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Examination of health promoting effect of fermented milk protein

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1. Introduction

Food, a fundamental necessity for human survival, has always been susceptible to spoilage and contamination. From the earliest civilizations, humans have employed various preservation techniques, fostering the development of fermentation. Lactic acid bacteria (LAB), a diverse group of Gram-positive, have played a central role in this endeavor (Adams & Moss, 1995). Their ability to convert carbohydrates, primarily sugars, into lactic acid not only contributes to the unique flavors and textures of fermented foods but also creates a hostile environment for spoilage and pathogenic bacteria (Leroy & De Vuyst, 2004). However, the significance of LAB extends far beyond its historical role in food preservation. Recent scientific discoveries have unveiled a treasure trove of additional functionalities associated with these versatile microbes. Research has increasingly focused on their potential as antioxidant and antimicrobial agents, opening exciting avenues for the food and health industries (Socrates et al., 2017). These versatile microbes are crucial in producing functional foods with improved health benefits, including better mineral absorption, protection against oxidative stress, and cardiovascular health enhancement (Satpute *et al*.,2022). The growing interest in lactic acid comes from the increasing demand for multiple aspects such as natural food preservation as consumers are increasingly opting for minimally processed, natural food products with extended shelf life. LAB offers a sustainable and safe alternative to synthetic preservatives, addressing concerns about potential health risks associated with chemical additives (Talon & Leroy, 2011), other reason is its presence as a safe and effective antimicrobial agent, the emergence of antibiotic-resistant pathogens poses a serious threat to global health. LAB, with their broad-spectrum antimicrobial activity, offers a promising avenue for developing natural alternatives to conventional antibiotics (Hamid *et al*.,2022). Moreover, the link between gut microbiota and overall health is gaining significant recognition. LAB strains exhibiting probiotic properties can enhance gut health by promoting beneficial bacterial populations, potentially aiding in digestion and boosting the immune system (Rasool *et al*., 2023). Oxidative stress, caused by the accumulation of free radicals, is implicated in various chronic diseases. LAB have demonstrated the ability to produce antioxidant compounds that can scavenge these harmful molecules, potentially mitigating the risk of chronic illnesses (Ruiz-Moyano et al., 2011). The effectiveness of LAB as food biopreservatives, probiotics, and functional food ingredients hinge on their ability to thrive and exert their beneficial properties under diverse environmental conditions. Several key factors influence LAB activity, including growth medium whereas different LAB strains exhibit varying preferences for growth substrates (Yang *et al*., 2018). The

acidity (pH) of the environment significantly impacts LAB growth and activity. Many LAB strains thrive in acidic environments, and their lactic acid production further lowers the pH, creating an inhospitable environment for undesirable microbes (Corsetti & Settanni, 2007). Understanding how different growth media influence LAB growth and activity can pave the way for the development of effective, targeted LAB-based biopreservatives for the food industry. By identifying the optimal conditions for specific LAB strains to produce antimicrobial compounds, their effectiveness in extending shelf life and ensuring food safety can be maximized (Talon & Leroy, 2011).

Aim of the study

This research aimed to comprehensively evaluate the potential of various lactic acid bacteria (LAB) cultures, specifically focusing on mixed cultures, as functional ingredients in the food industry. The study investigated the influence of three key factors:

Glucose Concentration: We explored how different glucose concentrations (0%, 1%, and 2.5%) impact the functionalities of LAB cultures, including their growth, baseline pH, and most importantly, their antioxidant and antimicrobial potential.

Mixed Cultures vs. Monocultures: We compared the performance of mixed LAB cultures to monocultures, hypothesizing that synergistic effects might enhance the overall bioactivity.

Impact of pH Environment: We investigated how modifying the external pH environment influences the microbial activity (particularly their antioxidant and antimicrobial potential) of LAB cultures. This analysis provides insights into their behavior within food matrices with varying pH levels.

By analyzing these factors, the research aimed to achieve the following objectives:

Identify Optimal Conditions for Bioactivity: Determine the optimal glucose concentration, baseline pH, and assess if mixed cultures exhibit superior antioxidant and antimicrobial activity compared to monocultures.

Develop Effective Biopreservatives: Establish the most effective LAB cultures and fermentation conditions for producing potent antimicrobial compounds, considering the impact of pH on their activity in food products.

2. Literature review

2.1. Fermented milks

Fermentation is any process in which the chemical and physical characteristics of raw material changes due to the activity of the microorganisms or their enzymes. The origins of milk fermentation can be attributed to the Middle East and Central and Eastern Europe (Frank and Marth, 1988).

Fermented milks have been known over centuries for their high nutritional value and health benefits. These products are made by acidifying pasteurized milk or cream with lactic acid bacteria, resulting in coagulation of milk protein and the production of lactic acid. The use of lactic acid bacteria to produce the acidity during the manufacture of fermented dairy products began before it was recognised that these bacteria caused the acidity and transformed milk into other products. This process will provide the resulted fermented milk products their desirable texture and characteristics. It is associated with different health benefits and increases their potential to be considered as functional foods. For intense, yogurt, plays a significant role in promoting gut health, which is a common target in the development of functional foods.

The nutritional value of fermented milk is attributed to the fact that it contains all the components of natural milk, except for lactose, which turns into lactic acid, when this acid formed it becomes the main preservation factor for these milks. With its presence, the growth of putrefactive and pathogenic bacteria is inhibited (Puiya 2015; de Oliveira, 2014). This process occurs in the presence of diverse types of microorganisms, which convert the lactose into lactic acid, resulting in a decrease in the pH level and the formation of the crud.

During this process the microorganisms tend to produce enzymes which is responsible for many changes such as releasing flavour and aroma compounds, such as aldehydes, ketones, and esters. Moreover, it increases the digestibility of the protein and improve the bioavailability of certain nutrients, such as minerals and vitamins, present in milk products (Cruz *et al.,* 2023).

Fermented milk products are considered functional foods due to their potential nutritional value and health benefits. They are rich in various nutrients such as calcium, phosphorus, potassium, vitamins, and high biological value proteins, these different components affect number of functions of the body in a positive way. Moreover, increasing scientific studies

confirm that the risk for many chronic diseases is reduced by the regular consumption of fermented milks and their supplementation with pro- and/or prebiotics (Hadjimbei *et al*., 2022).

2.1.1. Enhancement of health and gastrointestinal microbiota

Fermented foods especially dairy products are considered to be rich in probiotics, these probiotics promote intestinal health by regulating the diversity of the gut microbiota. These probiotic microorganisms are known for their ability to survive the digestive conditions including highly acidic pH, digestive enzymes, and bile salts (Bhagat *et al*., 2020). Moreover, pathogenic microbes such as bacteria, viruses, and fungi are inhibited by the fermented food's probiotics, which ensure their competitive survival and multiplication (Hernández-González et al., 2021). Lactic acid bacteria (LAB) strains from a variety of fermented foods exhibit antimicrobial qualities against opportunistic pathogens like *Staphylococcus aureus*, *Helicobacter pylori*, and *Escherichia coli* (Ren *et al.,* 2018). Their mechanical protection might be due to the release of substances resistant to microorganisms. These prevent their presence by blocking adhesion sites in the external tissues in the intestine of the host (Fooks *et al*., 1999).

2.1.2. Improvement of cardiovascular health

The consumption of cheese and fermented dairy products can reduce blood pressure and the risk of arterial stiffness. That's related to the bioactive peptides that are produced during fermentation, which inhibit the angiotensin-converting enzyme, reducing angiotensin II (Rai *et al.,* 2017). There is no question that the incidence of deadly heart attacks is correlated with plasma cholesterol levels, and the way to measure this is by measuring levels of low-density cholesterol (LDL). Schaafsma (1998) conducted experiments which demonstrated that the daily ingestion of 152 ml of probiotic-fortified milk is beneficial. It functions to lower both total cholesterol and this kind of cholesterol in the blood.

2.1.3. Improvement of neurological health

The gut-brain axis plays a crucial role in neurological health, affecting memory, cognition, sleep patterns, stress reactivity, and mood (Galland, 2014). Some studies show that disturbances in the gut-brain axis can lead to chronic noncommunicable diseases and

psychological disorders such as depression (Slyepchenko *et al*., 2017). Diversifying the gut microbiota through fermented foods can promote mental and neurological health by providing beneficial probiotics and bioactive molecules (Takada *et al.,* 2016).

2.1.4. Reducing the symptoms of lactose intolerance

Dairy products in addition to milk, are considered as a primary source of lactose, and are present in milk before the fermentation process in a percentage of about 4.6%. During the fermentation process, lactic acid bacteria digest 20-30% of it and break it down into the absorbable form, which is the monosaccharides glucose and galactose due to their secretion of a disaccharide-digesting enzyme, which is the enzyme lactase. Glucose is converted into lactic acid. This leads to decrease the symptoms of lactose intolerance, which include diarrhea and abdominal bloating when consuming milk. This condition affects many people and results from a hereditary deficiency of the enzyme responsible for digesting milk sugar in the intestine, which explains the ability of affected people to tolerate curd more than fresh milk (Savaiano *et al*.,1984).

2.1.5. Reduce and inhibit cancer

Colon cancer is the second most common type of cancer in Western societies, and it is related to age, making it more likely to appear as the person ages. There are mutations in one of the genes in the colon, and as a result, tumours form cancerous. Research on experimental animals has shown that dairy products fortified with probiotic bacteria can prevent and reduce cancer by reducing damage to DNA with the cancer-causing substance carcinogen (Stanton *et al*,. 2001). The summary of the therapeutic mechanism it exerts against cancer cells is as follows:

1. Dairy stimulates the immune system.
2. Breaking down carcinogenic compounds.
3. Reduces the numbers of pathogenic bacteria and thus their carcinogenic metabolic compounds.
4. Changing the physicochemical conditions in the colon and thus reducing the possibility of the formation of carcinogenic substances (Homaid, 2021).

Therapeutic bacteria have so-called anti-cancer effects, due to the presence of friendly bacteria or the metabolic substances that result from its activity that prevent the transformation of cancer

generators into carcinogenic substances. Confirmed by experiments and research the use of probiotics against cancer in humans who consume amounts of saturated fats has resulted in non-occurrence cancers. Experiments conducted on animals have also proven that providing diets containing cultures of *Bifidobacterium longum* suppressed the formation of substances responsible for colon cancer and stopped its development (Kulkarni and Reddy, 1994). These results were also confirmed in another experiment in which a strain of *Bifidobacterium longum* was added to inulin, which led to a decrease in the possibility of cancer (Rowland *et al*.,1998).

2.2. Functional ingredients of fermented milk

Fermented milks have been consumed for thousands of years, and the belief that they are healthy is almost as old as that. They have all the nutritional components that found in milk, but lactic acid bacteria (LAB) ferment these components, and it causes changes in it for a better form of nutrition.

2.2.1. Lactose

During the fermentation process, lactic acid bacteria ferment the lactose and turn it into lactic acid, this will reduce the pH, and influences the physical properties of the casein, which leads to promotes the digestibility and improves the utilization of the calcium and other minerals, also it will cause an inhibitor effect on the growth of harmful bacteria, it is also easier to be tolerated by the lactose intolerance people due to their lower lactose values (McBean, 1999).

2.2.2. Fat

The digestibility of fat is also improved during fermentation. Milk fat is known for its high proportion of saturated fatty acids therefore it is advisable most of the times to avoid it, because it contributes to an atherogenic blood profile and increased risk of coronary heart disease. But only three of them (lauric, palmitic, and myristic) rise the levels of blood cholesterol in the other hand one-third of milk fatty acids are unsaturated, so it's play cholesterol-lowering effect (Gurr, 1992). Moreover, fermented milks contain substances such as calcium, linoleic acid, conjugated linoleic acid (CLA), antioxidants, and lactic acid bacteria or probiotic bacteria that have hypocholesterolemic effects, if not protective ones (Rogelj, 2000). Also, components that

are considered to be anti-carcinogenic, like CLA, sphingomyelin, butyric acid, ether lipids, beta-carotene, and vitamins A and D (Jahreis *et al*., 1999).

2.2.3. Protein

The proteolytic activity of LAB gives rise to protein degradation; resulting in a few free amino acids and bioactive peptides. Bioactive peptides are a beneficial supplement to useful nourishments, and milk proteins are currently the main source of a range of organic dynamic peptides such as casomorphins, casokinins, immunopeptides, lactoferrin, lactoferricin, and phosphopeptides. Some of the milk protein-derived bioactive peptides are inert within the structure of the parent protein and can be released by enzymatic proteolysis during gastrointestinal assimilation or food processing, e.g. maturation. The most organic exercises of these peptides are immunomodulation, anti-microbial movement, antithrombotic action, blood weight control, and mineral or vitamin official (Tomé and Ledoux, 1998; Park, 2009). Fermented milks are moreover a wealthy source of whey proteins such as lactalbumin, lactoglobulin, lactoferrin, lactoperoxidase, immunoglobulins, and an assortment of development variables. These proteins have demonstrated a number of natural impacts extending from anti-carcinogenic action to distinctive impacts on the gastric function (McIntosh *et al*., 1998).

2.2.3.1. Bioactive peptides

Bioactive components are referred to specific ingredients found in foods in small amounts and it has beneficial physiological functions beyond their nutritional function. Among these components, bioactive peptides, which are protein fragments, play a crucial role presented by positively impacting various body functions and contributing to overall health (Kitts and Weiler, 2003). These bioactive molecules are usually found in the primary sequence of the protein and need to be released and free through processes such as hydrolysis by digestive enzymes, or enzymatic cleavage by proteases (from microorganisms or plants), or food manufacturing methods like acids, alkali, or heating. Sometimes these processes may overlap and the proteolytic action that started in the food continues in the body. When these particles are released, the bioactive peptides need to reach the receptors in the intestinal lumen or other peripheral organs by passing through the systemic circulation (Meisel and FitzGerald, 2003).

Bioactive peptides have been extensively studied for their health benefits, and due their antimicrobial and antioxidant properties.

2.2.3.1.1. The antimicrobial effect of bioactive peptides

For centuries, the growth of foodborne infections has been considered as a health and economic risk, because contaminated food may contain several types of pathogens and toxins linked to more than 200 diseases (León and Segura, 2020). Therefore, the idea of using preservatives to keep food in safe conditions, but nowadays, the demand for having normal fresh less processed food with fewer preservatives and artificial component keeps growing, which has led to the search for alternatives and here where the use of antimicrobial peptides (AMPs) arises in this scenario (Corrêa *et al.,* 2021), to address concerns about microbial resistance to synthetic compounds for intense, nisins, produced by *Lactococcus lactis* subsp. *lactis*, are well-characterized antimicrobial peptides and are widely used as food preservatives. They are classified as GRAS (generally recognized as safe) and regulated as a food additive in several countries (Khan, 2016).

2.2.3.1.2. Mechanism of antimicrobial activity of bioactive peptides

Antimicrobial peptides must be effectively applied in the agri-food sector, this requires an understanding of the molecular mechanisms underlying their activity which can be summarized as the following, AMPs vary wide in size, but they frequently have characteristics in common, such as hydrophobicity and net positive charge, which enable them to interact with the cytoplasm and/or membrane of microorganisms (Corrêa *et al.,* 2019). Receptor-mediated or nonreceptor-mediated mechanisms can be involved in the interaction between AMPs and microorganisms. Also, receptor-mediated AMPs, like bacteriocins like nisins, work against particular intracellular or membrane targets. Due to the fact that phospholipids and other membrane constituents are negatively charged, microorganisms usually have a net negative charge on their cell surface (Bhattacharya, *et al*., 2016). AMPs interact with the membrane to take advantage of their net positive charge, which is the basis of their nonreceptor-mediated mechanism. AMP activity is determined by various structural factors, including amino acid composition, conformation, charge, domain presence, and hydrophobicity (Corrêa, *et al.,* 2019). AMPs that target membranes can lead to cell death through the formation of pores and membrane destabilization (Shai, 1999).

Moreover, the immunomodulatory mechanisms of antimicrobial peptides and their modulation of the gut microbiota are closely linked to the health benefits they promote in humans and animals (Liébana *et al*., 2021). Researchers found that AMPs can bind to specific receptors in the consumer organism, leading to immune responses and affecting cellular functions. This immunomodulation can result in the suppression or stimulation of specific effectors, such as antibody production, cytokine expression, lymphocyte activation, or proliferation, as well as non-specific effectors like the activation of macrophages, natural killer cells, and granulocytes (Gauthier *et al*., 2006). AMPs with immunomodulatory activity are primarily produced by organisms to act on their own metabolism, but when consumed through food, they can have important immunomodulatory effects (Théolier *et al*., 2013).

2.2.3.1.3. The antioxidant effect of bioactive peptides

Bioactive peptides, derived from various sources including milk proteins, have garnered significant attention for their antioxidant properties. These peptides, originating from whey proteins like β-lactoglobulin, α-lactalbumin, and caseins such as α-, β-, and κ-casein, have demonstrated the ability to combat oxidative stress (Tonolo *et al*., 2020).

Studies have revealed that these peptides can prevent the peroxidation of essential fatty acids, a crucial mechanism in mitigating cellular damage caused by reactive oxygen species (ROS) and free radicals. Furthermore, modifications such as the addition of specific amino acids like leucine or proline can enhance their antioxidant activity, promoting synergy with non-peptide antioxidants (Sánchez and Vázquez, 2017).

Research has also shown that milk-derived peptides possess electron-donating capabilities, enabling them to neutralize free radicals and convert them into more stable compounds, these peptides, released either through enzymatic hydrolysis or fermentation, contribute to the oxidative stability of dairy products like yogurt (Li *et al*., 2015).

Furthermore, bioactive peptides exert their antioxidant effects through multiple mechanisms, including scavenging free radicals, preventing lipid peroxidation, and activating specific signaling pathways linked to antioxidant systems (Tonolo, 2023; Vibhute, 2023). The structure and amino acid sequence of these peptides are crucial determinants of their antioxidant activity (Sanapala and Pola, 2023). Additionally, they possess various other bioactivities such as antibacterial and antiaging properties (Ibrahim *et al.,* 2023), making them promising candidates for preventing and treating various diseases (Zhu et al., 2023).

The antioxidant potential of bioactive peptides derived from milk proteins offers a natural and effective means of combating oxidative stress, thereby contributing to food preservation and disease prevention (El-Sayed and Awad, 2019).

2.3. Probiotic microorganisms in fermented milk

The term "probiotic," which means "for life," refers to bacteria that have beneficial effects on humans and animals. The idea of using certain bacteria for their positive effects can be traced back to Eli Metchnikoff, a Nobel Prize-winning scientist from Russia who worked at the Pasteur Institute in the early 20th century. Metchnikoff proposed that by modifying the microbial population in our intestines, we could replace harmful bacteria with beneficial ones, based on their dependence on the food we consume. This concept has led to the development of functional foods, which are foods containing ingredients that have positive effects on health beyond basic nutrition. Probiotics are examples of such ingredients, containing biologically active components that promote health (Anukam, 2007; Ziemer & Gibson, 1998).

Originally, the term "probiotic" referred to substances produced by one microorganism that encouraged the growth of others. Over time, it also came to describe tissue extracts and animal feed supplements that supported the balance of intestinal flora in animals, promoting their well-being. A widely embraced definition, put forth by Fuller, defined probiotics as live microbial supplements in food that enhance the microbial balance within the host animal, aiding its health (Fuller, 1989).

Various microbial species demonstrate potential as probiotics. Lactic acid bacteria, a group crucial for nutrition, include important strains found in genera like Lactococcus and Bifidobacterium. Lactic acid bacteria are capable of producing lactic acid through carbohydrate fermentation. *Bifidobacterium*, while phylogenetically distinct, shares probiotic properties due to its metabolic pathway. In the food industry, particularly dairy, *Streptococcus thermophilus* and *Lactococcus lactis* are significant, though not strictly probiotics. These species are pivotal in dairy production, contributing to the fermentation process. (Holzapfel et al., 2001; Felis & Dellaglio, 2007).

2.3.1. The most important characteristics of probiotic strains

Probiotic bacteria are characterized by necessary and desirable characteristics, namely maintaining their vitality and effectiveness during treatment and storage, ease of application and use when manufacturing products, in addition to their resistance to physicochemical treatments of food (Prado et al, 2008). These bacteria must not be pathogenic, toxic, or carcinogenic, and must not generate mutations. In order for the host to act as an antidote to pathogens, it must not have a mechanism for transporting to the plasma and be able to resist antibiotics, in addition to its ability to survive the digestion process and have the ability to adhere and colonize in the mucous tract of the digestive system and increase immune stimulation without any irritant effect (Saarela et al., 2000).

A number of scientific references have stipulated special specifications for microbial strains in order for them to be considered a probiotic strain, as it is not enough for them to produce a probiotic effect. Rather, it is important for a large percentage of these microbes to reach the intestine alive so that they can create a vital balance, meaning that the beneficial strain should prevail at the expense of the unsuitable strain. The desired conditions (Ouwehand et al., 1999) and these references suggested the following conditions:

1) Able to tolerate high acidity.

2) Able to withstand juices, digestive enzymes, and digestion products.

3) Able to withstand antibiotics.

4) Tolerant to bile salts.

5) The ability to produce volatile fatty acids.

6) It should be safe and have no side effects and does not affect intestinal permeability.

7) It has the ability to adhere to the intestinal mucosa.

8) It can maintain its vitality in food that is used during pregnancy.

9) The ability to alert and activate the immune system (Homaid.2021).

2.3.2. Mechanism of action of beneficial bacteria or probiotics

Beneficial bacteria have been known to improve health, but the processes behind them have not been explained they use it for this (Holzapfel et al., 1998) many studies have presented these bacteria used by everyone (Rolfe, 2000), including:

1- Production of inhibitory substances: production of some organic forms, hydrogen peroxide, and bacteriocins, which are inhibitors of Gram-negative and Gram-positive bacteria.

2- It blocks adhesion sites: it works to compete with pathogenic bacteria for the winners' sites in the host, they usually inhibit microbes through their effect on the epithelial tissue, thus blocking them from admission sites.

3- Competition for nutrients: Probiotics eliminate the causes of diseases by consuming the nutrients that pathogenic bacteria need

4- Immune stimulation: Stimulating the host's immunity may be one of the most important mechanisms used by probiotics to protect it from intestinal diseases

5- Disabling toxin receptors: It disables toxin receptors located on the intestinal mucosa, thus protecting the host against intestinal diseases caused by *Clostridium*. There are also some other mechanisms, such as reducing the production of toxins or reducing the severity of microbial pathogens. (Fooks et al., 1999).

2.4. Overview of lactic acid bacteria

Lactic acid bacteria are a class of Gram-positive, non-spore-forming, fastidious, cocci or rods that are catalase-negative and have a high tolerance for low pH levels. One of the most significant types of microbes used in food fermentations. LAB also improve the texture and flavour of fermented foods. Their primary byproduct is lactic acid, which is produced from glucose and growth-inhibiting agents like hydrogen peroxide, bacteriocins, and diacyls, among others, which stop the spread of pathogens and bacteria that cause food spoiling (Mokoena, 2017).

Although lactic acid bacteria include more than 60 genera, the frequent genera that occur in food fermentation generally include *Lactobacillus*, *Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Enterococcus, Weissella*, etc. (Wang *et al*.,2021).

The growth optimum for LAB is at pH 5.5–5.8, and these microorganisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids, and carbohydrates. To date, several LAB isolates from the *Lactobacillus* genus and their bacteriocins have been applied in food preservation and in the control of human pathogens (Mokoena, 2017).

Lactic acid bacteria are present in different parts of our surroundings including dead plants, spoiled fruits, milk, sausages, fish and other fermented items, cereals, pickles, and other veggies, and so on. They are predominant in some human places like the oral cavity, ileum, colon, and vagina. To be more explicit, LAB are classified under Firmicutes, phylum class Bacilli and order *Lactobacillales*. However, the genus *Lactobacillus* including genera of *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus*, are the most studied and widely used. Lactic acid bacteria or *Lactobacillus* is the most numerous genera, enduing over 100 species with carbohydrate fermentation furnishing their preferred hospitable environment. While most LAB organisms are normal or even beneficial, some of them are used as probiotics to heal or improve human health. For a microorganism, however, to meet the qualification for a probiotic, certain criteria are critical. These include being virulent and clinically approved, withstanding changes in pH and bile concentration, being immune system-friendly and not ensuring transmission of antibiotic-resistance genes to other potential pathogens. Taking probiotics is either in the form of medications or some vegetable foods which makes the intestine healthy (Mokoena,2017).

2.4.1. Lactic acid bacteria as a probiotic

Discussion about the significance of *Lactobacillus* species in fermented foods necessitates consideration of their role as probiotics. Initially, *L. acidophilus* was favoured for its presumed dominance in the gut, but various lactobacilli strains have since been employed in probiotic preparations, including *L. delbrueckii subsp. bulgaricus, L. casei, L. brevis, L. cellobiosus*, *L. lactis, L. fermentum, L. plantarum,* and *L. reuteri* (Ali, 2010). There is growing interest in utilizing diverse lactic acid bacteria strains as probiotics to improve both human and animal health (Halász, 2021).

Recently, lactic acid bacteria have garnered attention for their potential in bolstering the human host system's defenses against foodborne pathogens. LAB is under scrutiny for its prospective application as a biopreservative in the food and dairy sectors and as an alternative to antibiotics

in medical treatments (Lashani et al., 2020). Probiotics have shown efficacy in managing a spectrum of health issues, including inflammatory bowel disease, irritable bowel syndrome, and antibiotic-associated diarrhea, particularly also in treating lactose intolerance. Effective probiotic strains must possess specific beneficial traits like gastrointestinal illness tolerance, epithelial cell adhesion, cholesterol absorption, bile salt hydrolysis, safety against virulence genes, non-hemolytic activity, antibiotic sensitivity, antibacterial properties, and viability during fermentation and storage (A'inurrofiqin et al., 2022).

2.4.2. Basic Metabolic Pathway of Lactic Acid Bacteria

2.4.2.1. Glucose Metabolism

Lactic acid bacteria use two metabolic pathways to break down glucose: homolactic fermentation and heterolactic fermentation. Glucose is primarily converted to lactic acid via the homolactic fermentation pathway. However, after the heterolactic fermentation pathway, glucose produces lactic acid as well as ethanol, acetic acid, and carbon dioxide (Wu and Zhao, 2019).

2.4.2.2. Fructose Metabolism

Fructose is phosphorylated by fructose kinase to form fructose 6-phosphate, fructose 6-phosphate produces pyruvate by EMP pathway, and finally is reduced to lactic acid, namely homolactic fermentation bacteria. Fructose, half of which is reduced as an electron acceptor for the final product of mannitol after mannitol dehydrogenase acts heterolactic fermentation bacteria, while the rest of the fructose is phosphorylated and forms glucose 6-phosphate; end products, such as lactic acid, ethanol, acetic acid, carbon dioxide, etc., are formed in the HMP pathway (Wu and Zhao, 2019).

2.4.2.3. Sucrose Metabolism

Once sucrose hydrolase has produced glucose and fructose, sucrose is transferred into cells via a permease or PTS system and enters the primary metabolic pathway of the cell. The EMP or HMP pathway is then used to degrade it into the finished product (Wu and Zhao, 2019).

2.4.2.4. Malate Metabolism

Lactic acid bacteria can decarboxylate malic acid to produce lactic acid and carbon dioxide by apple lactate. For some lactic acid bacteria, malic acid is metabolized to produce pyruvic acid and carbon dioxide, and pyruvic acid is metabolized to produce metabolites such as lactic acid. In addition, malic acid can also be reacted to 6 Metabolomics of Lactic Acid Bacteria 178 form fumaric acid, which is then converted to succinic acid to form another metabolic pathway (Wu and Zhao, 2019).

2.4.2.5. Citric Acid Metabolism

The creaminess flavour during the fermentation of dairy products arises from the metabolism of citric acid. Several factors have been identified that determine the products of citrate metabolism of lactic acid bacteria's genes and the conditions of their growth. When it is transported and originated within the cell, citric acid is decomposed into fermentation acetic acid and oxaloacetate lyase citrate. The resulting pyruvate is produced by the decarboxylase produced oxaloacetate. Pyruvate is then to lactic acid, fumaric acid, acetic acid, and diacetyl or acetoin. Based on these results, diacetyl, and acetoin, which are present in slight amounts, are among the most important regarding flavour regulation (Wu and Zhao, 2019).

2.4.2.6. Amino Acid Metabolism

Among the amino acids metabolized by lactic acid bacteria, transaminase and decarboxylase play an important role. In addition to these two enzymes, other enzymes such as lyase and dehydrogenase play a role in the amino acid metabolism of lactic acid bacteria. Under the action of decarboxylase, lactic acid bacteria can convert glutamic acid into γ-aminobutyric acid. Under the action of dehydrogenase, lactic acid bacteria can produce α-ketoglutaric acid, an important metabolic intermediate of glutamic acid, and glutamate are formed from α-ketoglutaric acid under the catalysis of a transaminase. Lactic acid bacteria can produce many flavour substances, such as ketones, aldehydes, and acids, due to incomplete metabolism of such amino acids, which directly act to enhance the flavour of the fermented food or as a substrate to produce flavour substances (Wu and Zhao, 2019). Protein degradation by lactic acid bacteria plays a pivotal role in ensuring food quality, safety, and nutrition, significantly impacting the allergenicity of dairy products and other fermented foods. This process encompasses multiple stages, including protein degradation, peptide transport, peptide

degradation, and amino acid catabolism. Lactic acid bacteria employ cell envelope proteinases (CEP) to initiate protein degradation, followed by the uptake of peptides into cells and their subsequent breakdown into amino acids facilitated by various peptidases. Moreover, lactic acid bacteria demonstrate proficiency in degrading allergenic proteins present in food, such as casein in dairy products and various proteins in wheat, thereby mitigating their allergenic potential (Wang *et al*.,2021)

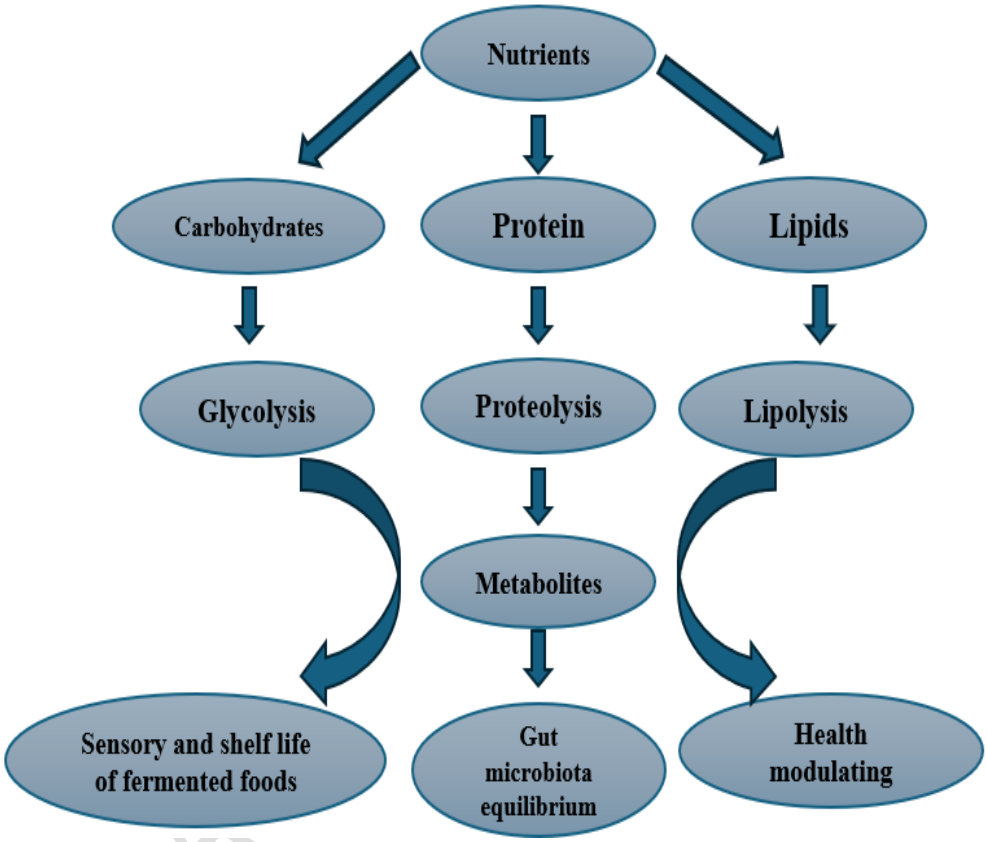

Figure 1: Metabolic pathway used by lactic acid bacteria to transform nutrients *(Mazguene,2023).*

2.4.2.7. Conversion of other non-nutritive and harmful substances in food

Lactic acid bacteria possess the ability not only to degrade major nutritional macromolecules like polysaccharides and proteins but also to break down various undesirable substances present in food. These capabilities extend to inhibiting mycotoxin accumulation, such as aflatoxin B1 and B2, during the preservation of cereal products, thereby reducing potential health risks associated with fungal contamination. Also decomposing harmful substances in

alcohol fermentation, such as ethyl carbamate, a potential carcinogen, thereby enhancing the safety of fermented alcoholic beverages. Lactic acid bacteria are capable of decomposing phytic acid, which affects the taste and digestibility of food. This degradation process occurs in yam-based foods, facilitated by phytase enzymes produced by specific lactic acid bacteria strains (Wang *et al*.,2021).

2.4.3. Application of metabolomics in the study of fermented dairy products:

Lactic acid bacteria are crucial in fermenting various dairy products like yogurt, cheese, and kefir, breaking down milk components into smaller molecules, thereby shaping their unique taste and flavour. In recent years, there has been a growing interest in employing metabolomics technology to precisely control the fermentation process of lactic acid bacteria in dairy fermentation. Researchers have employed metabolomics to investigate the interplay between texture, taste, and nutritional content in fermented dairy. For example, studies have unveiled differences in metabolite production during the fermentation of specific cheeses, enabling region-specific identification. Similarly, the role of lactic acid bacteria in cheese ripening has been examined, highlighting differences between naturally fermented and commercially initiated processes. Further studies have linked metabolite findings to sensory quality, demonstrating how specific lactic acid bacteria strains can enhance growth and affect flavour metabolites in milk. Investigations into selenium-enriched yogurt have suggested associations between selenium distribution and certain proteins, potentially influencing protein expression. As metabolomics advances, it offers deeper insights into biomarkers, metabolic profiles, and the intricate relationship between these components and lactic acid bacteria in fermented dairy. This knowledge lays a foundation for scientifically assessing and improving the utilization of lactic acid bacteria in fermented foods (Wu and Zhao,2019).

Moreover, the selection of appropriate lactic acid bacteria strains is essential for achieving desired properties in various fermented food products. Table (1) presents a comprehensive overview of the types of fermented products and the associated lactic acid bacteria strains commonly used in their production:

Table 1: Fermented foods and beverages and their associated lactic acid bacteria (*Ali, 2010)*

Types of fermented products	Lactic acid bacteria
Dairy products	
Hard cheeses without eyes	*L. lactis subsp. lactis, L. lactis subsp. cremoris*
Cheeses with small eyes	*L lactis subsp. lactis, L lactis subsp. lactis var. diacetylactis, L lactis subsp. cremoris, Leuc.mesenteroides subsp. cremoris*
Swiss- and Italian-type cheeses	*Lb. delbrueckii subsp. lactis, Lb. helveticus, Lb. casei, Lb. delbrueckii subsp. bulgaricus, S. thermophilus*
Butter and buttermilk	*L lactis subsp. lactis, L lactis subsp. lactis var. diacetylactis, L lactis subsp. cremoris, Leuc. mesenteroides subsp. Cremoris*
Yoghurt	*Lb. delbrueckii subsp. bulgaricus, S. thermophilus*
Fermented, probiotic milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii, B. lactis, B. bifidum, B. breve*
Kefir	*Lb. kefir, Lb. keftranofacies, Lb. brevis*
Fermented meats	
Fermented sausage (Europe)	*Lb. sakei, Lb. curvatus*
Fermented sausage (USA)	*P. acidilactici, P. pentosaceus*
Fermented fish products	*Lb. alimentarius, C. piscicola*
Fermented vegetables	
Sauerkraut	*Leuc. mesenteroides, Lb. plantarum, P. acidilactici*
Pickles	*Leuc. mesenteroides, P. cerevisiae, Lb. brevis, Lb. plantarum*
Fermented olives	*Leuc. mesenteroides, Lb. pentosus, Lb. plantarum, P. acidilactici, P. pentosaceus, Lb. plantarum, Lb.fermentum*
Fermented vegetables Soy sauce	*T. halophilus*
Fermented cereals Sourdough	*Lb. sanfransiscensis, Lb. farciminis, Lb. fermentum, Lb. brevis, Lb. plantarum, Lb. amylovorus, Lb.reuteri, Lb. pontis, Lb. panis, Lb. alimentarius, W. cibaria*
Alcoholic beverages	
Wine (malolacticfermentation)	*0. oeni*
Rice wine	*Lb. sakei*

B: *Bifidobacterium*, C: *Carnobacterium*, L: *Lactococcus*, Lb: *Lactobacillus*, Leuc: *Leuconostoc*, O: *Oenococcus*, P: *Pediococcus*, S: *Streptococcus*, T: *Tetragenococcus*, W: *Weissella*

This table illustrates the diversity of lactic acid bacteria strains utilized in fermenting various food products, reflecting the importance of strain selection in achieving desired product attributes (Caplice & Fitzgerald, 1999; Wood, 1997)

2.4.4. Products synthesized by lactic acid bacteria

2.4.4.1. Organic Acids

Lactic acid bacteria are proficient in synthesizing various organic acids, notably lactic acid, which holds significant industrial importance across agriculture, food, medicine, and environmental sectors.

Apart from lactic acid, lactic acid bacteria also produce acetate, propionate, 3-hydroxypropionate, formate, and succinate, contributing to flavour complexity. These organic acids are synthesized through hetero-lactic fermentation pathways and amino acid metabolism. This mechanism generates organic acids like 2-ketoisocaproic acid (Wang *et al*.,2021).

2.4.4.2. Bacteriocin

Bacteriocins are antibacterial compounds produced by bacteria using ribosomes. They inhibit the growth and reproduction of various bacteria.

Bacteriocins, categorized into two main types, Category I lantibiotics containing lanthionine, and Category II bacteriocins without lanthionine, are pivotal in bacterial defense mechanisms. Additionally, Type III bacteriocins, such as helveticin M and helveticin J, further enrich the classification. For these bacteriocins to fulfill their biological function, they must be secreted out of the cell. This secretion typically occurs through specific transport systems, including the double glycine guide sequence transport system or the ABC transporter system. In the food industry, bacteriocins find extensive application as bacteriostats and preservatives due to their safety for human consumption. They effectively inhibit various foodborne pathogens, thereby extending the shelf life of food products. Moreover, bacteriocins synthesized by lactic acid bacteria play a crucial role in intestinal health by contributing to the balance of intestinal microbiota and enhancing resistance to infectious diseases (Wang *et al*.,2021).

2.4.4.3. Vitamins

Lactic acid bacteria exhibit the capability to synthesize various vitamins, including folic acid, riboflavin, vitamin C, pyridoxal, and cobalamin, expanding their application in the food industry for nutritional fortification. Folic acid, essential for nucleotide and protein biosynthesis, is synthesized by strains belonging to *Streptococcus, Lactobacillus*, and *Lactococcu*s genera through Pterin and pABA branches. Riboflavin production involves genes clustered on a rib operon, and genetically engineered strains show increased riboflavin synthesis. These findings underscore the potential of lactic acid bacteria in fortifying fermented foods with essential vitamins, catering to the needs of diverse populations (Wang *et al*.,2021).

2.4.4.4. Exopolysaccharides

Exopolysaccharides are synthesized by the lactic acid bacteria and have very important physical properties along with probiotic feature which help in improving the functionality and characteristics of fermented foods. For example, these polymers facilitate bacterial attachment and ultimately help to impart new features to fermented foods. The gram-negative bacteria synthesis of exopolysaccharides is governed by the specific gene clusters within the bacterial genome, which regulates the processes, sugarnucleotide synthesis, glycosyltransferase activity, and polymerization. These polysaccharides find their use in the food industry, especially in fermented dairy products. As an additive, they ensure texture stability and desirable sensory properties. On the other hand, there are certain lactic acid bacteria strains, like *Streptococcus thermophilus* and *Lactobacillus delbrueckii*. The strains *Lactobacillus bulgaricus* in yogurt produces more exopolysaccharide, less syneresis and hence better sensory perception. Moreover, new exopolysaccharides with functional characteristics as antioxidant or antibacterial are created in strains like *Lactococcus lactis* F-mou, they can be used to expand the range of food applications (Wang *et al*., 2021).

2.4.4.5. Gamma-Aminobutyric Acid

Gamma-aminobutyric acid (GABA) is one of the natural amino acids widely distributed in nature, acting as an important and consciously distributed neurotransmitter in the central nervous system, with researches on its implications on different diseases. The GABA concentrations are primarily produced by the enzyme glutamate decarboxylase, which decarboxylates the amino acid L-glutamate. Existing the gene GadA and transcriptional regulator gene gadR in *Levilactobacillus brevis*, live them to synthesize more GABA. High performance *Levilactobacillus brevis* strains can be used in fermentation field for GABA production which can achieve remarkably good GABA titers and bioconversion ratios. Lactic acid bacteria are harnessed in the food industry to add and improve GABA levels of cereals, cheese, yogurt and fermented milk thereby offering functional benefits. The production of traditional Chinese soybean products also benefits from the symbiotic effect of co-inoculation with *Levilactobacillus brevis* and *Bacillus subtilis*, which results in an increase in GABA concentration in this fermented product and a reduction of harmful compounds (Wang *et al*.,2021).

2.4.4.6. Antioxidant Substances

Lactic acid bacteria which inhabit in the gut are known to synthesize antioxidant substances that make food consumption safe and give the human body great benefits. In case of phenol substance, certain lactic acid bacteria are metabolizing them through decarboxylase protein and reductase enzymes, these chemical reactions then lead to degradation of hydroxycinnamic and hydroxybenzoic acids into active phenol metabolites that are increasingly considered to be more powerful than those before and having antioxidant activity. Besides this, proteins are broken down better by it and they are also protected from high level of acidity. Fermently, while the antioxidants that the lactic acid bacteria synthesize play a vital role in this respect, they are also essential in explaining the probiotic mechanism by their ability to scavenge free radicals, moderate inflammatory reactions and enhance the level of expression of intracellular tight junction proteins. (Wang *et al*.,2021).

3. Materials and Methods

The experiments were conducted at the Hungarian University of Agriculture and Life Sciences (MATE), Buda Campus, Budapest, Hungary.

3.1. Materials

Applied strains

For the experiments, a set of starter cultures was chosen (Table 2). The strains were stored at -18°C as freeze-dried Direct Vat Set (DVS) cultures.

Table 2 The chosen set of lactic acid bacteria strains and starter cultures

Starter cultures	Producer	Properties
YoFlex Mild 1.0	Chr. Hansen	Thermophilic starter culture *Lactobacillus delbrueckii subsp. bulgaricus* *Streptococcus thermophilus*
ALC-01	Chr. Hansen	*L. plantarum*
CHN-22	Chr. Hansen	Mezophilic aroma producing culture *Lactococcus lactis subsp. cremoris,* *Lactococcus lactis subsp.lactis*, *Leuconostoc mesenteroides subsp. cremoris,* *Lactococcus lactis subsp. lactis biovar diacetylactis*
ABY-1		*Bifidobacterium,* *Lactobacillus acidophilus,* *Streptococcus thermophilus,* *Lactobacillus bulgaricus*
Lacto 7	commercial	*Lactobacillus plantarum Lp*-115 *Lactobacillus rhamnosus GG.* *Lactococcus lactis LI*-23. *Lactobacillus salivarius Ls*-33. *Lactobacillus gasseri Lg*-36. *Lactobacillus rhamnosus Lr*-32 *Lactobacillus reuteri* 1E1
Lactobacillus acidophilus 150	Chr. Hansen	Mezophilic Monoculture
Lactobacillus acidophilus LA-5	Chr. Hansen	Mezophilic Monoculture
Mix*:*	own mix	Mezophilic Monocultures *Lactobacillus acidophilus* 150 *Lactobacillus acidophilus* LA-5 *Lactobacillus acidophilus* N2

Pathogen strains

A panel of pathogenic strains including *E. cloacae, Listeria monocytogenes* 4ab, *E. coli* 0157:H7, *E. coli* 8739, and *E. faecalis* were chosen to complete the experiments.

MRS agar

MRS agar was used for growing LAB strains and was prepared according to the following Table 3.

Table 3: Composition of MRS broth

Component	Amount
Proteose peptone	10 g
Beef extract	8 g
Yeast extract	4 g
Glucose	20 g
Sodium acetate	5 g
Triammonium citrate	2 g
Magnesium sulfate	0.2 g
K_2HPO_4	2 g
Tween 80	1 mL
Manganese sulfate	0.05 g
Distilled water	1000 mL

The medium autoclaved at 121ºC for 15 min.

Skimmed milk agar

To prepare the 1% skimmed milk agar media 25 g of the non-fat dry milk was reconstituted into 250 mL of distilled water we added to the previous amount 1.3% agar-agar (1.3g/100g). The mixture was stirred thoroughly and autoclaved at 121ºC for 15 min.

Tryptone Soy Agar

To examine the antimicrobial activity, we need to prepare the TSA media.

Tryptone Soy Agar was prepared by suspending 30 g of tryptone soy broth dehydrated media in 1 liter of purified filtered water and then adding agar in a ratio of 13 g per 1 liter to give it the ability to solidify. The mixture was sterilized at 121°C for 15 minutes and cooled to 45-50°C.

Milk protein concentrate solution

To make a 5% Milk Protein Concentrate (MPC) solution, 5 grams of MPC80 powder was measured and mixed with 100 millilitres of distilled water. This mixture was then divided into 14 separate flasks, each containing 50 millilitres. In 7 flasks, the desired amount of glucose solution, specifically 2.5%, was added, while the other flasks remained without any sugar added for the first experiment. while 2.5% and 1% of glucose were examined for the second experiment. The mixture was stirred thoroughly and autoclaved at 121ºC for 15 min.
150 microliter of each LAB strain was inoculated in flasks containing the MPC solutions.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) solution

DPPH solution has been prepared by adding 0.00142 g of DPPH to 100 ml of methanol, and further used in determining the antioxidant activity of the cultures.

3.2. Methods

3.2.1. Strain maintenance

Before starting the experiments the starter cultures were inoculated in MRS (de Man Rogosa Sharpe) broth and incubated at 37 °C for 24 h under aerobic conditions. The pathogen strains were maintained on tryptone soy broth at 37 °C for 24 h.

3.2.2. Screening for proteolytic activities

Screening for proteolytic activities on skimmed milk agar
Proteolytic activity was determined using skimmed milk agar medium. The warm agar was poured into Petri dishes and allowed to solidify. After solidification, holes of 8 mm diameter were made into the media (Fung et al.,1999). Strains were grown in MRS broth at 37°C for 24

h. 150 µl of one day old ferment broth were pipetted to the holes. After incubating the Petri dishes for 24 hours, the result can be determined as an empty clear zone around the holes.

3.2.3. Fermentation of milk protein concentrate

After inoculating the LAB strains into the MPC solutions with different glucose concentrations (1% and 2.5 %), the flasks were incubated at 37º C for multiple durations of 8, 16, 20, and 24 hours. After that sample from these flasks were taken for antimicrobial and antioxidant experiments.

3.2.4. Antioxidant activity measurement

The DPPH method is a technique used to measure the antioxidant activity of compounds. It involves the use of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, which reacts with antioxidants to produce a colour change that can be measured spectrophotometrically. Antioxidant activity in obtained fermented aqueous solutions of Milk Protein Concentrate (MPC) was determined using this method as the following:

3.9 mL of diphenyl-2-picrylhydrazyl solution were combined with 100 µl of the sample. The mixture was then left in darkness for 30 minutes before being centrifuged. Subsequently, the absorbance of the samples was measured at a wavelength of 517 nm using a spectrophotometer.

The antioxidant activity was determined using the formula:

$$A\% = \left(A/(A - A')\right) \times 100$$

Where:

- A represents the initial absorbance of the control at 517 nm.
- A' represents the final absorbance of the tested sample at 517 nm.

3.2.5. pH measurement

A Mettler-Toledo GmbH, Switzerland pH meter was used to measure the pH of the prepared samples, at room temperature

3.2.6. Antimicrobial activity assay

Tryptone Soy Agar was used to determine the antimicrobial activity of the fermented samples. The 45-46°C medium was dispensed into sterile Petri dishes containing 200 µl of the pathogen strain. After solidification holes with a diameter of 8 mm were prepared and filled with 150 µl of the LAB-tested strain. Because of the diffusion, the Petri dishes was placed to 4ºC for 1 hour. After incubating the Petri dishes for 24 hours and the result can be determined as an empty clear zone around the holes.

3.2.7. pH Modifying

This procedure was carried out to neutralize the acidic environment, which could potentially hinder the growth of pathogenic strains. It aimed to confirm that the antimicrobial effect and inhibition of pathogens were indeed due to the activity of the formed peptides, Sodium hydroxide (NaOH) solution with a concentration of 1N was used to reach a pH of around 6.

4. Results and Discussion

4.1. The results for mono and mixed cultures of LAB

4.1.1. Screening for proteolytic activities on skim milk agar

The principle is that some microorganisms have the ability to degrade the casein protein by producing a proteolytic exoenzyme, called proteinase (caseinase). For demonstration of such an activity, in the lab, skim milk agar is used. Milk proteins form a colloidal suspension that gives the medium its colour and opacity because it deflects light rays rather than transmitting them. Following inoculation and incubation of the agar plate cultures, organisms secreting proteases will exhibit a zone of proteolysis, which is demonstrated by a clear area surrounding the holes. This loss of opacity is the result of a hydrolytic reaction yielding soluble, non-colloidal amino acids, and it represents a positive reaction. In the absence of protease activity, the medium organism remains opaque, which is a negative reaction.

We did two replicates of the experiment with two different temperatures to see the effect of changing the temperature on the growth, each experiment also included two different incubation time 8 hours and 24 hours. In observations, rather than clear zones indicating proteolytic activity, a widespread growth was evident around bacterial colonies. This implies that the cultured bacteria lack proteolytic characteristics. Several factors could contribute to this, such as the absence of enzymes necessary for protein breakdown within the bacteria. Not all lactic acid bacteria (LAB) possess proteolytic activity, which is crucial for peptide production. The capability to hydrolyze proteins into peptides varies among LAB strains. While some strains have proteolytic enzymes facilitating this process, others lack this ability (Lee *et al*.,2023). Secondly, variations in skim milk agar composition or the presence of additional nutrients could influence the extent of protein breakdown by any residual proteolytic activity in LAB (Perwendha *et al*.,2020).

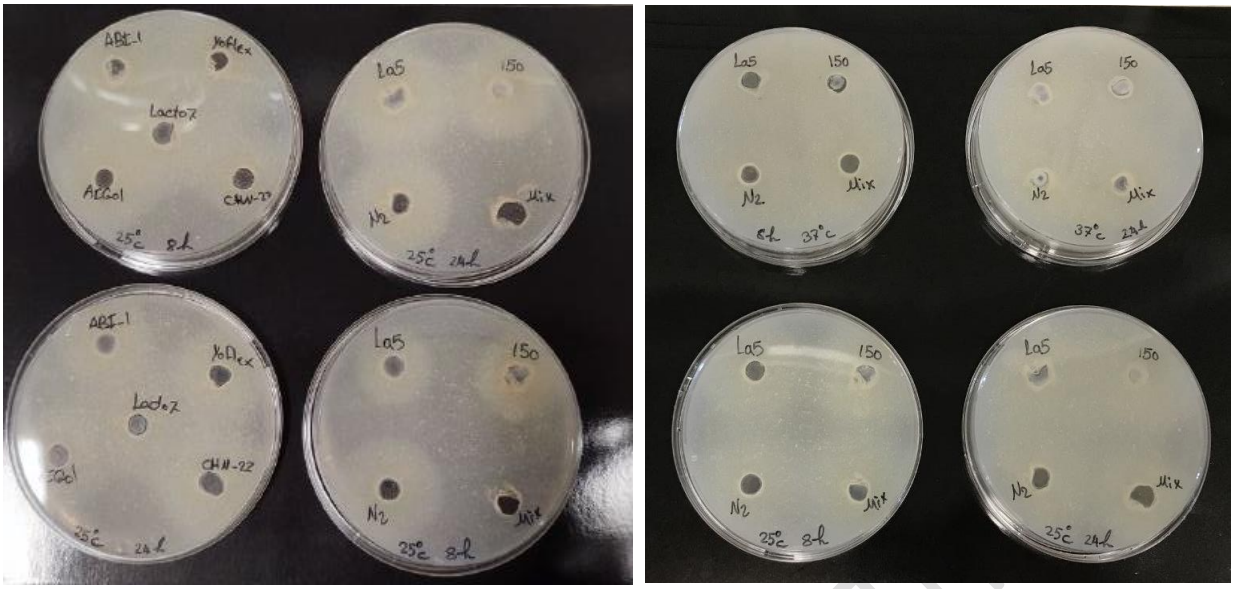

Figure 2 Screening of proteolytic activity on skim milk agar plate after 8 and 24 hours at different temperatures

4.1.2. Antioxidant activity of fermented milk protein concentrate

This approach helps assess the potential of LAB strains to act as antioxidants and contribute to human health by combating free radical damage. A stable free radical compound, such as DPPH (2,2-diphenyl-1-picrylhydrazyl) or ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), is used. These radicals exhibit a distinct colour. The LAB broth extract is added to the free radical solution. If the extract contains antioxidant compounds, they will scavenge the free radicals, neutralizing their harmful effects. The reaction mixture is monitored, often by measuring changes in colour intensity. As free radicals are neutralized, the solution's colour intensity typically diminishes. The degree of this change is proportional to the extract's antioxidant activity. In this experiment mono and mixed cultures of LAB which were added to the MPC solution and were left to ferment at 37ºC to have hopefully bioactive peptides will be formed. Then it was tested for its antioxidant effect, with glucose concentrations of 2.5% and with no glucose, and the results were as the following in Figures 3,4.

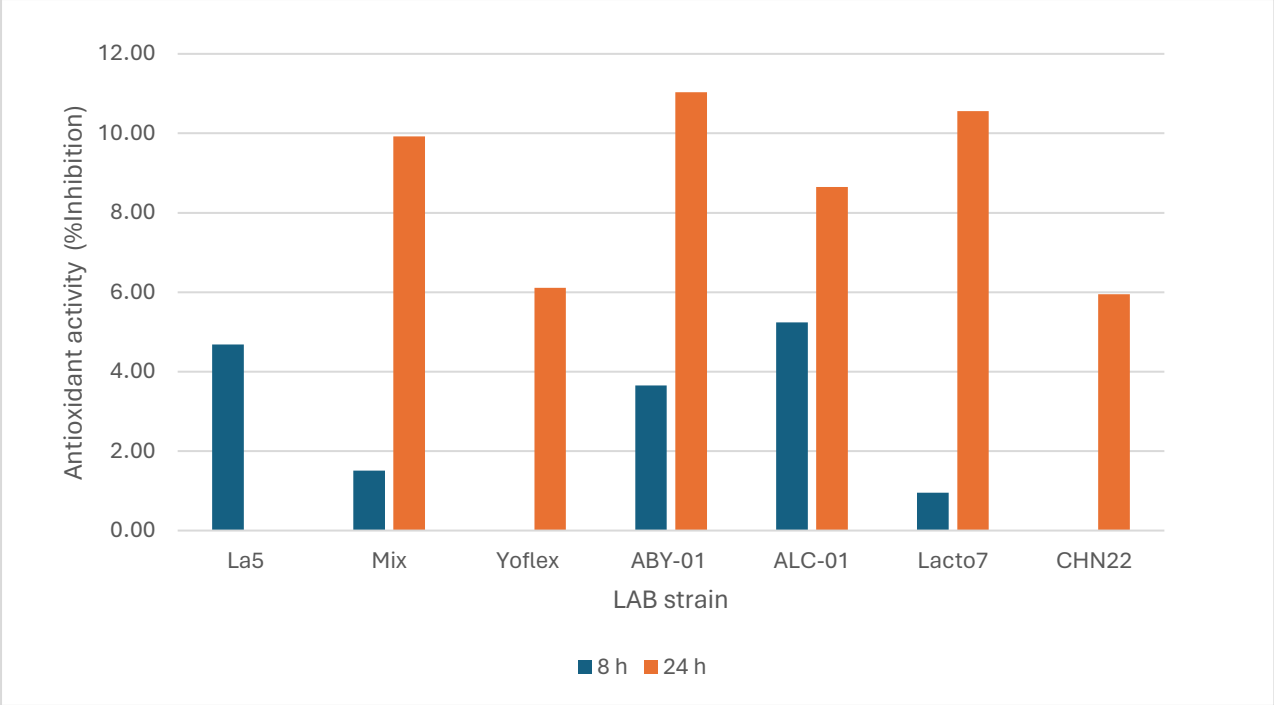

Figure 3: Antioxidant activity over time of LAB with the absence of glucose

From the Figure 3 we can notice the following:

For the 8 hours incubation with the absence of glucose the highest antioxidant activity was observed for the cultures ALC-0l, La5, ABY-1 with radical scavenging activity (RSA %) values of 5.24, 4.68, 3.65.

For the 24 hours incubation with the absence of glucose the highest antioxidant activity was observed for the cultures ABY-01, Lacto7, Mix 24, with RSA %values of 11.03, 10.56, 9.92.

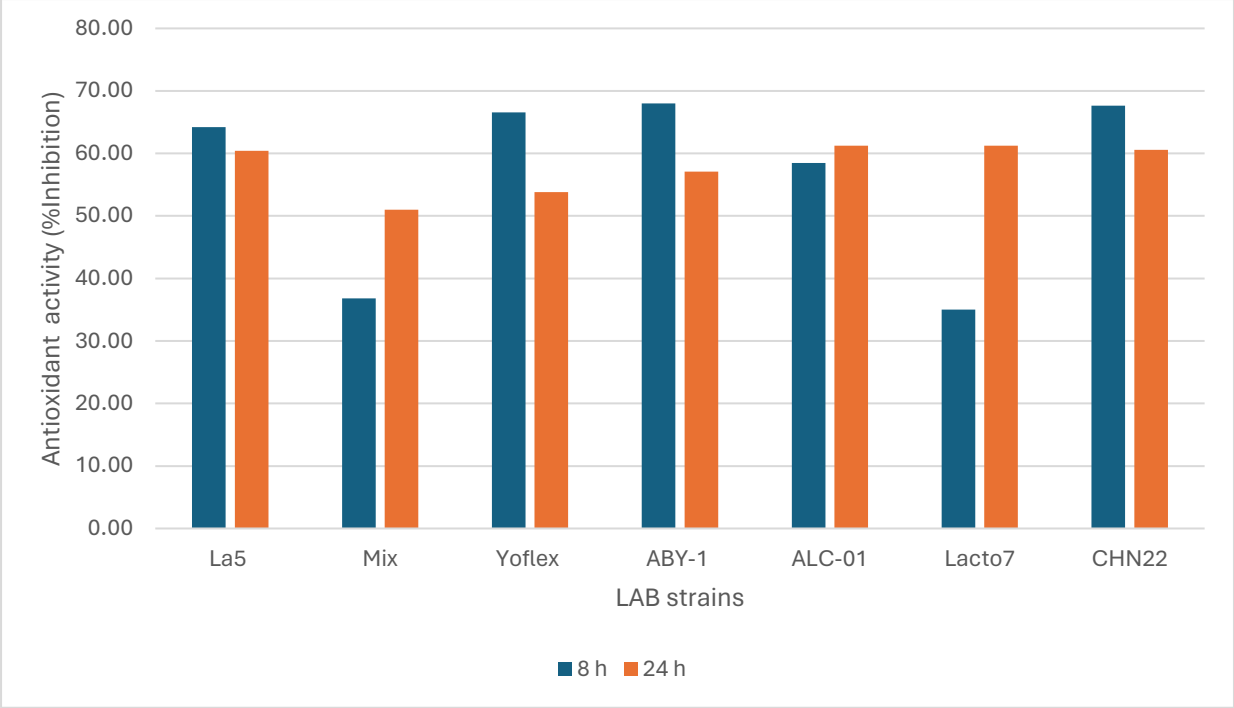

Figure 4: Antioxidant activity of LAB over time with the presence of glucose 2.5%

From the Figure 4, we can conclude that the highest antioxidant activity for the 8 hours of incubation with the presence of glucose was for the strains ABY-1, CHN22, YoFlex with radical scavenging activity of 68.02, 67.625, 66.59.

For the 24 hours incubation with the presence of glucose the highest antioxidant activity was observed for the strains ALC-01, CHN22, LA-5 with RSA % of 61.23, 60.60, 60.40.

We can notice that the antioxidant activity was higher in the presence of glucose. The connection between antioxidants and glucose lies in the Maillard reaction, where glucose reacts with protein hydrolysates to form Maillard reaction products (MRP). These MRPs have been found to exhibit antioxidant properties, showing strong scavenging activity against reactive oxygen species like hydroxyl radicals and superoxide anions. When protein hydrolysates are combined with glucose in the Maillard reaction, the antioxidative effect is significantly enhanced, leading to improved antioxidant properties (Guérard, & Sumaya-Martinez,2003).

4.1.3. Antimicrobial activity

An antimicrobial test was conducted on all of the mixed cultures of LAB TSB agar against a panel of pathogenic strains including *E. cloacae, Listeria monocytogenes*, *E. coli* 0157:H7, *E.*

coli 8739, and *E. faecalis.* The results were noticed as a clear zone around the colonies of bacteria and was measured in mm. The results are shown in Table 4 and Table 5.

Table 4: Antimicrobial activity of the different LAB cultures with the absence of glucose present by mm clear zone

Strain/ pathogen	***Listeria***		***E.cloacae***		***E.coli* 8739**		***E.coli* 0157:H7**		***E.faecalis***		**sum**
Time	**8 h**	**24 h**	**8 h**	**24 h**	**8 h**	**24 h**	**8 h**	**24 h**	**8 h**	**24 h**	
LA-5	1	0	0	0	0	0	0	0	0	0	1
Mix	1	0	0	0	0	0	0	0	0	0	1
YoFlex	1	0	1	0	0	0	0	0	0	0	**2**
ABY-01	1	0	1	0	0	1	0	2	0	2	**7**
Lacto7	1	0	0	0	0	0	0	0	0	0	1
ALC-01	1	0	0	0	0	0	0	0	0	0	1
CHN22	1	1	0	0	0	1	0	0	0	0	**3**
sum	7	1	2	0	0	2	0	2	0	2	-

Based on the results from Table 4 we can conclude that the strongest culture to inhibit the growth of pathogens was ABY-1 followed by CHN22 and then YoFlex.

And the most resistant pathogenes were *E.coli* 0157:H7, *E.coli* 8739 and *E.faecalis* for 8 hours of incubation and the absance of glucose, and the least resistant pathogen was *Listeria*.

The most resistance pathogens for 24 hour incubation with the absence of glucose was *E.cloaceae,* followed by *Listeria* and there weren't least resistant pathogenes because they are all had the same results.

Table 5: Antimicrobial activity of the different LAB strains with the presence of glucose present by mm clear zone

Strain/ pathogen	***Listeria***		***E.cloacae***		***E.coli* 8739**		***E.coli* 0157:H7**		***E.faecalis***		**sum**
Time	8 h	24 h	8 h	24 h	8 h	24 h	8 h	24 h	8 h	24 h	
La5	1	1	0	1	1	2	0	1	0	1	8
Mix	1	3	1	2	0	2	1	3	1	0	**14**
YoFlex	0	2	0	1	0	1	1	3	0	1	9
ABY	0	3	1	1	1	1	1	3	1	1	13
Lacto7	1	3	1	3	0	2	2	2	1	1	**16**
ALC-01	1	3	1	3	1	3	2	3	2	1	**20**
CHN22	1	1	0	0	0	1	1	0	0	0	4
Sum	13	16	4	11	3	12	8	15	5	5	-

From the Table 5 we can conclude that the strongest strain to inhibit the growth of pathogens was ALC-01 followed by Lacto7 and the Mix.

And the most resistant pathogenes were *E.coli* 8739 and *E.cloacae* for 8 hours of incubation and the presence of glucose, and the least resistant pathogen was *Listeria*.

The most resistance pathogens for 24 hour incubation with the presence of glucose was *E.faecalis,* and the least resistant pathogenes were *Listeria* and *E.coli* 0157:H7.

Lactic acid bacteria (LAB) strains possess probiotic potential, enabling them to inhibit pathogens (Unban et al., 2021). LAB-produced lactic acid demonstrates antimicrobial effects against various pathogens (Hossain et al., 2022). Additionally, LAB-derived lactic acid and its metabolites, including organic acids and bacteriocins, effectively suppress the growth of pathogenic bacteria and viruses, as observed in the study by Daliri *et al*. (2020). Furthermore, LAB are recognized for efficiently inhibiting the growth of multidrug-resistant bacteria, a process that can be further stimulated by glucose. Glucose enhances the production of lactic acid, acetic acid, hydrogen peroxide, and bacteriocins by LAB, all of which possess potent antimicrobial properties (Balabekyan *et al*., 2018; Shivsharan *et al*., 2013).

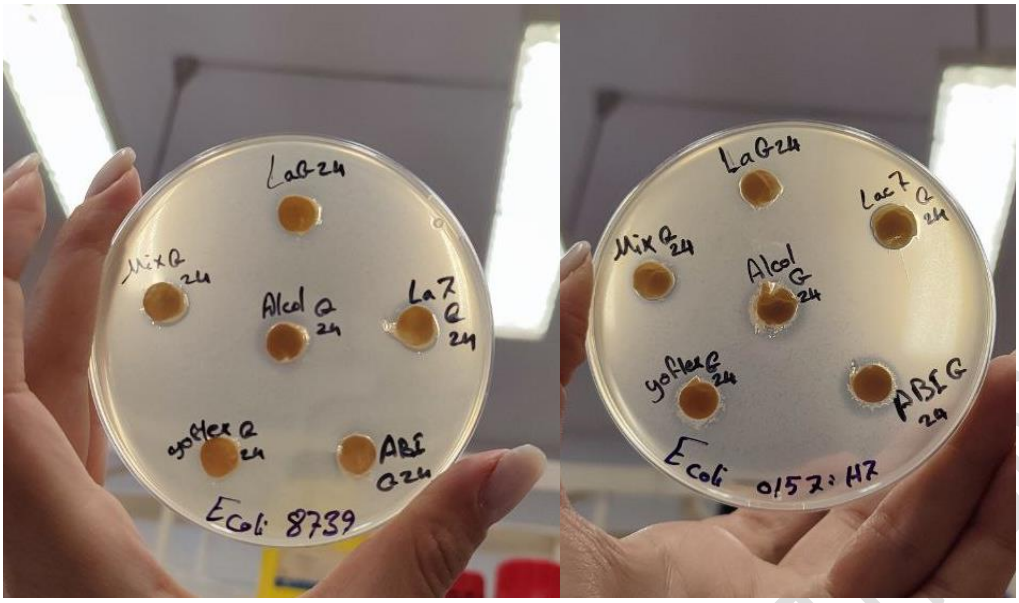

Figure 5: Clear zone around the colonies of LAB cultures from fermented MPC.

4.2. Effect of glucose concentration on MPC fermentation

In this experiment, 3 cultures of LAB (Mix, Lacto7, and ALC-01) were selected based on their superior performance in the prior experiments with a glucose concentration of 1% and 2.5%. These cultures were then evaluated for their pH, antioxidant properties and antimicrobial activity.

4.2.1. pH evaluation

The pH values of the MPC solution with glucose fermented by previously chosen mixed cultures of lactic acid bacteria were monitored over a 24-hour period to assess their acidification potential. The results reveal distinct patterns of acidification among the tested strains and concentrations. pH was measured using a digital pH meter, and the result is shown in Table 6.

Table 6: pH values of LAB strains over time with the presence of 1% glucose

Strain/Time	8 h	16 h	20 h	24 h
Mix	3.92	4.05	4.05	4.00
***Lacto*7**	5.60	5.00	4.22	4.12
***ALC*-01**	4.29	4.12	4.13	4.08

Table 7: pH values of LAB strains over time with the presence of 2.5% glucose

Strain/ Time	8 h	16 h	20 h	24 h
Mix	3.85	3.67	3.59	3.54
***Lacto*7**	5.56	4.96	4.18	4.09
***ALC*-01**	4.16	3.71	3.65	3.58

- At both 1% and 2.5% glucose concentrations, Mix and ALC-01 strains exhibited relatively lower pH values compared to Lacto7 strains. Mix at MPC with 2.5% glucose concentration consistently demonstrated the lowest pH values across all time points, indicating strong acidification potential. After 8 hour of fermentation the pH was under 4 and reach pH 3.54 at the end of the fermentation. ALC-01 strains also showed notable acidification, especially at a higher concentration of glucose 2.5%. For example, ALC-01 at 2.5% glucose showed a pH of 3.65 at the 20-hour mark while Lacto7 showed a value of 4.18.
- In contrast, Lacto7 strains exhibited higher pH values, indicating weaker acidification compared to Mix and ALC-01 strains.
- The findings suggest that Mix and ALC-01 strains, particularly at higher concentrations, possess stronger acidification abilities compared to Lacto7 strains.
- The rise in samples following the addition of glucose can be attributed to its role as a substrate for lactic acid bacteria, facilitating the production of lactic acid. As more sugar is introduced, the lactic acid bacteria utilize it, leading to increased acidity (Hartati *et al*.,2012) and these results are consistent with the results of Dimitrellou *et al*., (2020) were they noticed that higher glucose concentrations addition led to lower pH values. The pH decreased inversely, as the greater the percentage of sugar used by the lactic acid bacteria, the higher the acidity and the lower the pH value, and this corresponds to (Hartati *et al.,*2012)

4.3. Results of the MPC fermentation with the most promising LAB cultures

4.3.1. Antioxidant activity

The outcomes of the tests were as follows:

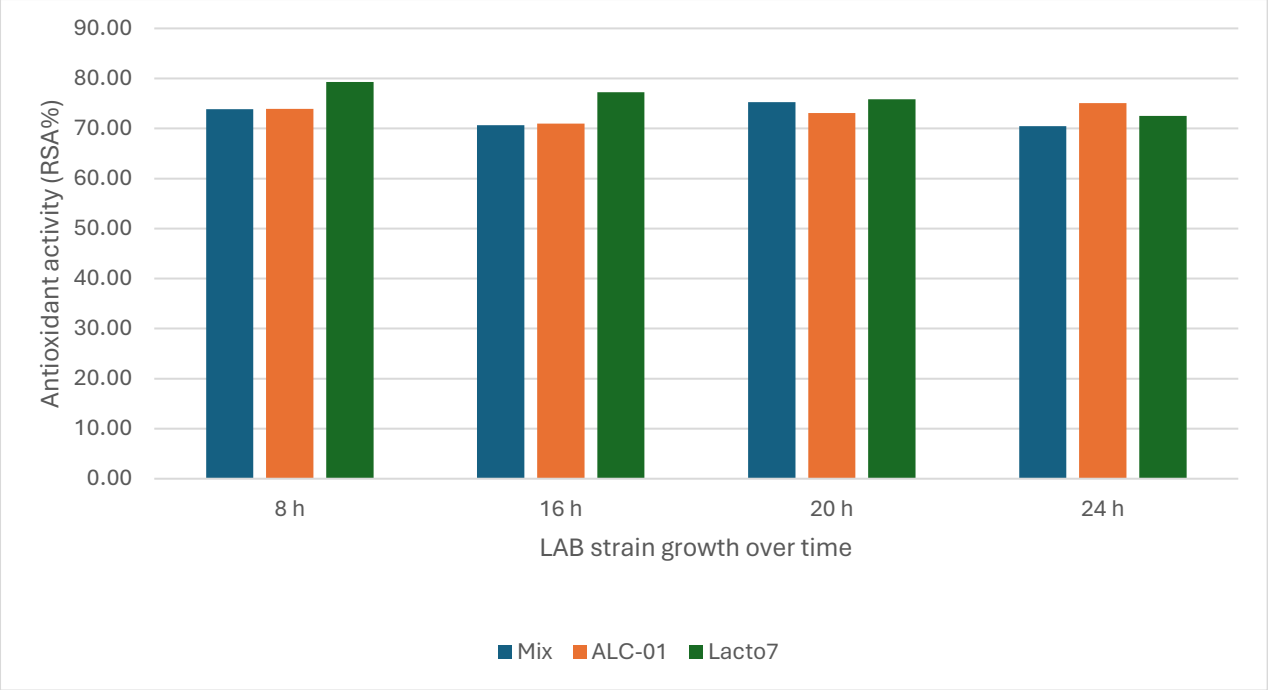

Figure 6: Antioxidant activity of LAB over time with the presence of glucose 1 %

Based on the data depicted in the Figure 6 for the 1% glucose concentration above, several conclusions can be drawn:

- At time point 8- hour fermentation, the antioxidant values for the LAB cultures were observed to vary. The cultures with the highest antioxidant value at this time point was Lacto7, with a value of (79.33 RSA%). Following closely behind was ALC-01 with an antioxidant value of (73.93 RSA%), and Mix with a value of (73.85 RSA%).
- Moving to time 16- hour fermentation, there was a shift in the rankings. Mix exhibited the lowest antioxidant value of (70.67 RSA%), followed by ALC-01 with (70.95 RSA%). Lacto7 had the highest value at this time point, measuring (77.26 RSA%).
- At 20- hour fermentation ALC-01 demonstrated the lowest antioxidant value of (73.10 RSA%), followed by Mix with (75.28 RSA%), were Lacto7 displayed the highest antioxidant value among the strains at this time point, with a value of (75.83 RSA%).
- Finally, at time point 24, Mix had the lowest antioxidant value of (70.48 RSA%). followed by Lacto7 with a value of (72.50 RSA%), and ALC-01 exhibited the highest antioxidant value of (75.08 RSA%) at this time point.

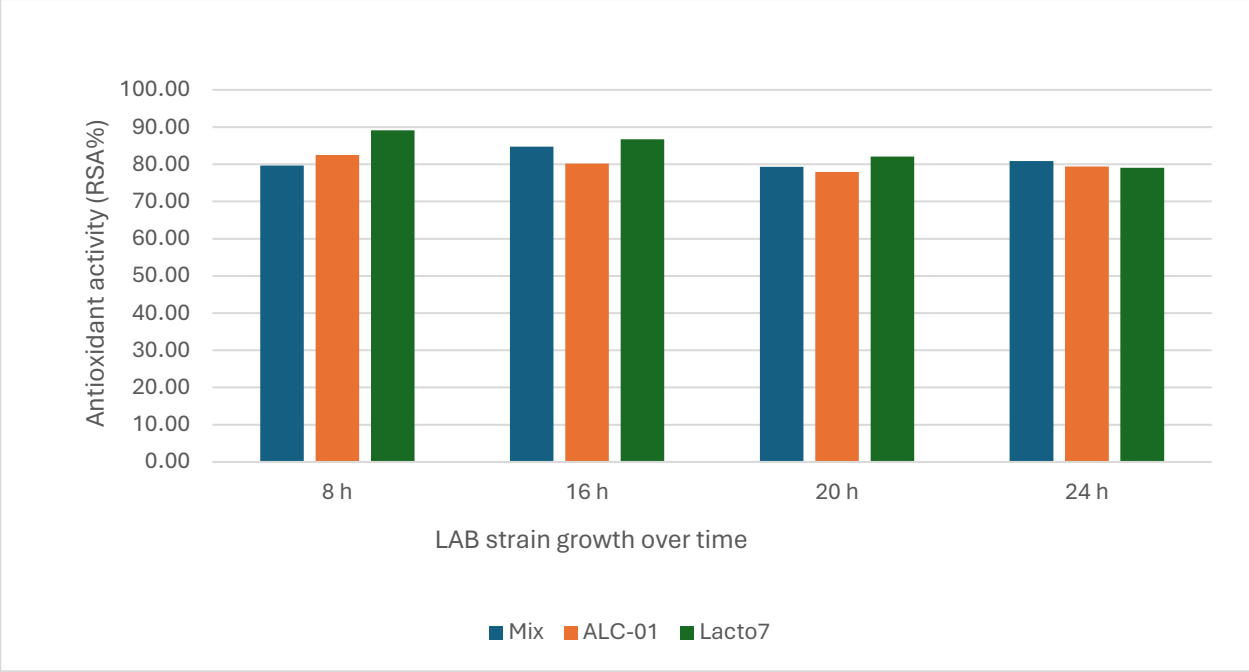

Figure 7: Antioxidant activity of LAB over time with the presence of glucose 2.5%

- It was noticed that the antioxidant activity was higher in the presence of glucose.

Moreover, higher sugar content available for lactic acid bacteria can lead to increased antioxidant activity due to that the fermentation process enhancing phenolic compounds (De Montijo-Prieto *et al.*, 2023).

Based on the data depicted in the Figure 7 for the 2.5% glucose concentration above, several conclusions can be drawn:

- At the 8-hour fermentation with a glucose concentration of 2.5%, the Lacto7 strain exhibited the highest radical scavenging activity (RSA %) value 89.16 RSA%, indicating its superior antioxidant activity during this incubation period. This was followed by the ALC-01 strain with an absorbance value of 82.5 RSA%, and then the Mix strain with a value of (79.70 RSA%).
- During the 16-hour incubation period, Lacto7 maintained its superiority with a radical scavenging activity (RSA %) value of 86.70 RSA%, followed by the Mix culture (84.76 RSA%), and then ALC-01.
- By the 20-hour mark, Lacto7 continued to outperform the other strains with a radical scavenging activity (RSA %) value of (82.10 RSA%), followed by the Mix (79.36 RSA%), and then ALC-01(77.93 RSA%).

- At the 24-hour mark, a slight shift was observed where the Mix strain showed superiority with an absorbance value of (80.91 RSA%). The absorbance values for ALC-01 and Lacto7 were close (79.44 RSA% and 79.04 RSA%, respectively) during this incubation period.

We can conclude that :

- At 1% and 2.5% glucose, Lacto7 consistently showed the highest absorbance radical scavenging activity throughout most of the incubation period (except at 24 hours).

4.3.2. Antimicrobial activity

The experiment to assess the antimicrobial activity was continued on the previously chosen strains (Mix, Lacto7, and ALC-01) against a panel of pathogenic strains. The results were noticed as a clear zone around the colonies of bacteria and was measured (mm) as shown in Tables 8, 9.

Table (8): Antimicrobial activity of ALC-01 with the presence of 1% and 2.5% glucose presented by mm clear zone

ALC-01 1% / Time	**mm**				**Sum**
	8 h	**16 h**	**20 h**	**24 h**	
E.cloacae	1	1	1	1	4
Listeria	0	1	1	1	3
***E. coli* 0157**	1	0	1	0	2
***E.coli* 8739**	0	0	0	0	0
E.faecalis	0	1	0	0	1
sum					**10**
ALC-01 2.5% /Time	**mm**				**Sum**
	8 h	**16 h**	**20 h**	**24 h**	
E.cloacae	2	1	3	2	8
Listeria	1	2	2	1	6
***E.coli* 0157**	2	2	2	3	9
***E.coli* 8739**	1	1	1	3	6
E.faecalis	2	1	1	1	5
sum					**34**

Table (9): Antimicrobial activity of the Mix with the presence of 1% and 2.5% glucose presented by mm clear zone

Mix 1% /Time	**mm**				**Sum**
	8 h	**16 h**	**20 h**	**24 h**	
E.cloacae	1	1	1	0	3
Listeria	0	0	0	1	1
***E.coli* 0157**	1	0	1	0	2
***E.coli* 8739**	0	0	0	1	1
E.faecalis	0	0	0	0	0
sum					**7**
Mix 2.5% /Time	**mm**				**Sum**
	8 h	**16 h**	**20 h**	**24 h**	
E.cloacae	2	2	2	3	9
Listeria	1	2	1	1	5
***E.coli* 0157**	1	2	2	2	7
***E.coli* 8739**	1	1	1	2	5
E.faecalis	1	1	1	2	5
sum					**31**

Table (10): Antimicrobial activity of Lacto7 with the presence of 1% and 2.5% glucose presented by mm clear zone

Lacto7 with 1% glucose	**mm**				**Sum**
	8 hour	**16 hour**	**20 hour**	**24 hour**	
E.cloacae	0	0	0	1	1+
Listeria	0	0	1	0	1
***E.coli* 0157**	0	0	0	0	0
***E.coli* 8739**	0	0	0	0	0
E.faecalis	0	0	0	0	0
sum					**2**
Lacto7 2.5% /Time	**mm**				**sum**
	8	**16**	**20**	**24**	
E.cloacae	1	1	1	1	4
Listeria	1	1	1	1	4
***E.coli* 0157**	0	0	0	1	1
***E.coli* 8739**	0	0	0	0	0
E.faecalis	1	0	0	1	2
sum					**11**

Based on the data shown on the Tables 8,9 and 10 we concluded the following :

- The antimicrobial activity of different LAB cultures against various pathogens was assessed over a period of 24 hours. The results indicate varying degrees of effectiveness among the tested strains and concentrations.
- The most sensitive pathogen to lactic acid strains is *E. coli* 8739. This can be seen in the sum column where *E. coli* 8739 has the lowest overall values, followed by *E.faecalis.*
- Among the tested strains, ALC-01 MPC with 2,5% glucose exhibited the highest overall antimicrobial activity with a sum score of 34, followed by Mix 2.5% with a sum score of 31. These two strains, particularly at the higher concentration of 2.5%, consistently demonstrated significant inhibition against all tested pathogens over the 24-hour period.
- On the other hand, Lacto7 strains showed limited antimicrobial activity, especially at the lower concentration of glucose, with sum scores of only 2 and 11 for Lacto7 at MPC with 1% and 2.5% glucose, respectively.
- Overall, the findings suggest that AlC-0l and Mix strains, particularly at a glucose concentration of 2.5%, hold promise as potential candidates for antimicrobial applications against the tested pathogens.
- That maybe can be referred to the fact that mixed cultures of lactic acid bacteria strains can inhibit pathogens due to their probiotic potential and antioxidant properties (Unban et al., 2021) leading to their growth suppression.

Figure 8: Clear zone around the colonies of LAB strains

4.3.3. Antimicrobial activity assess after modifying the pH

The experiment to assess the antimicrobial activity was continued on the previously chosen strains (Mix, Lacto7, and ALC-01) after modifying the pH of 20 and 24 hours samples were the lower pH values was conducted.

.This procedure was carried out to neutralize the acidic environment, which could potentially hinder the growth of pathogenic strains. The results were noticed as a clear zone around the colonies of bacteria and was measured (mm) as shown in Table 11,12, and 13.

Table 11: Antimicrobial activity of the Mix with the presence of 1% and 2.5 % glucose after modifying the pH presented by mm clear zone.

strain Mix	**MPC with 1% glucose**		**MPC with 2.5 % glucose**	
	20 h	**24 h**	**20 h**	**24 h**
E.faecalis	**1**	**1**	**1**	**1**
E.cloacae	**0**	**1**	**1**	**1**
Listeria	**1**	**0**	**1**	**1**
***E.coli* 0157:H7**	**0**	**0**	**1**	**1**
***E.coli* 8739**	**1**	**1**	**0**	**1**

Table 12: Antimicrobial activity of the Lacto 7 with the presence of 1% and 2.5 % glucose after modifying the pH presented by mm clear zone

strain Lacto 7	**MPC with 1% glucose**		**MPC with 2.5 % glucose**	
	20 h	**24 h**	**20 h**	**24 h**
E.faecalis	1	0	1	1
E.cloacae	0	0	1	1
Listeria	1	0	1	1
***E.coli* 0157:H7**	0	1	1	1
***E.coli* 8739**	0	0	1	1

Table 13: Antimicrobial activity of the ALC-01 with the presence of 1% and 2.5 % glucose after modifying the pH presented by mm clear zone

strain ALC-01				
	MPC with 1% glucose		MPC with 2.5 % glucose	
	20 h	24 h	20 h	24 h
E.faecalis	0	1	0	0
E.cloacae	0	0	0	1
Listeria	0	0	0	0
***E.coli* 0157**	0	0	0	0
***E.coli* 8739**	0	0	0	0

Based on the antimicrobial effect results of lactic acid strains against pathogens, we can draw the following conclusions:

- The most sensitive pathogen to lactic acid strains at 20 hours was *Listeria* and *E.faecalis* while *E.cloacae*, *E. coli* 0157:H7, and *E.coli* 8739 showed more resistance.
- The less sensitive pathogen to lactic acid strains at 24 hours was *Listeria* followed by *Ecoli* 0157:H7 and *E.coli* 8739. While *E.faecalis* and *E.cloacae* were the most sensitive.
- After 20 Hours of Incubation:

  Mix 1% and Mix 2.5% showed consistent inhibition against *E.faecalis, Listeria,* and *E.coli* 0157:H7.

  Lacto7 strains at both concentrations (1% and 2.5%) exhibited inhibition against *E.faecalis* and *Listeria.*

  ALC-01 strains did not demonstrate significant inhibition against any of the tested pathogens at either concentration.
- After 24 Hours of Incubation:

  Mix 1% and Mix 2.5% maintained their inhibition against *E.faecalis, Listeria*, and *E.coli* 8739, with Mix 2.5% additionally inhibiting *E.coli* 0157.

  Lacto7 strains showed varied results: Lacto7 1% exhibited inhibition only against *E.coli* 0157:H7, while Lacto7 2.5% showed inhibition against all tested pathogens.

  ALC-01 1% demonstrated inhibition against *E.faecalis*, whereas ALC-01 2.5% inhibited only *E.coli 8739.*
- Overall:

Mix strains, especially at the higher concentration of 2.5%, consistently demonstrated broad-spectrum antimicrobial activity against the tested pathogens.

Lacto7 strains showed moderate antimicrobial activity, with Lacto7 at MPC with 2.5% glucose exhibiting better inhibition compared to Lacto7 at MPC with 1% glucose.

ALC-01 strains exhibited minimal antimicrobial activity, with ALC-01 1% showing slight inhibition against *E.faecalis*.

Conclusion and Suggestions

In this comprehensive study, I delved into the antioxidant and antimicrobial potentials of various lactic acid bacteria (LAB) cultures, shedding light on their applicability in food and pharmaceutical industries. The investigation encompassed the influence of glucose concentration and incubation time on these properties, offering valuable insights for future research and practical applications.

- It was found that the presence of glucose generally bolstered the antioxidant activity of LAB cultures, with Lacto7 emerging as a standout performer across different glucose concentrations and incubation times. This underscores the significance of understanding the interplay between fermentation conditions and antioxidant production in harnessing the full potential of LAB cultures.
- Moreover, my findings highlighted the substantial antimicrobial efficacy of ALC-01 and Mix strains, particularly at higher glucose concentrations. This suggests their potential utility in combating pathogenic bacteria, paving the way for the development of natural antimicrobial agents with broad-spectrum activity.
- Additionally, the distinct acidification patterns observed among LAB cultures underscored the intricate relationship between acid production, antioxidant, and antimicrobial properties. This opens avenues for further exploration into the mechanistic underpinnings of LAB-mediated inhibition of pathogen growth and the production of bioactive compounds.

Suggestions

Building upon these findings, several avenues for further research emerge:

1. **Mechanism of Action:** Investigate the specific compounds responsible for the antioxidant and antimicrobial properties of LAB cultures. Understanding the mechanisms underlying their activity could facilitate targeted interventions to enhance their efficacy.
2. **Food Applications:** Assess the feasibility of incorporating LAB cultures into food products, considering their impact on sensory attributes, shelf life, and overall functionality. This could unlock opportunities for the development of functional foods with enhanced nutritional value and extended shelf life.
3. **Synergy and Combination Effects:** Explore the synergistic effects of combining LAB cultures with complementary properties. Investigating potential synergies between

strains with high antioxidant activity and strong antimicrobial activity could lead to the development of novel formulations with enhanced bioactivity.

4. **Long-Term Stability and Safety:** Conduct rigorous studies to ensure the long-term stability and safety of LAB cultures for industrial applications. Evaluating their viability and safety profile under various storage conditions is crucial for their successful integration into commercial products.

Addressing these research directions could deepen our understanding of the therapeutic potential of LAB cultures and accelerate their translation into valuable functional ingredients for diverse applications.

Summary

In summary, this study elucidated the antioxidant and antimicrobial potentials of lactic acid bacteria (LAB) cultures under varying fermentation conditions. The findings underscored the importance of glucose concentration and incubation time in modulating the bioactivity of LAB cultures, with implications for their practical applications in food preservation and healthcare.

The most important Key Findings

- **Antioxidant Activity:**
    - Glucose presence generally enhanced antioxidant activity for all LAB cultures.
    - Lacto7 displayed the highest overall antioxidant activity at 1% and 2.5% glucose concentration (except for Mix at 24 hours with 2.5% glucose).
- **Antimicrobial Activity:**
    - ALC-01 with 2.5% glucose exhibited the strongest overall antimicrobial activity.
    - Mix strains, particularly at 2.5% glucose, demonstrated broad-spectrum antimicrobial activity.
    - Lacto7 showed moderate antimicrobial activity, with better performance at 2.5% glucose concentration.
    - E. *coli* 8739 was the most sensitive pathogen to lactic acid strains.
    - Neutralizing the acidic environment (via pH adjustment) generally reduced the zone of inhibition for most strains against most pathogens.

By harnessing the antioxidant and antimicrobial properties of LAB cultures, we can pave the way for the development of innovative solutions to address pressing challenges in food safety and public health. Through continued research and collaboration, we can unlock the full potential of LAB cultures as valuable resources for promoting human health and well-being.

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DECLARATION

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