ^{D}

Notes: Results are shown as means ± standard deviation, columns with different uppercase letters differ statistically ($p \leq 0.05$).

These results demonstrate that chickpea MRPs generally exhibit higher antimicrobial activity than faba bean MRPs across the tested microorganisms. The significant log reductions, particularly against *Lactococcus lactis* and *Candida albicans*, suggest the potential application of chickpea MRPs as effective natural antimicrobial agents in food preservation and safety.

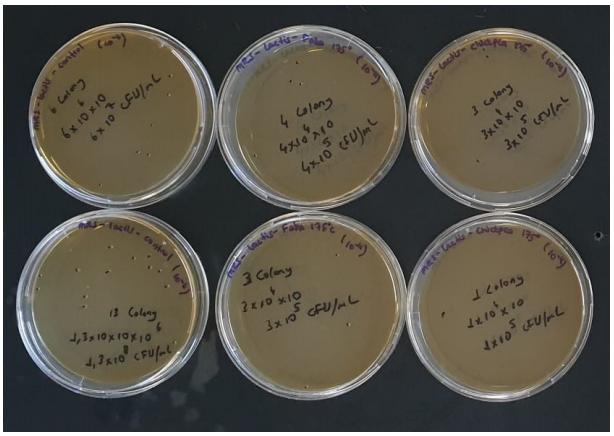

Figure 9. *L.lactis* and Chickpea Hydrolysate and Faba bean Hydrolysate MRP samples colony count.

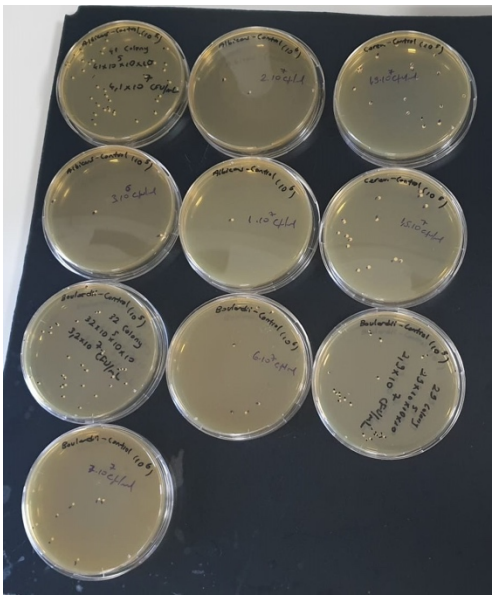

Figure 10. *C.albicans, S.cerevisiae,* and *S.boulardii* control groups' colony count.

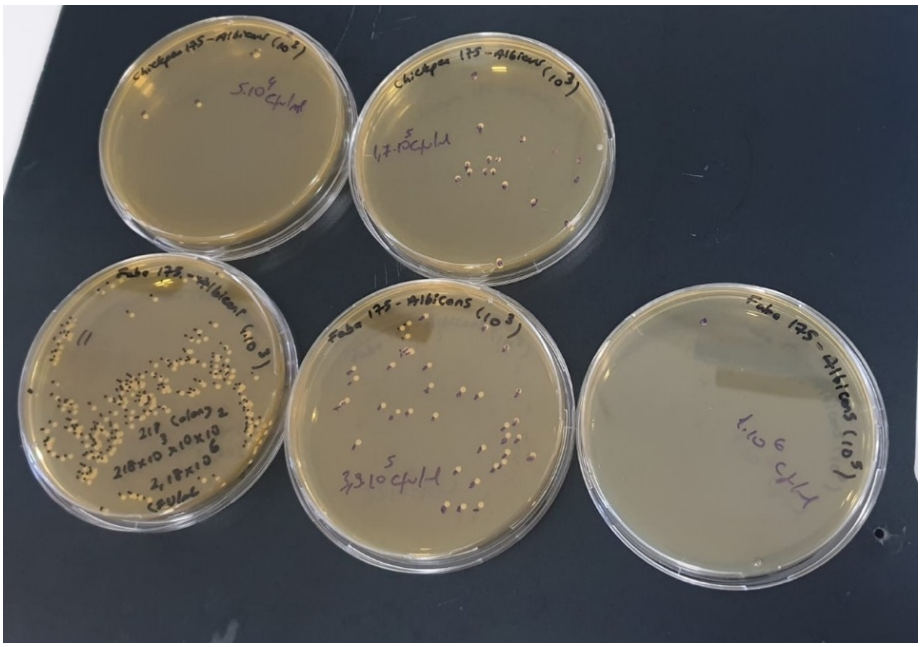

Figure 11. *C.albicans* - Chickpea hydrolysate MRP and Faba bean hydrolysate MRP colony count.

Figure 12. *S. cerevisiae*- Chickpea hydrolysate MRP and Faba bean Hydrolysate MRP colony count.

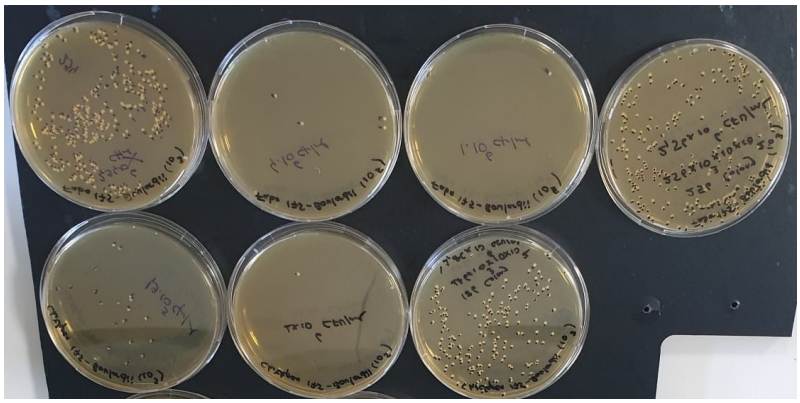

Figure 13. *S. boulardii*- Chickpea hydrolysate MRP and Faba bean hydrolysate MRP colony count.

3.6. Well Diffusion Antimicrobial Test

This lack of zone formation indicates that both Chickpea-D-glucose MRPs and Broad Bean-D-glucose MRPs did not exhibit detectable antimicrobial activity against the selected bacterial and yeast strains under the conditions tested.

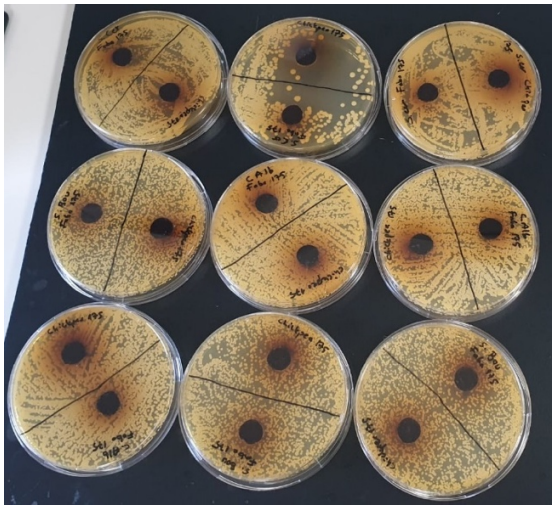

Figure 14. Well diffusion antimicrobial activity of Faba bean hydrolysate - glucose MRP and Chickpea hydrolysate - glucose MRP on *C.albicans, S. cerevisiae* var. *boulardii,* and *S. cerevisiae.*

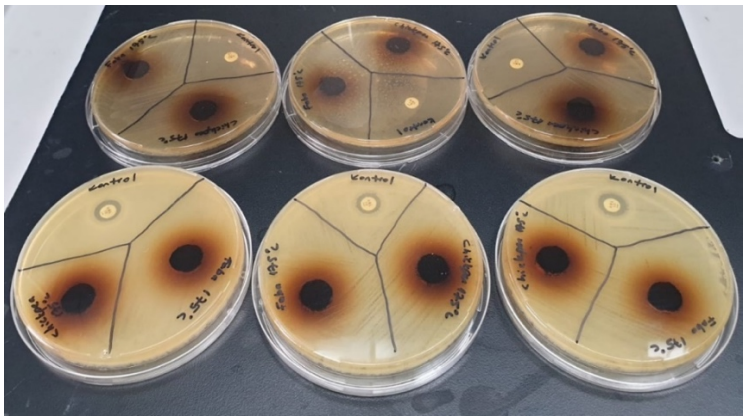

Figure 15. Well diffusion antimicrobial activity of Faba bean hydrolysate - glucose MRP and Chickpea hydrolysate - glucose MRP on *L.lactis* and *S. aureus.*

3.7. HPLC Analysis

HPLC chromatograms of chickpea-MRP samples shown in Figure 16 and faba bean-MRP sample shown in Figure 17. For the chickpea-MRP samples, significant peaks were observed at retention times of approximately 2.5, 4.25, 5.6, 6.0, 7.5, and 9.75 minutes. The most prominent peak appeared at 7.5 minutes, followed by another major peak at 9.75 minutes. The faba bean-MRP samples exhibited peaks at retention times of 2.5, 4.25, 7.5, and 9.5 minutes, with the largest peak occurring at 9.5 minutes, which was slightly different from the chickpea-MRP profile. These differences in retention times and peak intensities suggest variations in the melanoidin composition between chickpea and faba bean MRPs, indicating that faba bean MRPs might contain a higher concentration or diversity of melanoidins.

Comparatively, standard compounds in Maillard browning, such as furans, furanone, pyrroles, and pyrazines, have known retention times and maximum absorbance wavelengths (λmax) that can be used as references for identifying the compounds present in MRPs (Bailey et al., 1996; Tressl et al., 1995). The study by Bailey et al. (1996) further classifies the HPLC-DAD chromatographic behavior of Maillard reaction products into unretained peaks, convex broad bands, tailing broad bands, and resolved peaks. The observed chromatograms in our analysis show resolved peaks and some broad bands, suggesting the presence of high molecular weight melanoidin polymers.

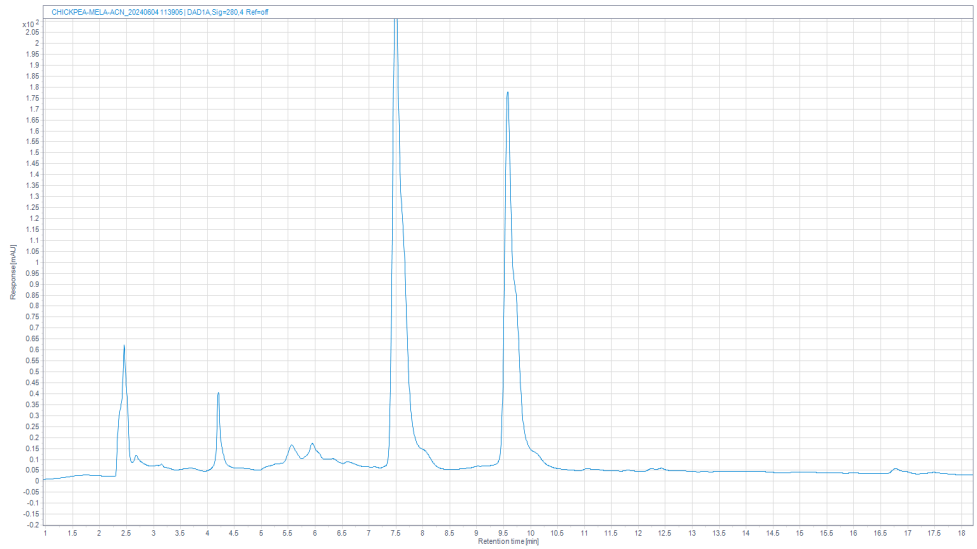

Figure 16. HPLC chromatogram of chickpea-MRP sample.

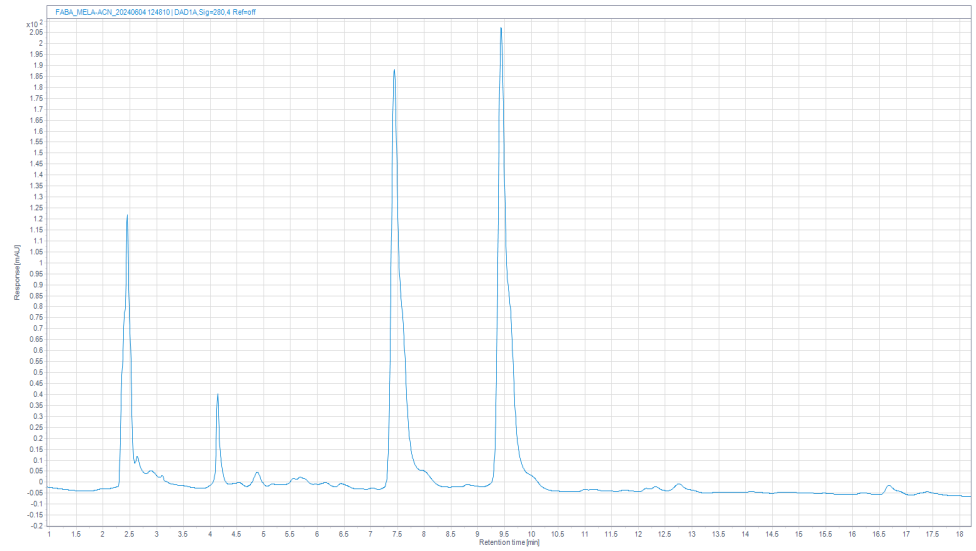

Figure 17. HPLC chromatogram of faba bean-MRP sample.

Our findings align with previous studies indicating the formation of complex mixtures of melanoidin compounds during the Maillard reaction. For instance, the major resolved peak at approximately 10 minutes in both aqueous and ethanolic solutions reported by Bailey et al. (1996) corresponds to the peaks observed in our analysis,

suggesting similar reaction pathways and compound formations. The presence of these compounds, particularly the high molecular weight melanoidin polymers, underscores the potential functional properties of chickpea and faba bean MRPs in food applications, contributing to enhanced color, flavor, and possibly antioxidant activities.

3.8. UV-Vis Spectrophotometer

The results can be seen in Figure 18, revealing that both faba bean-MRP and chickpea-MRP exhibited similar absorption peaks, with the highest peak observed at approximately 285 nm. However, the absorbance intensity of faba bean-MRP was notably higher than that of chickpea-MRP, indicating a greater concentration of Maillard reaction products in the faba bean samples. The calculated melanoidin concentrations from the standard curve were 5.1 mg/mL for faba bean-MRP and 4.2 mg/mL for chickpea-MRP, demonstrating that faba bean hydrolysates produce a higher yield of melanoidins than chickpea hydrolysates.

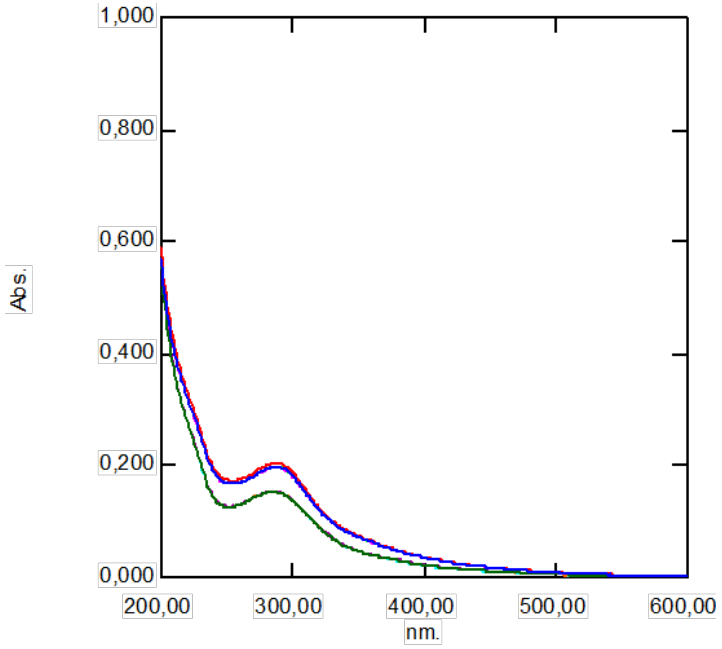

Figure 18. UV-Vis results of Maillard reaction products (Blue peak: Faba bean-MRP, Black peak: Chickpea-MRP).

These findings are consistent with those reported in the literature. For instance, the UV-Vis spectra of MRPs from various sugar-amino acid models typically show absorption peaks around 265 nm and 285 nm, which are indicative of melanoidin formation (Yu et al., 2012). Moreover, it has been observed that MRPs from hexoses (such as glucose and fructose) display a broad absorption peak around 265 nm, whereas MRPs from pentoses (like ribose and xylose) exhibit sharper peaks at the same wavelength, often with significantly higher absorbance (Hwang et al., 2011).

The comparative analysis with literature highlights the distinct UV-Vis spectral patterns associated with different sugar and amino acid combinations, underscoring the variability in MRP composition based on the reactants. In our study, the pronounced peak at 285 nm and the higher absorbance in faba bean-MRP suggest a more extensive Maillard reaction, possibly due to the presence of more reactive amino acids or higher initial sugar concentrations in faba beans compared to chickpeas.

3.9. FTIR Analyzes

FTIR spectroscopy was used as the method of choice for analyzing the MRPs at the functional group level. The results can be seen in Figures 19, 20, 21 and 22. The whole spectra between the wavenumbers of 4000 and 400 cm^{-1} showed that the chickpea protein MRPs underwent structural changes before 175 C^o as seen in Figure 19. As the temperature rose to 275 C^o, the structural changes became more apparent. However, the structural changes were induced for the 250 C^o and 275 C^o applications for the common bean samples due to the changes in the overall shapes of the amide I and amid II bands between 1700 cm^{-1} and 1480 cm^{-1} as seen in Figure 20. Similar results were observed for the faba bean protein hydrolysates in Figure 21 and for the soybean protein hydrolysates in Figure 22. If the spectra were investigated for different spectral regions, the changes were apparent in the aliphatic region between 3000 cm^{-1} and 2800 cm^{-1}. Increasing the temperature weakened the bands at 2927 cm-1 for the 250 C^o and 275 C^o applications in the chickpea MRP samples. In addition, the bands at 2981 cm^{-1} and 2972 cm^{-1}. The band at 2855 cm^{-1} weakened also.

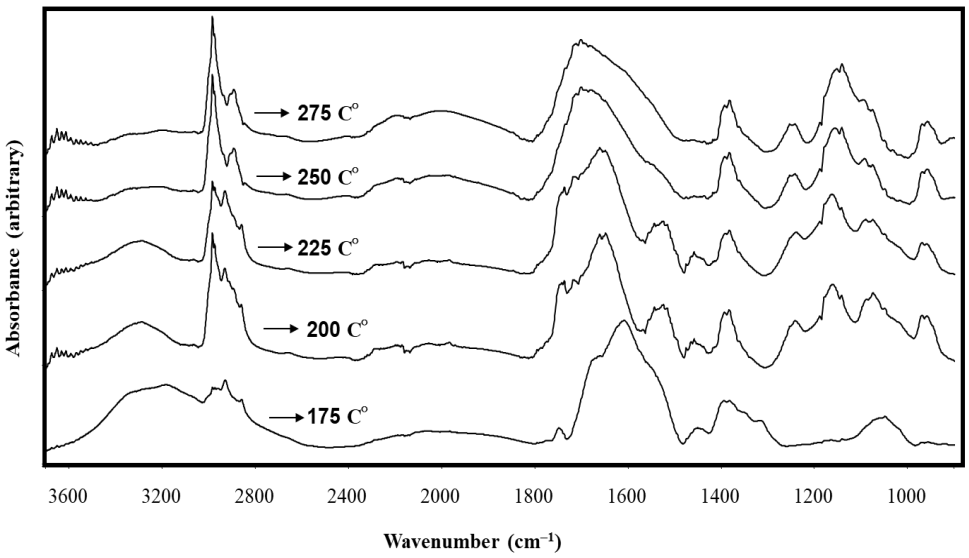

Figure 19. Chickpea protein hydrolysate - glucose MRP at different temperatures.

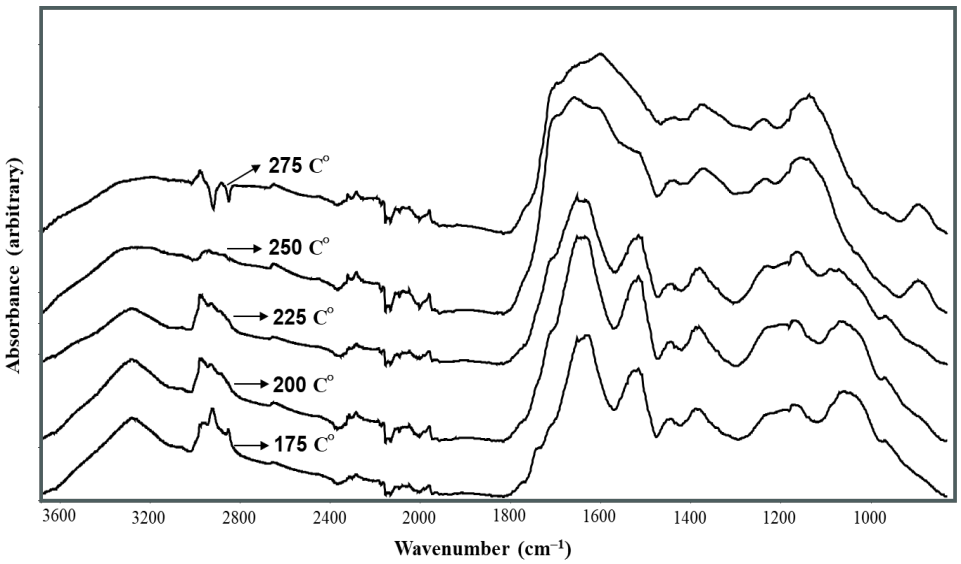

Figure 20. Common bean protein hydrolysate - glucose MRP at different temperatures.

In the common bean MRP samples, the amid A band between 3700 cm^{-1} and 3000 cm^{-1} disappeared gradually with the increasing temperature, indicating the loss of C-O

stretching vibrations in the protein structure. The same is true for the faba bean MRP samples in Figure 21 and the soybean MRP samples in Figure 22.

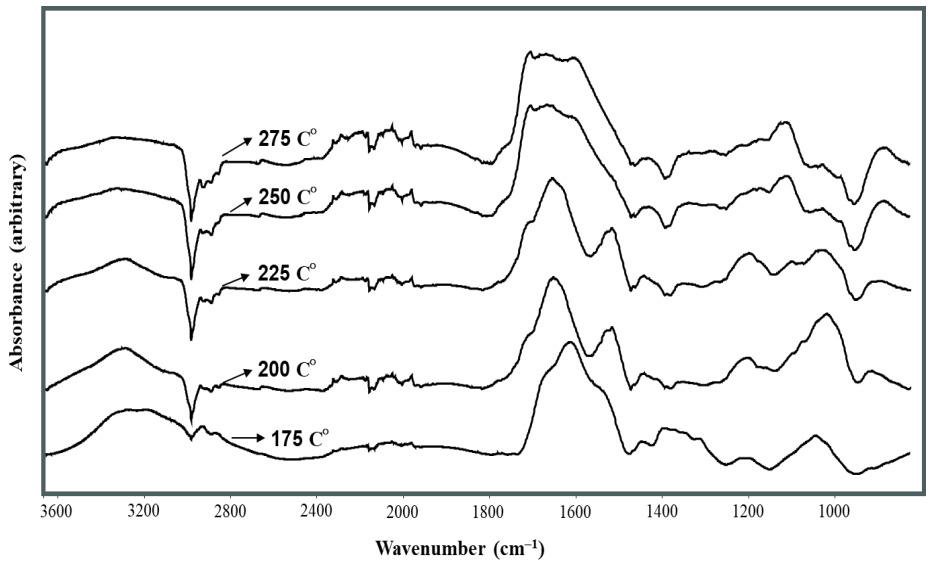

Figure 21. Faba bean protein hydrolysate - glucose MRP at different temperatures.

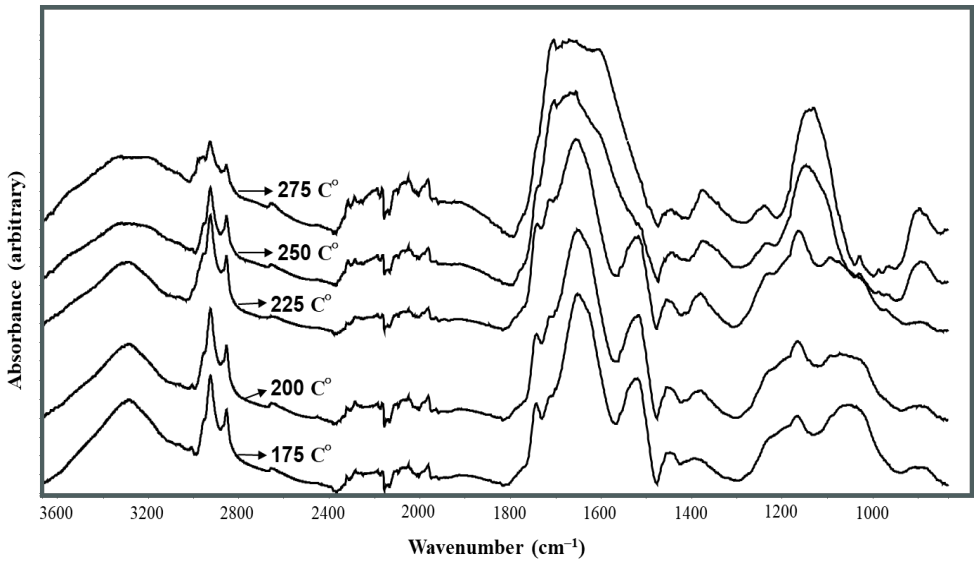

Figure 22. Soybean protein hydrolysate - glucose MRP at different temperatures.

Important changes were also observed in the carbonyl compounds. In the chickpea MRP samples, the carbonyl band at 1744 cm-1 disappeared for the 250 C° and 275 C° samples, indicating the loss of the stability of these compounds with the increasing temperature. The same is true for the soybean and common bean MRP samples.

The changes in the fingerprint region of the FTIR spectra were also observed, such as the CH_3 and CH_2 bands at around 1457 cm^{-1} and 1380 cm^{-1}, respectively. These bands are the side chain vibration bands of the amino acids. The band at 1380 cm^{-1} disappeared, indicating the changes in the amino acid level at elevated temperature levels. However, some bands appeared, such as the one at 1238 cm^{-1} in the chickpea and soybean MRP samples and the band at 1138 cm^{-1} for the common bean MRP samples. In addition, some extra bands appeared with the application of elevated temperatures in the soybean MRP samples, such as the ones at 1162 cm^{-1}, 1030 $cm^{-1,}$ and 898 cm^{-1}.

MRPs are important antimicrobials. Therefore, experiments were designed to investigate the effect of these MRPs on the cellular macromolecules and, hence, their structure and composition. The effect of chickpea protein hydrolysate on *C. albican*s as an important human pathogen in Figure 23.

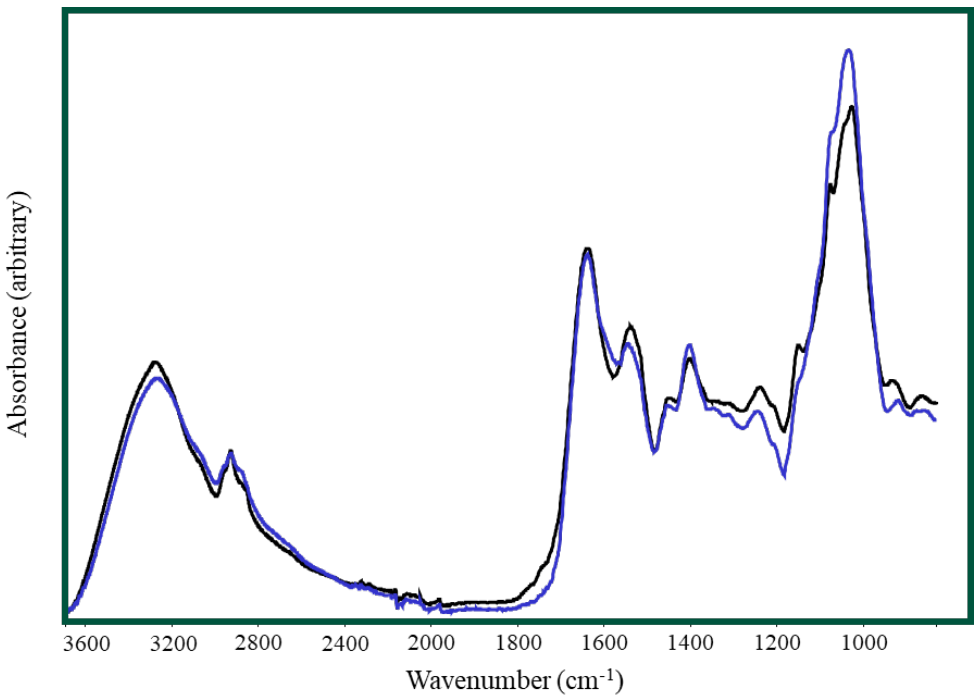

Figure 23. Blue peak is control (only *C.albicans*), Black peak is *C.albicans* treated with Chickpea MRP.

When the spectral regions were analyzed individually, it can be said that chickpea MRP increased the lipid/ protein ratio, as seen in Figure 24. In addition, the changes in the cellular proteome were apparent based on the results obtained in the amide I and amid II bands, as seen in Figure 25. In the fingerprint region, *C. albicans* cells treated with chickpea MRPs, the band at 1030 cm^{-1} showed decreases when the bands in this region were normalized with respect to the PO_2^{-2} asymmetric stretching band as seen in Figure 26, which might be due to the decreases in the synthesis of glycogen stores within the cellular cytoplasm.

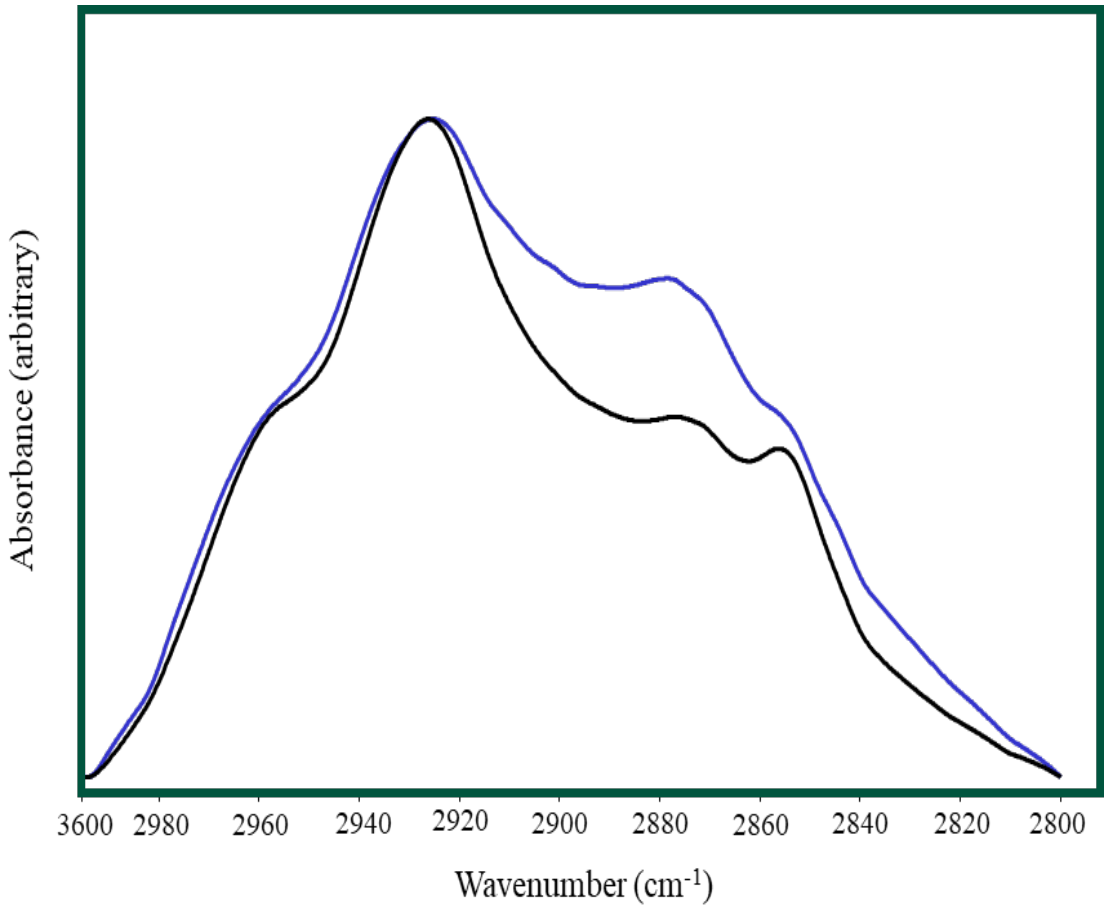

Figure 24. Blue peak is control (only *C.albicans*), Black peak is *C.albicans* treated with Chickpea MRP.

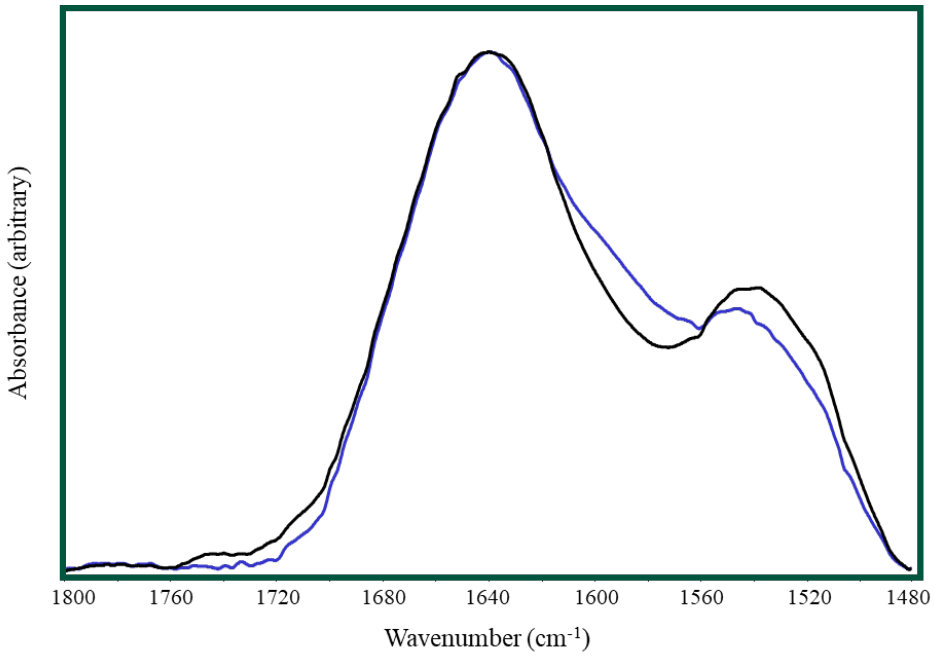

Figure 25. Blue peak is control (only *C.albicans*), Black peak is *C.albicans* treated with Chickpea MRP.

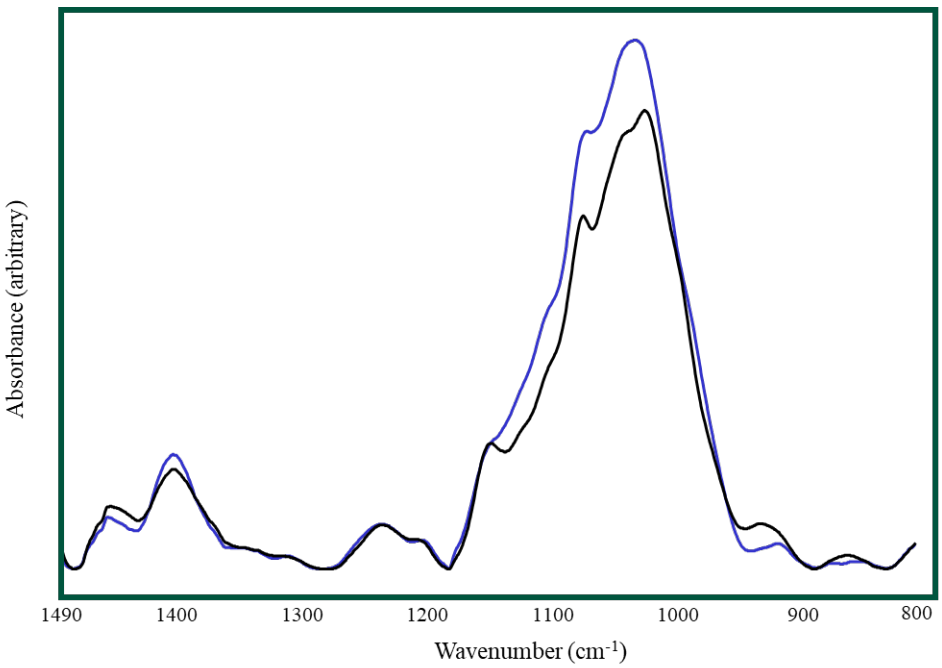

Figure 26. Black peak is *C.albicans* and Blue peak is *C.albicans* treated with Chickpea MRP.

The effect of chickpea protein hydrolysate on *L. lactis* as an important procaryote is presented in Figure 27, 28, 29, and 30. In Figure 27 the effect of chickpea hydrolysate MRP on *L. lactis* cells was investigated in the aliphatic spectral region. In this region, CH_3 asymmetric stretching at around 2960 cm^{-1} decreased when normalized with respect to CH_2 asymmetric stretching vibration. This corresponds to an increase in the chain length of the aliphatic chains in the cellular membranes due to the increased actions of elongates because of the cellular-physiological stress due to the presence of the chickpea MRPs. This indicates another mode of action of this protein-hydrolysate-based MRP.

The basic mode of action of melanoidins is due to their iron-quenching capacities. However, our results indicated a clear change in the cell membrane physiology. The effect of chickpea protein hydrolysate MRPs on protein metabolism and content was investigated in Figures 28 and 29. The amide A band in Figure 28 and the amide I and II bands did not show significant changes. Therefore, we concluded that the chickpea hydrolysates did not induce changes in the cellular protein physiology considerably. However, the same is not true for the fingerprint region. The PO_2^{-2} symmetric stretching vibration downshifted to lower band numbers upon treatment with the chickpea protein hydrolysate-based MRPs, indicating changes in the structure and coiling of the bacterial nucleic acids, as seen in Figure 30.

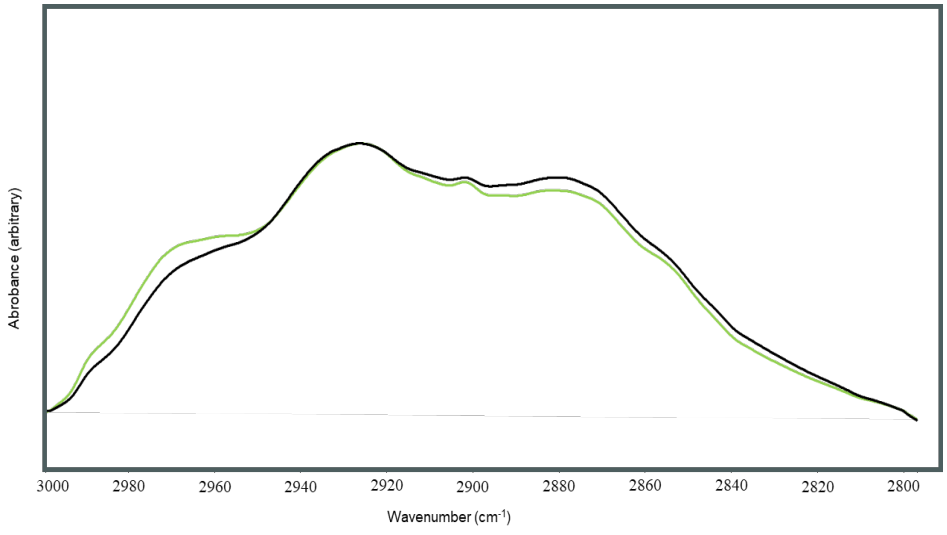

Figure 27. Green peak is control (*L.lactis* only), Black peak is *L.lactis* treated with Chickpea MRP.

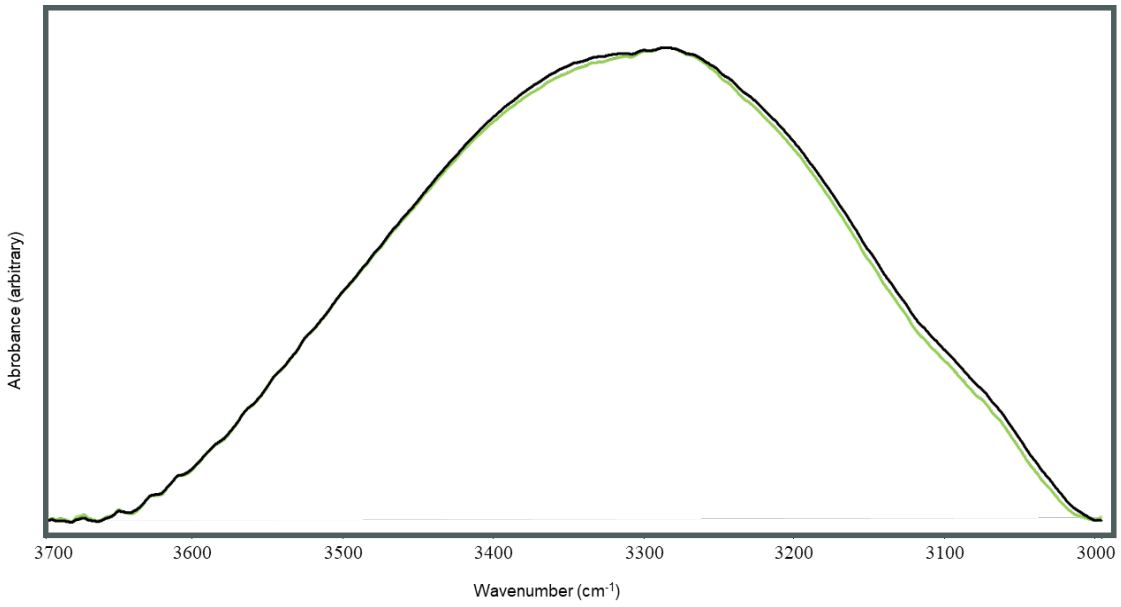

Figure 28. Green peak is control (only *L.lactis*), Black peak is *L.lactis* treated with Chickpea MRP.

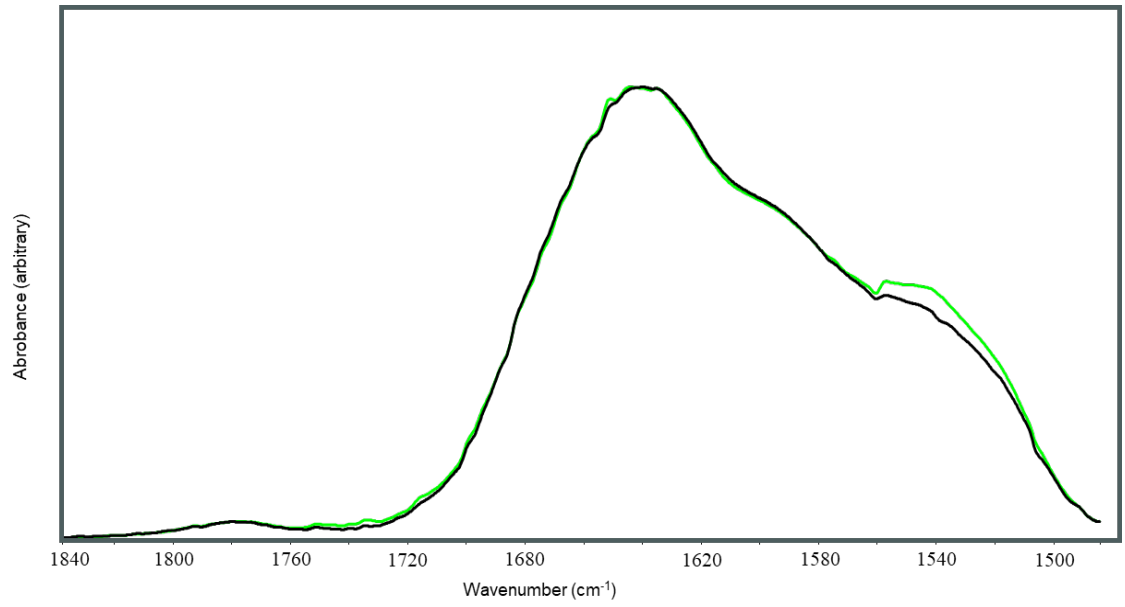

Figure 29. Green peak is control (only *L.lactis*), Black peak is *L.lactis* treated with Chickpea MRP.

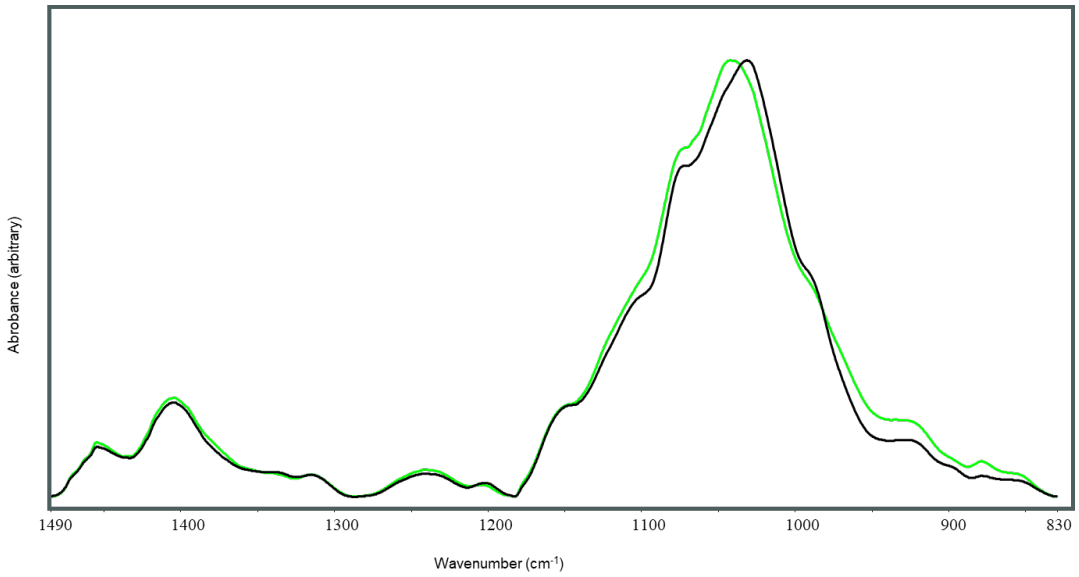

Figure 30. Green peak is control (only *L.lactis*), Black peak is *L.lactis* treated with Chickpea MRP.

3.10. ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum of the faba bean-MRP exhibited several distinct peaks. The peak at δ 2.0 ppm was attributed to the methyl protons of N-acetyl glucosamine (HN-$COCH_3$), indicating the presence of this amino sugar derivative. Additional peaks at δ 1.86 ppm (dimethyl-formamide, CH_3), δ 1.21 ppm (tert-butyl methyl ether, CCH_3), and δ 1.23 ppm (n-pentane, CH_2) were also observed, suggesting the incorporation of various aliphatic compounds. The region between δ 3.0 to 4.0 ppm displayed multiple peaks, indicative of glucose moieties, which is consistent with the formation of sugar derivatives during the Maillard reaction. The largest peak observed was due to D_2O is the solvent used in the analysis.

Similarly, the ^{1}H-NMR spectrum of the chickpea-MRP revealed comparable structural features. The peaks at δ 2.0 ppm (methyl protons of N-acetyl glucosamine, HN-$COCH_3$), δ 1.86 ppm (dimethyl-formamide, CH_3), and the region between δ 3.0 to 4.0 ppm (glucose moieties) were also present, highlighting the similarities in the chemical composition of MRPs derived from different legume sources. The largest peak, as expected, corresponded to D_2O.

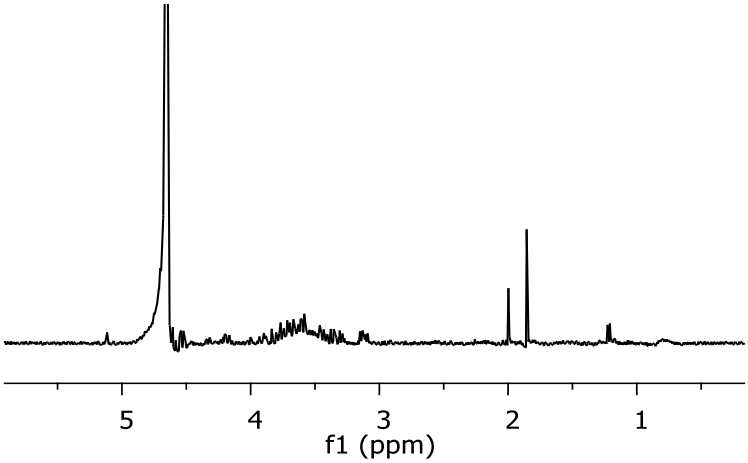

Figure 31. ^{1}H-NMR result of Faba bean MRP.

These findings align with previous studies, such as those by Arata Badano et al. (2019) and Kasaai (2010), which characterized similar peaks in the NMR spectra of chitosan derivatives. The consistent observation of peaks corresponding to N-acetyl glucosamine and glucose moieties in both faba bean and chickpea MRPs underscores the involvement of these components in the Maillard reaction, contributing to the formation of complex melanoidin structures.

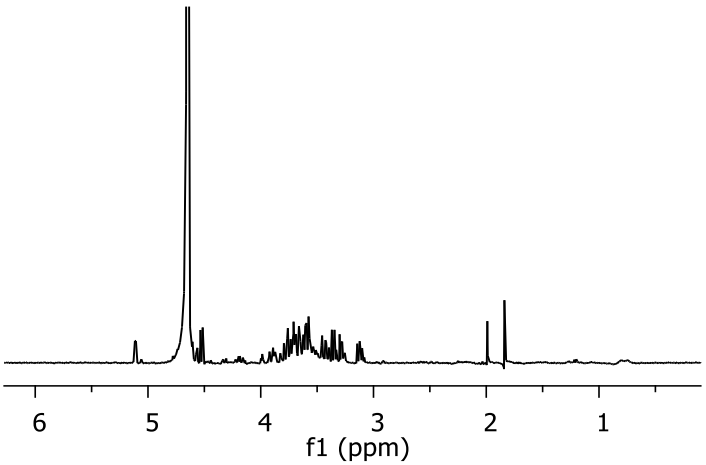

Figure 32. ^{1}H -NMR result of Chickpea MRP.

3.11. Discussion

The chemical composition analysis of legume protein isolates in this study demonstrated significant changes from their respective pretreatment states, highlighting the efficiency of the alkaline extraction-acid precipitation method in producing high-protein isolates. The protein content of chickpea, faba bean, common bean, and soybean isolates significantly increased to 90.297 ± 0.531%, 91.621 ± 0.348%, 89.273 ± 0.327%, and 91.884 ± 0.259%, respectively, compared to their pretreatment values of 20.497 ± 0.855%, 22.283 ± 0.384%, 19.243 ± 0.437%, and 22.747 ± 0.372%. This significant increase in protein content is consistent with findings from other studies, where protein levels of legume protein isolates prepared by isoelectric precipitation ranged from 80.8% to 97.1% on a dry weight basis (Karaca et al., 2011; Sánchez-Vioque et al., 1999).

Carbohydrate content in the protein isolates was notably reduced, with chickpea protein isolate containing 5.308 ± 0.570%, faba bean 5.367 ± 0.752%, common bean 7.113 ± 0.844%, and soybean 3.407 ± 0.429%, compared to their pretreatment levels of 45.510 ± 0.501%, 46.601 ± 0.209%, 47.743 ± 0.836%, and 40.893 ± 0.713%. These findings align with the results reported by Du et al. (2014) and Santiago-Ramos et al. (2018), where carbohydrate contents in bean flour varied between 60.1% and 64.2%.

The fat content of the legume protein isolates was also significantly lower than in their pretreatment states. Chickpea, faba bean, common bean, and soybean isolates exhibited fat contents of 2.730 ± 0.229%, 1.420 ± 0.437%, 1.517 ± 0.401%, and 3.427 ± 0.117%, respectively, compared to their respective pretreatment values of 18.707 ± 0.741%, 16.183 ± 0.350%, 16.937 ± 0.502%, and 20.997 ± 0.435%. This reduction is consistent with the findings of other studies where defatting procedures resulted in low lipid levels in protein isolates, typically less than 1% (Leyva-Lopez et al., 1995; Du et al., 2014).

Ash content was measured following the AOCS Official Method, and a decrease in the protein isolates was observed compared to their pretreatment samples. Chickpea protein isolate exhibited an ash content of 1.697 ± 0.215%, faba bean 1.593 ± 0.102%, common bean 2.487 ± 0.199%, and soybean 1.287 ± 0.191%, while their pretreatment ash contents were 15.287 ± 0.475%, 14.933 ± 0.427%, 16.077 ± 0.450%, and 15.457 ± 0.189%, respectively. These values are comparable to those reported for protein isolates

from mung bean, black bean, and black gram bean, which ranged from 2.2% to 4.0% (Wani et al., 2015; Kudre et al., 2013).

The substantial increase in protein content and reduction in carbohydrate, fat, and ash contents highlight the effectiveness of the alkaline extraction-acid precipitation method in isolating high-purity proteins. These protein isolates hold significant potential for application in food formulations where high protein content is desirable, such as in protein supplements and nutraceutical products. The reduced carbohydrate and fat contents make these isolates suitable for inclusion in low-carb and low-fat dietary products. This study's findings align with existing literature, affirming the robustness and applicability of the extraction methods employed (Can Karaca et al., 2011; Sánchez-Vioque et al., 1999; Du et al., 2014).

The enzymatic hydrolysis of legume proteins using Alcalase enzyme revealed significant insights into the degree of hydrolysis (DH) over time for chickpea, faba bean, common bean, and soybean protein isolates. The results demonstrated a progressive increase in DH with extended hydrolysis times across all legume samples. Initially, at 30 minutes, the DH values were relatively low, with chickpea showing 7.24 ± 0.44%, faba bean 6.83 ± 0.29%, common bean 5.25 ± 0.35%, and soybean 5.27 ± 0.27%. However, as hydrolysis proceeded, these values increased significantly, reaching 26.41 ± 0.47% for chickpea, 22.50 ± 0.41% for faba bean, 20.00 ± 0.47% for common bean, and 19.62 ± 0.35% for soybean after 180 minutes. This pattern of increasing DH indicates the effective enzymatic breakdown of protein structures, releasing α-amino groups.

These findings align with the observations of Ghribi et al. (2015), who reported a similar sigmoidal response in DH for chickpea proteins hydrolyzed with Alcalase, attributing this to enzyme inhibition and inaccessibility of cleavage sites. Meinlschmidt et al. (2015) also found a DH of 13.6% for soy protein hydrolyzed with Alcalase after 120 minutes, which is within the range observed in our study for soybean at 19.62 ± 0.35% after 180 minutes. Nguyen et al. (2016) reported a much higher DH of around 50% using a higher ratio of Alcalase to soy protein, indicating that enzyme concentration significantly impacts the hydrolysis efficiency.

The results of our study also resonate with those of del Mar Yust et al. (2013), who showed a more linear DH response over time for chickpea protein hydrolyzed with immobilized Flavourzyme, reaching a maximum of 10% DH after 300 minutes. The lower DH in their study is likely due to the lower enzyme-to-substrate ratios used. Additionally, the SDS-PAGE analysis by Warsame et al. (2020) demonstrated the

reduction of protein molecular weights with increased hydrolysis time, which corresponds with the increasing DH observed in our study. The SDS-PAGE patterns revealed that Alcalase effectively cleaved proteins in FBPC (faba bean protein concentrate), resulting in protein bands below 15 kDa with prolonged hydrolysis times.

The physicochemical properties of the hydrolysates also reflected the impact of enzymatic hydrolysis. The solubility of faba bean protein concentrates increased with higher DH, achieving the highest solubility at DH (35 min) (Akbari et al., 2020; Pan et al., 2019). This increased solubility is attributed to the formation of smaller peptides that form stronger hydrogen bonds with water. The increased electronegativity, as indicated by the ζ-potential, was due to the exposure of more ionizable amino acids and the release of carboxylate ions (COO^-), which enhanced protein solubility due to higher repulsive electrostatic forces between molecules (Eckert et al., 2019). Additionally, prolonged hydrolysis significantly reduced surface hydrophobicity, likely due to the cleavage of hydrophobic areas and refolding of exposed residues (Zang et al., 2019).

Overall, the results of our study are consistent with those reported in the literature, affirming the robustness of Alcalase in hydrolyzing legume proteins and enhancing their functional properties. The observed increases in DH, and the corresponding changes in solubility, electronegativity, and surface hydrophobicity underscore the potential applications of these hydrolysates in food formulations where enhanced solubility and reduced hydrophobicity are desirable. These findings contribute to the growing body of knowledge on the enzymatic hydrolysis of legume proteins and their potential applications in food science and technology.

These results of color determination of Maillard reactions align with findings from other studies, such as those by Chiang et al. (2020) and Song et al. (2020), which highlighted the impact of the Maillard Reaction on color and flavor development in food products. The Maillard Reaction produces a wide range of sensory properties, including flavor, color, and odor, which are critical in the food industry for enhancing the functional properties of amino acids, peptides, and proteins. Chen et al. (2020) also demonstrated how the Maillard Reaction could improve the overall flavor by reducing off-flavor compounds and increasing desirable aroma compounds.

The observed color changes in MRPs indicate the complex reactions taking place during the Maillard Reaction. As temperature increases, the extent of browning and the formation of colored compounds such as melanoidins also increase, which are responsible for the darker and more intense colors observed. This is consistent with the findings of

Meinlschmidt et al. (2015) and Nguyen et al. (2016), who reported similar trends in the degree of hydrolysis and color development in protein hydrolysates.

The variability in color parameters among different legume MRPs suggests that the type of legume and the specific conditions of the Maillard Reaction significantly influence the outcome. This highlights the importance of optimizing reaction conditions to achieve desired sensory attributes in food products. The differences in L*, a*, and b* values also reflect the diversity of chemical reactions, including forming various pigments and browning compounds.

Antimicrobial activity findings align with the results of other studies, such as the work by Meinlschmidt et al. (2015) and Nguyen et al. (2016), which reported varying degrees of antimicrobial activity for MRPs derived from different protein sources. For example, sunflower MRPs (SF-MRPs) demonstrated significant antimicrobial effects against *Escherichia coli* and *Staphylococcus aureus*, with minimum inhibitory concentrations (MICs) of 86 mg/mL and 70 mg/mL, respectively. In contrast, soybean and corn meal MRPs (SB-MRPs and CN-MRPs) did not exhibit significant antimicrobial activity, highlighting the variability in antimicrobial efficacy depending on the source of the MRPs.

Rufián-Henares and de la Cueva (2009) study further supports these findings, demonstrating that MRPs can disrupt bacterial cell membranes by chelating Mg^{2+} ions, leading to destabilization and cell death. This mechanism of action, combined with the observed antimicrobial effects, suggests that MRPs have the potential to serve as natural preservatives in various food systems.

Additionally, the study by Monente et al. (2015) highlighted the role of ultrasonication in enhancing the antimicrobial properties of MRPs by extending protein molecules to form stable network structures that adsorb on the surface of microbial cells, forming a polymer membrane that prevents nutrient transport. This method could be further explored to enhance the efficacy of chickpea and faba bean MRPs.

The antimicrobial activity of MRPs derived from chickpea and faba bean hydrolysates demonstrates their potential as effective natural antimicrobial agents. The significant log reductions in microbial populations, particularly for *Lactococcus lactis* and *Candida albicans*, highlight the potential applications of these MRPs in food preservation. Further research and optimization of MRP production processes could enhance their antimicrobial efficacy and expand their use in the food industry. These findings contribute to the growing body of evidence supporting the use of MRPs as

functional food ingredients with antimicrobial properties, providing a natural alternative to synthetic preservatives.

This observation aligns with findings from other studies that have used UV-Vis spectroscopy to analyze MRPs. In a study investigating the UV-Vis spectra of MRPs derived from asparagine and sugars, distinct absorption maxima were observed at 234, 277, 294, and 307 nm in different fractions (Iannou et al., 2007). The changes in absorbance at 294 nm were particularly noted as a measure of intermediate products of the Maillard reaction, reflecting the presence of uncolored intermediate products (UIPs) such as aldehydes and small molecule ketones. The detection of advance stage soluble pre-melanoidins in the range of 320-350 nm and high molecular weight melanoidins in the range of 420-450 nm, although not observed in this study, is a common characteristic of MRPs in other model systems .

The higher reactivity of fructose compared to glucose in Maillard reactions, as observed in the asparagine-sugar model systems, may provide insights into the reactivity differences between Faba bean and chickpea hydrolysates with D-glucose. Fructose has a higher proportion of open-chain form than glucose, leading to more pronounced changes in absorption maxima and more reactive Maillard products. This principle can be extrapolated to the current study, suggesting that the chemical nature and structural composition of Faba bean hydrolysate make it more reactive with glucose, resulting in higher MRP concentrations and stronger UV-Vis absorption peaks.

The ^{1}H-NMR analysis provided additional molecular-level details, revealing distinct proton signals corresponding to various functional groups. Both the faba bean and chickpea MRP spectra displayed significant peaks in the aromatic region (δ 6.0-8.0 ppm), indicative of aromatic compounds formed during the Maillard reaction. The aliphatic region (δ 0.5-4.0 ppm) showed signals corresponding to aliphatic chains and sugar moieties, suggesting the presence of complex mixtures of melanoidin compounds. These findings highlight the structural diversity and complexity of MRPs, which include various aromatic compounds and sugar derivatives.

CHAPTER 4

CONCLUSION

This study has successfully demonstrated the significant antimicrobial properties and biochemical characteristics of Maillard Reaction Products (MRPs) derived from legume protein hydrolysates reacted with D-glucose. The investigation focused on MRPs from chickpea, faba bean, common bean, and soybean, subjected to various thermal conditions ranging from 175°C to 275°C.

The antimicrobial activity assays revealed that chickpea MRPs exhibited the highest antimicrobial efficacy, followed by faba bean MRPs. The study showed significant reductions in the counts of *Escherichia coli, Staphylococcus aureus, Lactococcus lactis, Candida albicans, Saccharomyces cerevisiae var. boulardii,* and *Saccharomyces cerevisiae* upon treatment with these MRPs. Notably, the antimicrobial efficacy was found to vary with the processing temperatures, indicating that specific thermal conditions optimize the antimicrobial activity of the MRPs. This finding underscores the importance of fine-tuning processing parameters to maximize the antimicrobial potential of MRPs.

Biochemical characterization of the MRPs through UV-Vis spectroscopy, FTIR, HPLC, and NMR analyses revealed distinct differences in melanoidin content and structural properties based on the legume source and thermal treatment. The UV-Vis spectra indicated variations in absorbance patterns, corresponding to differences in melanoidin concentration. FTIR analysis provided insights into the functional groups present in the MRPs, highlighting the complexity and diversity of the chemical structures formed during the Maillard reaction. HPLC and NMR analyses further elucidated the molecular composition and structural intricacies of the MRPs, offering a comprehensive understanding of their biochemical properties.

Colorimetric analysis and browning index determination demonstrated that higher processing temperatures resulted in darker MRPs with increased browning indices. This correlation between temperature and browning suggests that thermal treatment not only

the antimicrobial properties but also the visual and sensory attributes of the MRPs. The darker color and higher browning index indicate more extensive Maillard reactions associated with enhanced antimicrobial activity.

The findings of this study suggest that legume-based MRPs have substantial potential as natural antimicrobial agents in food preservation. Their ability to significantly reduce microbial counts positions them as promising alternatives to synthetic preservatives. Using natural antimicrobials aligns with the growing consumer demand for clean-label products and offers a pathway to enhance the safety and shelf-life of food products without compromising health.

Furthermore, the comprehensive biochemical characterization of the MRPs provides valuable insights into their structural properties, which can inform future research and application in various industrial contexts. The study lays the groundwork for developing novel food products with enhanced health benefits, utilizing legume-based MRPs as key functional ingredients.

In conclusion, this research contributes to understanding the antimicrobial and biochemical properties of MRPs, highlighting their potential applications in food science and technology. The results advocate for further exploration into optimizing the production and application of MRPs to fully harness their benefits in food preservation and other industrial applications.

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APPENDIX A

Figure A1. Lipid extraction from Legumes with n-Hexane

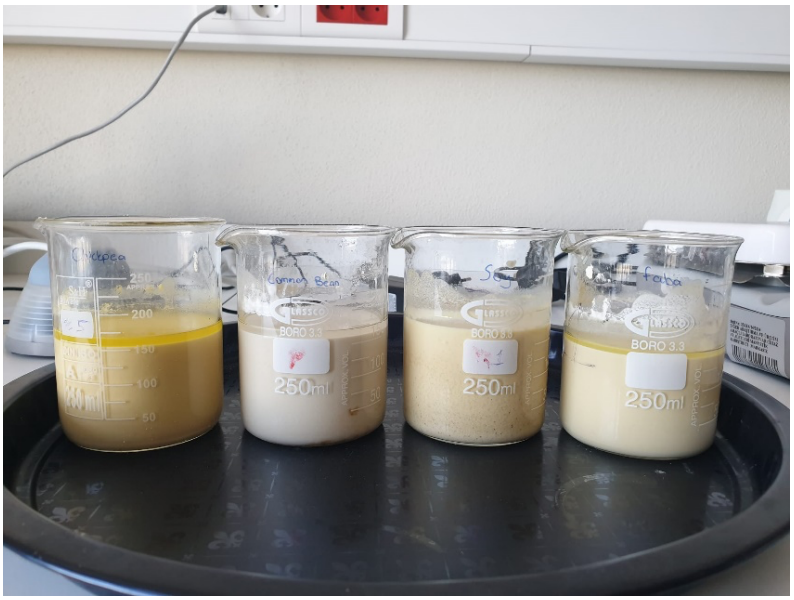

Figure A2. After lipid extraction from Legumes

Figure A3. Enzymatic hydrolysis of legume protein isolates with Alcalase enzyme

APPENDIX B

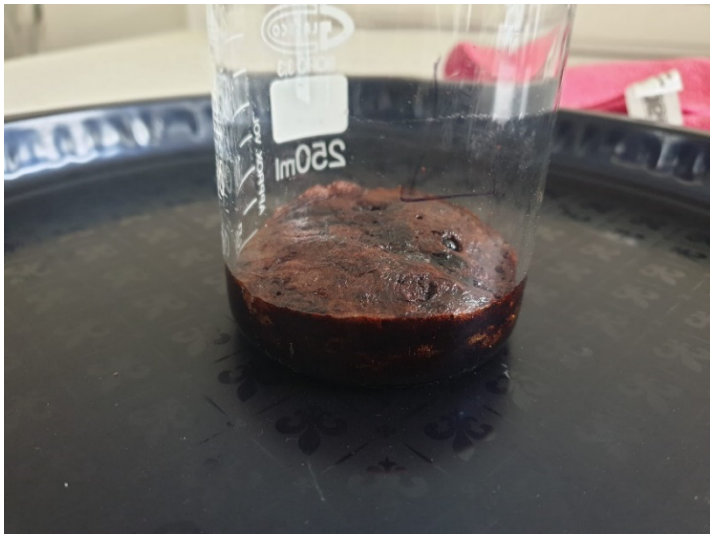

Figure B1. Maillard reaction product after heat treatment

Figure B2. Maillard reaction products filtered with Whatman filter paper.

Figure B3. Ultrafiltration setup for Maillard reaction products

APPENDIX C

Figure C1. After ultrafiltration process of Maillard reaction products (Left: Retentate, Right: Permeate)