

**PRODUCTION AND NUTRIENT ANALYSIS OF NOVEL MILLET AND
FRUIT JUICE-BASED PROBIOTIC BEVERAGE USING PROBIOTIC
LACTIC ACID BACTERIAL STRAINS**

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

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I at this moment declare that the presented work in the thesis entitled “**Production and nutrient analysis of novel millet and fruit juice based probiotic beverage using probiotic lactic acid bacterial strains**” in fulfillment of the degree of **Doctor of Philosophy (Ph.D.)** is the outcome of research work carried out by me under the supervision Dr. Arun Karnwal, working as Professor in the Department of Microbiology of Lovely Professional University, Punjab, India. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made whenever the work described here has been based on the findings of another investigator. The work has not been submitted in part or whole to any other University or Institute for the award of any degree.

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CERTIFICATE

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ABSTRACT

In recent years, the popularity of probiotic-rich foods has surged due to their potential health benefits. This study focuses on creating a millet-based probiotic fermented beverage by isolating, characterizing, and utilizing lactic acid bacteria (LAB) strains to produce a nutritious product with health-promoting properties. Sixty LAB isolates were obtained from various food sources and screened for specific characteristics associated with probiotic properties. Sixteen isolates were further analyzed for metabolic capacities under different temperature and salt content conditions, showing features typical of LAB.

Twelve isolates were evaluated for probiotic attributes, including tolerance to low pH, bile salt concentrations, and antimicrobial activity. Two isolates, CM1 and OS1, demonstrated promising probiotic characteristics and were identified as *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*, respectively, through molecular identification using 16S rRNA sequencing. These strains were chosen as starter cultures for beverage development due to their resilience and potential health benefits.

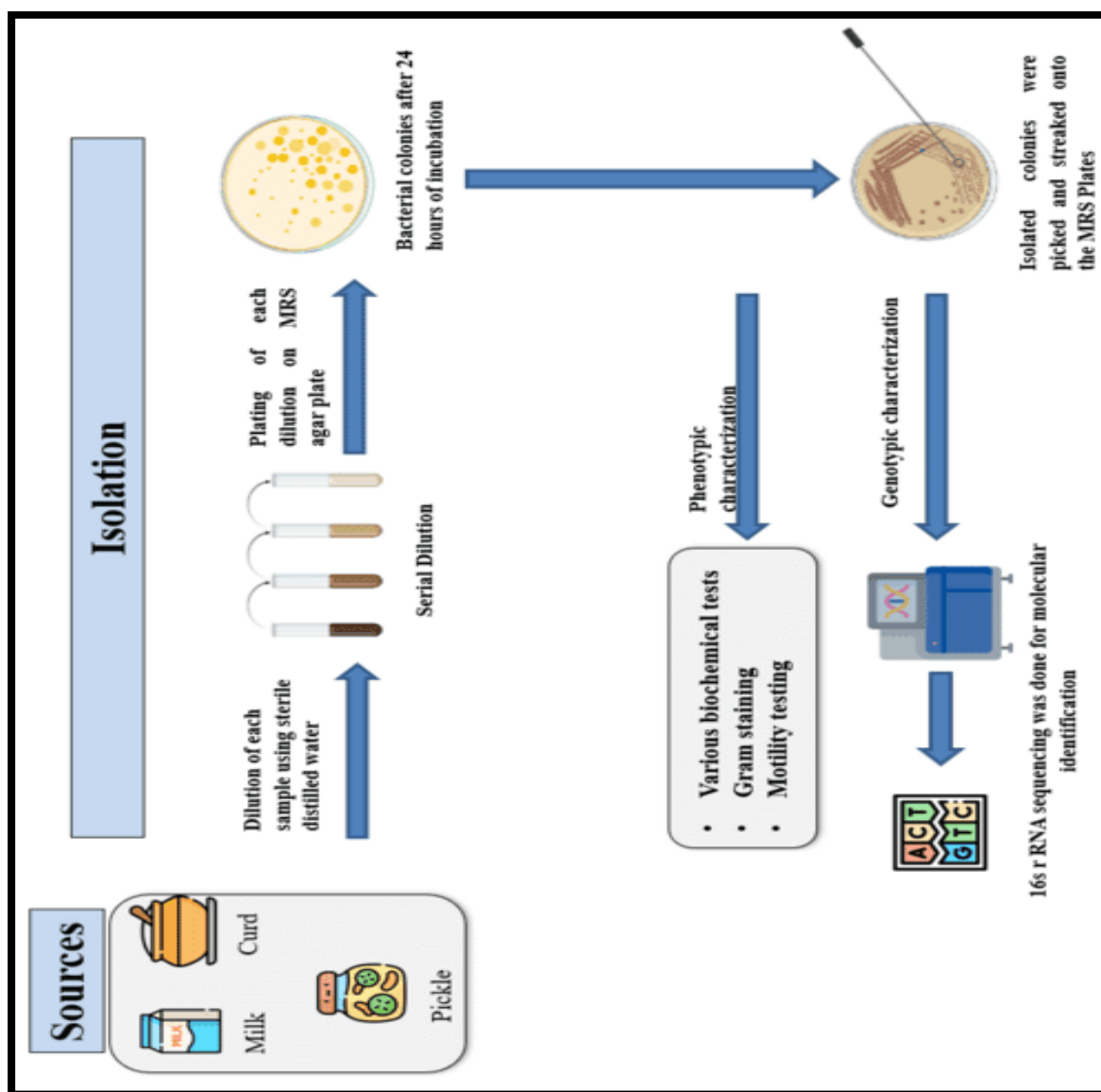
The selected isolates' media components and growth optimization were carried out using the Plackett-Burman design and Response Surface Methodology. Three millet varieties (finger millet, foxtail millet, and barnyard millet) were evaluated for their physicochemical characteristics, showing promise for beverage formulation. Different blends of millet with pineapple and apple juices were prepared, and fermentation with the selected LAB strains was conducted at 37°C for 48 hours.

During fermentation, minimal changes were observed in protein, ash, and fiber content, while significant alterations were noted in viable LAB count and pH, indicating metabolic activity. Quality assessment of the beverage formulations based on chemical characteristics, sensory attributes, and microbial viability revealed that a 50:50 blend of foxtail millet milk and apple juice performed the best, exhibiting higher protein, ash, fiber content, viable LAB count, and better sensory scores.

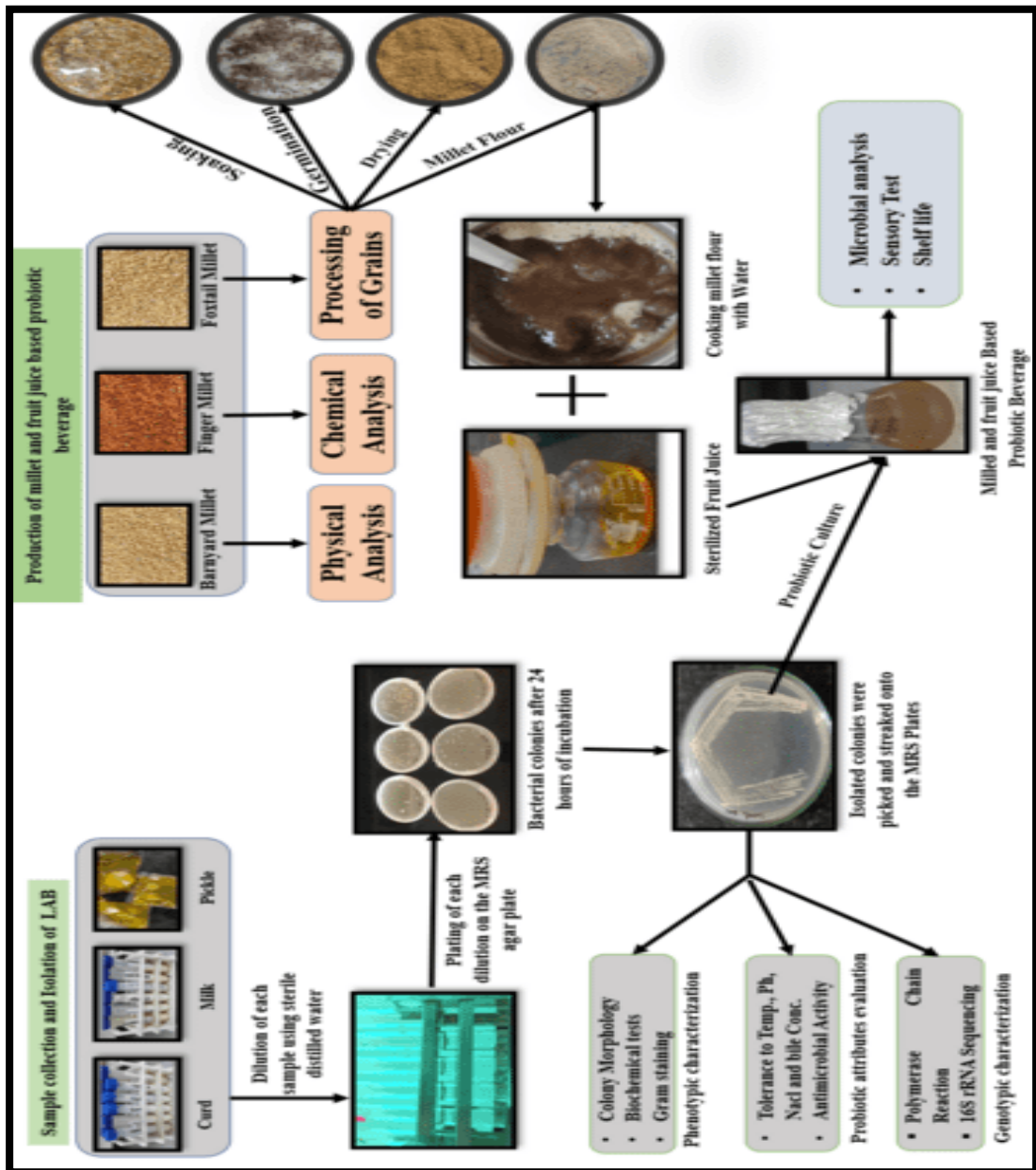
Packaging and storage conditions were evaluated, with glass bottles stored under refrigerated conditions deemed optimal for maintaining product quality and microbial viability over time.

In conclusion, this study successfully developed a millet-based probiotic fermented beverage through a comprehensive process involving LAB isolation, characterization, strain selection, and formulation optimization. The chosen beverage formulation, consisting of a blend of foxtail millet milk and apple juice fermented with *Lactobacillus acidophilus* and *Lactobacillus delrueckii*, demonstrated superior quality and acceptability. These findings highlight the potential of utilizing local millet varieties and selected LAB strains to create nutritious and health-promoting beverages. Furthermore, the study emphasizes the importance of packaging and storage conditions in maintaining product quality and microbial viability. This research contributes to developing sustainable and nutritious food options with potential consumer health benefits.

GRAPHICAL ABSTRACT 1



GRAPHICAL ABSTRACT 2



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ABBREVIATION USED

| S.No. | Abbreviation | Full Form |
|-------|--------------|---|
| 1. | LAB | Lactic Acid Bacteria |
| 2. | L. | Lactobacillus |
| 3. | CM | Cow Milk |
| 5. | BM | Buffalo Milk |
| 6. | C | Curd |
| 7. | OS | Oral Cavity |
| 8. | FB | Fermented batter |
| 9. | MR | Methyl Red |
| 10. | VP | Voges Proskauer |
| 11. | KIA | Kliger's Iron Agar |
| 12. | PBD | Plackett-Burman Design |
| 13. | RSM | Response Surface Methodology |
| 14. | CCD | Central Composite Design |
| 15. | DOE | Design of Experiments |
| 16. | SED | Statistical Experimental Design |
| 17. | ONPG | ortho-Nitrophenyl- β -galactoside |
| 18. | DNA | Deoxyribonucleic acid |
| 19. | RPM | Rotation Per Minute |
| 20. | PCR | Polymerase Chain reaction |

| | | |
|-----|-------|--------------------------------|
| 21. | DNTPs | Deoxy-nucleoside triphosphate |
| 22. | SPC | Standard Plate Count |
| 23. | CFU | Colony Forming Unit |
| 24. | MRS | De Man, Rogosa and Sharpe agar |
| 25. | OD | Optical Density |
| 26. | GIT | Gastrointestinal Tract |
| 27. | IBD | Inflammatory Bowel Disease |
| 28. | IBS | Irritable bowel syndrome |
| 29. | LDL | Low-density Lipoprotein |
| 30. | HDL | High-Density Lipoprotein |

Chapter – 1

INTRODUCTION

1. INTRODUCTION

Plant-based beverages have gained significant traction in the food market year after year, mainly due to their popularity as health-conscious alternatives to traditional dairy products. Several factors drive this trend, including increasing awareness of the health benefits associated with plant-based diets and concerns about the environmental impact of animal agriculture (Shori, 2015). Additionally, individuals with dietary allergies or intolerances often turn to plant-based options as substitutes for cow's milk. These beverages offer diverse ingredients, including grains, legumes, fruits, and vegetables, making them an appealing choice for consumers seeking variety in their diets. One specific area of interest is the growing demand for non-dairy probiotic beverages. With rising concerns about cholesterol levels in dairy-derived probiotic products, consumers are turning to alternatives made from grains, organic produce, and vegetable juices (Sridharan and Das, 2019). These beverages are cost-effective and rich in phytochemicals, therapeutic substances, and dietary fiber. They appeal to individuals with lactose intolerance and are often fortified with cancer-preventive agents, making them part of a healthy diet (Blandino et al., 2003).

Millet-based beverages, however, have yet to gain widespread acceptance despite their nutritional benefits. Millet, known for its resilience and gluten-free properties, is rich in polyphenols, fiber, minerals, and vitamins (Sarita & Singh, 2016). Incorporating millet into probiotic beverages can enhance their nutritional value and support digestive health (Vasudha & Mishra, 2013). Probiotic millet beverages offer a tangy flavor profile and provide numerous health benefits, including improved intestinal health and support for individuals with specific dietary restrictions (De Stefano et al., 2017).

To produce non-dairy probiotic beverages, a formulation combining beneficial bacterial strains with a suitable liquid base is required (Hassan et al., 2012). This involves selecting a non-dairy liquid, such as millet or almond milk, sterilizing the base, culturing the chosen probiotic strains, fermenting as necessary, adding flavorings, and ensuring proper packaging and quality control measures (Kumar et al., 2020). These beverages offer a wholesome and gut-friendly alternative to traditional drinks, supporting balanced nutrition and overall wellness (Navyashree et al., 2022).

Millets

Millets, a collection of small-seeded grasses, are cultivated worldwide as cereal crops and provide sustenance for one-third of the world's population. They are edible crops and the sixth most significant cereal crop in agricultural production worldwide (McDonough et al., 2000). Various types of millet grains exist, with popular varieties including finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), barnyard millet (*Echinochloa utilis*), pearl millet (*Pennisetum glaucum*), Kodo millet (*Paspalum setaceum*), proso millet (*Panicum miliaceum*), and little millet (*Panicum sumatrense*). These millets exhibit notable characteristics, particularly their resilience as drought-resistant crops. They also possess natural resistance to pests and diseases, feature short growth periods, and demonstrate productivity in harsh climatic conditions, surpassing major cereals (Saleh et al., 2013). Millets hold immense potential for expanding genetic diversity within the food market and enhancing food security and nutrition (Mal et al., 2010). There are several types of millet, each with distinct characteristics and culinary uses. Here are some commonly known millet types:

- **Pearl Millet (*Pennisetum glaucum*):** This millet is a most grown variety. It has large, round grains and a mild, nutty flavor. Pearl millet is highly drought-resistant and is a main crop in arid regions of Africa and India. It is primarily used for making flatbreads, porridge, and fermented beverages (Rani et al., 2018).
- **Finger Millet (*Eleusine coracana*):** It is also known as ragi and is an essential crop in Africa and Asia. The grains are small and finger-shaped, hence the name. Finger millet is rich in fiber, iron, and calcium, and it is commonly used to make porridge, rotis (Indian bread), and other dishes (Chandra et al., 2020).
- **Foxtail Millet (*Setaria italica*):** This small-grain millet is cultivated in East Asia, India, and Africa. Foxtail millet is gluten-free and rich in protein and fiber. It is used in various preparations, such as porridge, rice substitutes, and baked goods (Sharma et al., 2018).
- **Proso Millet (*Panicum miliaceum*):** An ancient grain widely cultivated in Europe, Asia, and North America. It has small, round grains and a mild, nutty flavor. Proso millet is versatile and can be used in soups, salads, side dishes, and even rice substitutes (Das et al., 2019).

- **Barnyard Millet (*Echinochloa frumentacea*):** It is also identified as Sanwa millet and is commonly grown in Asia. The grains are small, white, and round. Barnyard millet has a slightly sweet flavor and is often used in porridge, upma, and rice substitutes in various dishes (Renganathan et al., 2020)
- **Little Millet (*Panicum sumatrense*):** Little Millet, also recognized as Samai or Kutki, is a type of Millet that has its place in the Poaceae family. It is one of the small-grain millet varieties widely cultivated in India, Southeast Asia, and parts of Africa. Little Millet has a nutty flavor and a slightly chewy texture. It can be cooked and used as a rice substitute or added to various dishes such as porridge, pilaf, salads, and desserts. In some regions, it is also used to make flour for baking bread, cookies, and other baked goods (Johnson et al., 2019).
- **Kodo millet (*Paspalum setaceum*):** Kodo millet is a small-seeded cereal grain. It comes from the millet family (Poaceae) and is primarily cultivated in India and other parts of Asia. Kodo millet is recognized for its nutritious properties and has been consumed for centuries. It is an excellent source of protein, nutritional fiber, and necessary minerals like iron, potassium, magnesium, calcium, and zinc. Because it does not contain gluten, it is an excellent alternative grain for people with celiac disease or gluten intolerance. (Johnson et al., 2019).

Due to their exceptional nutritional value and high energy content, millets are highly recommended for various groups, including children, convalescents, and older adults. The carbohydrate content ranges from 71.82% to 81.02% in millet grains (Cheik et al., 2006), making it a significant energy source. The protein content of millets is abundant and of good quality, except for a deficiency in lysine (Singh & Raghuvanshi, 2012). Different varieties of millet exhibit varying protein ranges from 12.25% to 13.09% and fat content from 4.32% to 5.11%. (Zhu, 2014). The protein digestibility in millet ranges from 56.29% to 71.15% (Anju, 2010). Additionally, millet contains total sugars ranging from 2.66% to 2.89%, reducing sugars (0.34% to 0.39%) and non-reducing sugars (2.15% to 2.57%). In a study conducted by Abdalla et al. in 2009, the nutritional composition of millet was analyzed. The results showed that millet contained 3.21-6.14% crude fiber, 1.53-2.00% ash, and various minerals in the following amounts per 100 grams: 10.80 mg calcium, 5.3-7.0 mg zinc, 7-18.0 mg iron, 1.8-2.3 mg manganese,

450-990 mg phosphorus, and 1.0-1.8 mg copper. The protein and oil contents of millet were equal to or superior to those of rice, corn, and sorghum.

Probiotics

The term "probiotic" was coined by an expert commission in 2002 to refer to living microorganisms that provide health benefits beyond essential nutrition when consumed in specific quantities (Isolauri et al., 2002). Typically, probiotics are consumed in quantities of around 10^9 cells per day to achieve the desired effects (Schrezenmeir & de Vrese, 2001). Prebiotics are non-digestible dietary components that promote the growth and activity of beneficial microorganisms in the host's gut, conferring health benefits. Synbiotics combine probiotics and prebiotics to enhance the survival and proliferation of consumed microorganisms in the small intestines.

The term "probiotics" was initially introduced in 1965 by Lilley and Stillwell and later redefined by Fuller in 1992 to include living bacterial supplements that improve microbial balance within the host. Havenaar and Huid further expanded the concept to include simple or complex cultures of viable microbes, administered as dehydrated cultures or fermented products, which beneficially impact the properties of existing microflora. For probiotics to be effective, they must be non-pathogenic, non-toxic, adhere to the gut, metabolize nutrients, and survive gastrointestinal conditions. Throughout history, extensive research has been conducted on live microbial food supplements' probiotic properties, emphasizing their potential to affect host health positively (Williams et al., 2010).

Probiotics, crucial for health, must remain stable and viable under diverse conditions. Recommended bacterial probiotics, such as Lactobacillus species (e.g., *Streptococcus lactis*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus GG*, and *L. casei*), offer numerous health benefits (Socol et al., 2010). Bacterial proteases in probiotics enhance the production of free amino acids, aiding nutrition, especially when endogenous protease production is lacking. Enzymatic hydrolysis by bacteria improves the bioavailability of fats and proteins, while lactic acid bacteria increase vitamin B complex levels in fermented foods. Probiotic fermentation boosts mineral bioavailability, lowers anti-nutrient levels, and enhances the digestibility of proteins and carbohydrates (Ouweland et al., 2002).

Probiotics consist of live beneficial bacteria and yeasts naturally present in the body, counteracting harmful bacteria and promoting well-being. They are vital components of the microbiome, a diverse community of microorganisms working synergistically to maintain body health (Singh et al., 2011). The microbiome encompasses bacteria, fungi, viruses, and protozoa, with each person harboring a unique microbiome composition. Understanding the complexity of the microbiome is akin to visualizing a diverse forest ecosystem, which is crucial for sustaining overall body health and functionality (Fasano, 2022).

To qualify as a probiotic, a microbe must possess specific characteristics, including the ability to:

- Be obtained from a human source.
- Survive the journey through your digestive system after being consumed.
- Demonstrate proven benefits to your health.
- Be safe for consumption.
- Probiotics are beneficial and friendly micro-organisms.
- They could compete with harmful microbes and establish colonies in our digestive system.
- Probiotics aid food fermentation into simpler byproducts and can promote health through various mechanisms.
- Factors such as improper diet, alcohol consumption, and aging can deplete the levels of probiotics in our system. That is why it is essential to include them in a regular diet.
- In specific cases, such as after taking antibiotics that may severely affect probiotic levels, consuming them orally in significant quantities or with food is advisable.
- Probiotics contribute to our health by:
 - a. Counteracting the adverse effects of harmful microbes.
 - b. Providing the body with beneficial byproducts.
 - c. Reducing the workload on the digestive system.

- d. Shielding our digestive system from the initial impact of harmful compounds through their biofilm acts as a protective barrier.
- e. Enhancing digestion and metabolism reduces the amount of food our bodies require.

How do probiotics function?

Probiotics, or beneficial bacteria, preserve a harmonious equilibrium within your body. Imagine it as sustaining a neutral state for the body. When a person falls ill, harmful bacteria penetrate the body and multiply, disrupting the balance (Reid et al., 2017). Good bacteria step in to combat the harmful bacteria and restore balance, resulting in improved well-being. Good bacteria contribute to overall health by supporting immune function and regulating inflammation. Additionally, specific types of beneficial bacteria can:

- Support in the digestion of food.
- Prevent the overgrowth of harmful microorganisms, thus preventing illness.
- Produce essential vitamins.
- Support the integrity of the gut lining, acting as a barrier to prevent harmful bacteria from food or beverages entering your bloodstream.
- Break down and facilitate the absorption of medications.

This balancing act naturally occurs continuously in your body. Probiotic supplements are unnecessary to facilitate this process, as good bacteria are already a natural component of your body. However, consuming a well-rounded diet of fiber daily helps maintain appropriate levels of good bacteria.

Why do we need non-dairy probiotics?

The landscape of probiotic consumption is evolving rapidly, driven by diverse dietary preferences and health considerations. While traditional dairy-based probiotics offer numerous health benefits, they pose challenges for individuals with lactose intolerance or dairy allergies. Non-dairy probiotics are emerging as a vital alternative, catering to these populations' needs while accommodating various dietary choices, such as veganism and plant-based eating.

Lactose intolerance, characterized by insufficient lactase enzyme production, impedes the digestion of lactose in dairy products, causing gastrointestinal discomfort. Non-dairy probiotics provide a solution for individuals with lactose intolerance, offering beneficial bacteria without lactose content. Similarly, dairy allergies, often triggered by specific proteins like casein or whey, necessitate alternatives to dairy-based probiotics to prevent adverse reactions. Non-dairy probiotics serve as a safe option for individuals with dairy allergies, enabling them to access the health benefits of probiotics without compromising their well-being.

Moreover, the popularity of vegan and plant-based diets continues to rise, driven by ethical, environmental, and health considerations. Non-dairy probiotics align with these dietary patterns, ensuring that individuals following vegan or plant-based lifestyles can maintain a healthy gut flora. By offering alternatives to dairy-based sources, non-dairy probiotics enhance accessibility and inclusivity within the probiotics market, catering to a broader spectrum of consumers.

In the United States, a notable trend in the functional food market distinguishes it from Europe by emphasizing nutraceuticals and botanical dietary supplements over fortified foods. However, growing awareness regarding immunity, cancer prevention, and heart health is reshaping consumer preferences toward functional foods. This trend mirrors developments in countries like the United Kingdom, indicating a global shift towards functional food consumption. Plant-based foods dominate the diet in Asia, where dairy and meat consumption are relatively low. Lactose intolerance and cultural dietary habits further limit milk consumption in many Asian populations. Consequently, fruits, vegetables, and cereals emerge as potential substrates for probiotic bacteria, offering alternative avenues for probiotic consumption.

While dairy-based probiotics currently dominate the market, there is a growing demand for non-dairy alternatives. Vegetable-based dietary supplements with minimal cholesterol content are gaining traction, reflecting evolving consumer preferences for healthier options. Advanced technologies enable the modification of structural properties of fruits and vegetables to create suitable substrates for probiotic production, eliminating allergens present in dairy products.

The transition towards non-dairy probiotics addresses several drawbacks associated with dairy consumption, including lactose intolerance, elevated cholesterol levels, and allergenic milk proteins. As the functional food sector continues to expand globally, there is immense potential for developing innovative probiotic dietary alternatives, particularly those derived from plant-based sources. The increasing prevalence of lactose intolerance, dairy allergies, and diverse dietary preferences necessitates the development of non-dairy probiotics. These alternatives accommodate specific dietary restrictions and promote gut health and overall well-being. With growing consumer awareness and technological advancements, the future of probiotics lies in a diverse range of non-dairy options that cater to the evolving needs of consumers worldwide.

Why Millet as a Substrate?

Millets are used in probiotic and prebiotic products to support the existing microbial community in the body or to aid in re-establishing gut bacteria after disruptions caused by antibiotics, chemotherapy, or illness. According to FAO and WHO (2001), probiotics are live microorganisms that provide several health benefits to the host when consumed sufficiently. Probiotic diets typically include fatty acids, vitamins, and other essential nutrients that enhance the body's ability to resist pathogenic micro-organisms. Fermented foods play a significant role in human diets worldwide, accounting for approximately 20 to 40 percent of the global food supply.

In Northern Ghana, Lei and Michaelsen (2006) conducted a fascinating exploratory investigation using naturally fermented millet foods as a natural remedy for gastrointestinal diseases in young children. In addition to their nutritional benefits, probiotic organisms play a significant role in enhancing metabolism. They contribute to various positive effects, such as stimulating the immune system, detoxifying potential carcinogens, reducing constipation, increasing phenol tolerance, and lowering cholesterol levels in the blood (Smoragiewicz et al., 1993). These advantages arise from the growth of viable lactic acid bacteria in probiotic fermented foods. These bacteria produce a diverse range of antimicrobial agents that inhibit the growth of food spoilage and pathogenic bacteria (Shahani et al., 1979).

Moreover, the combination of prebiotics and probiotics has been extensively studied for its impact on various health benefits. However, there have been limited efforts to develop a symbiotic product combining probiotics and prebiotics (Gibson & Roberfroid, 1995). Fermented products utilizing probiotic bacteria have typically been prepared using milk, whey, or juices. Still, no similar work has been conducted using raw and germinated coarse cereals and millets with *L. acidophilus* and *L. delbrueckii* inoculation. The combined effect of germination and fermentation, particularly with probiotic microorganisms, offers additional advantages. This approach not only improves the nutritional quality of the products but also provides therapeutic benefits. Furthermore, adding apple juice to the coarse cereals will enhance their nutrient content.

Chapter – 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Probiotics are live microorganisms, mainly bacteria, that are beneficial to the human body when consumed in adequate amounts. The term "probiotic" comes from the Greek words "pro" (meaning "for") and "bios" (meaning "life"). These microorganisms are commonly found in fermented foods and supplements and have gained significant attention for their potential health benefits. The history of probiotics dates back thousands of years to ancient cultures that recognized the positive effects of fermented foods on human health. However, in the early 20th century, Russian scientist Elie Metchnikoff coined "probiotics" and popularized that certain bacteria could confer health benefits. Metchnikoff observed that people in Bulgaria who consumed large amounts of fermented milk had longer lifespans and attributed this to beneficial bacteria in the fermented milk. According to Lilly and Stillwell, probiotics are microorganisms that promote the growth of other microorganisms. They are highly effective traditional medications characterized as living microbial supplements that can positively impact the host by enhancing its microbial balance in the intestines. In 1953, the term "probiotic" was first introduced by Kollath, who used it to describe natural, synthetic, and inorganic food and nutrient systems that could enhance dietary supplements rather than relying on harmful antimicrobials. Another expert, Vergio, in his publication on gut microbiology titled "Anti-and Probiotika," distinguished between the adverse effects of antibiotics and other antimicrobials under certain circumstances. Probiotics primarily belong to lactic acid bacteria (LAB) and Bifidobacteria. These bacteria are naturally present in our digestive system and are crucial in maintaining gut health and overall well-being. The beneficial effects of probiotics are attributed to their various mechanisms of action. They can improve gut microbiota composition by restoring the balance of beneficial bacteria and preventing the growth of pathogenic bacteria. Probiotics also produce antimicrobial compounds, strengthen the intestinal barrier, modulate the immune system, and aid in digesting and absorbing nutrients.

In recent years, probiotic research has significantly expanded, leading to numerous studies exploring their potential health benefits. Probiotics have been investigated for their role in promoting digestive health, alleviating gastrointestinal disorders such as

IBD and IBS, enhancing immune function, mitigating antibiotic-associated diarrhea, reducing susceptibility to specific allergies, and positively impacting mental health. While probiotics are generally considered safe for most individuals, there may be associated risks, particularly for those with compromised immune systems or significant underlying health conditions. Therefore, seeking advice from a healthcare professional before starting a probiotic regimen is advisable. Overall, probiotics have gained significant popularity and are widely available in various forms, including capsules, tablets, powders, and fermented foods like kefir, yogurt, kimchi, and sauerkraut. Ongoing research aims to uncover additional benefits and applications of probiotics in improving human health (Fuller, 1989). A wealth of evidence suggests that incorporating probiotics into food offers many health benefits. These include reduced serum cholesterol levels, enhanced gastrointestinal function, improved immune system function, prevention of gastroenteritis in young children, and reduced colon cancer risk (Berner & Donnel, 1998). Traditionally, probiotics were primarily associated with lactic acid bacteria and found predominantly in dairy products. However, contemporary consumer preferences have shifted towards non-dairy-based probiotic alternatives, such as vegetables, fruits, and cereal-based products. This shift is due to concerns about cholesterol, lactose intolerance, and the growing trend of vegetarianism, leading to increased demand for alternative probiotic sources.

2.1 History and Background

In the latter half of the 19th century, extensive experimental research on microorganisms shed light on their interactions with the human host. These studies highlighted the beneficial effects of microorganisms in processes such as their interaction with vaginal microbes through the production of lactic acid from sugars, which inhibited the growth of pathogenic microorganisms. The findings and historical records from this period, focusing on the use of essential metabolic agents, were commonly referred to as "Lactic Acid Microorganisms" (LAB) (Escherich et al., 1885). Recent research has further emphasized the importance of a "vital" and "stable" microbial community in the gastrointestinal tract (GIT). Over the past three decades, research has reinforced the symbiotic relationship between LAB and the human host.

One advocate of this idea was Metchnikoff, who proposed that LAB played a crucial role in the longevity of Caucasians who consumed fermented milk. Metchnikoff also suggested that the fermentation of sugars by LAB produced lactic acid. In 1889, another group of microorganisms capable of producing lactic acid, known as Bifidobacteria, was discovered. Although phylogenetically distinct, Bifidobacteria were often recognized as part of the LAB community.

Lactobacillus spp. and *Bifidobacterium* spp. are the predominant probiotic microorganisms used in the food industry. These microorganisms have earned the "Generally Recognized as Safe" (GRAS) designation from certain regulatory bodies. In addition, specific yeast strains like *S. boulardii* and *S. cerevisiae* have demonstrated promising probiotic characteristics (Figuroa-Gonzalez et al., 2011). To integrate probiotics into plant-based foods, strains like *L. plantarum*, *L. casei*, *L. acidophilus*, *L. rhamnosus*, and *Bifidobacterium lactis* can be employed (Martins et al., 2013). Probiotic strains isolated from food sources exhibit greater resilience to temperature and pH fluctuations during production. However, their survival rates in the gastrointestinal tract may be lower than those of strains obtained from the intestine (Klein et al., 1998). When using probiotic microorganisms individually or in combination, it is essential to consider the potential variations in the concentrations and types of metabolites produced during fermentation. These factors can significantly affect the final attributes of the product. For a probiotic food product, it is recommended to incorporate at least 10^6 colony-forming units (CFU) per gram or milliliter, providing a daily dosage of 10^8 – 10^9 live cells (Champagne et al., 2011). However, ensuring the viability of probiotic cells during food processing, transport, and storage poses a significant challenge in probiotic production. Exploring the interactions between food substrates and probiotic bacteria could enhance the viability and efficacy of probiotic food products (Shori, 2016).

Probiotics promote host well-being through several essential mechanisms, including regulating mucosal membrane activity, reducing epithelial cell apoptosis, and promoting mucin production (Saad et al., 2013). They also contribute to synthesizing antimicrobial peptides like defensins and cathelicidins, producing antimicrobial agents

such as bacteriocins and microcins, and creating an inhospitable environment for pathogens by lowering the pH. Probiotics engage in competitive adherence to epithelial cells, directly or indirectly preventing pathogen attachment (Wu et al., 2008). Additionally, probiotics modulate the immune system by inhibiting pro-inflammatory pathways, enhancing mucin production, and interacting with quorum-sensing signals that regulate interactions among pathogenic microorganisms (Medellin-Peña et al., 2007). Numerous scientific investigations have explored the beneficial impacts of probiotics on gastrointestinal health, including fighting infections, demonstrating antimicrobial properties, enhancing lactose metabolism, lowering serum cholesterol levels, boosting immune system activation, exhibiting antimutagenic and anticarcinogenic properties, alleviating diarrhea, mitigating inflammatory bowel disease, and addressing *Helicobacter pylori* infections through the incorporation of specific strains into food products (Heller, 2001). Probiotics are commonly found in natural foods, particularly dairy items like curd, yogurt, and fortified dairy products (Vijayendra & Gupta, 2012). Globally, probiotics are predominantly available in fermented foods and dairy products, serving as primary probiotic carriers (Sanders et al., 2013). However, growing concerns about lactose intolerance, high cholesterol levels, milk protein allergies, high blood pressure, and the saturated fat content of dairy products have led to a shift towards non-dairy alternatives, including fermented cereals and fruit and vegetable juices enriched with probiotics (Vijaya Kumar et al., 2013). Despite these concerns, the perceived health benefits of probiotics have maintained interest among dairy and non-dairy consumers (Ranadheera et al., 2010). As a result, non-dairy probiotic products are becoming a part of daily life. It is important to note that non-dairy probiotic preparations are not a new concept, as various non-dairy forms like cereals and soy have been in production for decades worldwide. The microorganisms used in probiotic formulations often originate from humans or animals, and studies suggest that probiotic strains are commonly found in fermented non-dairy substrates (Schrezenmeir & de Vrese, 2001).

Probiotic formulations intended for consumers generally maintain their safety throughout their storage period and the medium in which they play a crucial role in their viability and interactions with other microbes. However, there is limited

understanding of how the surrounding food matrix and potential drug interactions affect the functionality of probiotics. Additionally, the type of food product significantly influences the growth, physiology, and effectiveness of probiotics in the formulation. When developing functional probiotic diets, selecting an appropriate food system for delivering probiotics is essential (Rouhi et al., 2013). The success of such products in the market depends on maintaining profitability and sensory qualities (Ranadheera et al., 2010). The technological aspects involved in producing probiotic foods directly impact the viability and stability of probiotic cells due to challenges like high temperature, physical stress, or osmotic stress-related cell damage (Bustos & Bórquez, 2013). Research into processing fermented foods, including cereals, soy, and meat, has been ongoing for many years. These fermentation processes often involve mixed cultures of yeast, fungi, and bacteria (LAB). Traditional fermented products serve as the primary host for these microorganisms, showcasing probiotic properties. However, compared to dairy-based options, there is considerably less knowledge about using non-dairy matrices as substrates for probiotic microorganisms. Regarding non-dairy food matrices, understanding the survival mechanisms of microorganisms against stressors, fermentation parameters, their role as initiators or starters, and their interactions with other microorganisms is still limited (Schrezenmeir & de Vrese, 2001). The condition of probiotic cells, the form of the food matrix, and the moisture content all play critical roles in ensuring the survival of probiotics during prolonged storage and processing, especially under extreme heat stress.

In the second quarter of the 19th century, research on microorganisms revealed their interactions with the human host and highlighted their potential benefits for digestion. The positive role of vaginal bacteria was noted for producing lactic acid from sugars, which prevented the growth of harmful pathogens. These findings, along with early developments in biotherapeutic principles and the use of lactic acid bacteria (LAB), emphasized the beneficial role of these microorganisms (Escherich et al., 1886). Recent studies have underscored the importance of maintaining a balanced microbial community in the gastrointestinal tract (GIT). Over the past three decades, research has solidified the understanding of LAB's positive interactions within the host body. Metchnikoff's proposition that the robust health of Caucasians was linked to their

consumption of fermented milk highlighted the beneficial aspect of lactic acid production via LAB fermentation. Another group of lactic acid-producing microorganisms, Bifidobacteria, although taxonomically distinct from LAB, was discovered in 1889 and identified by Tissier in the early 1900s, specifically in the feces of breastfed infants. Infants fed breast milk experienced fewer gastrointestinal disturbances than those fed with non-dairy formulas, indicating a positive correlation between Bifidobacteria and human gastrointestinal health.

2.2 Probiotic explanation and the relevant words

2.2.1 Probiotic

The term "probiotic" originates from Greek, signifying 'for life.' In 1965, Lilly and Stillwell were the first to employ this term, describing it as "substances secreted by one microorganism that fosters the growth of another," drawing a comparison with the term "antibiotic." This optimistic and expansive definition likely contributed to broadening the term "probiotic" to encompass various contexts and assume a more general meaning. In 1971, Sperti applied the term to refer to tissue extracts that stimulate the growth of microbes. The contemporary usage of the term "probiotic" began in 1974 with Parker, who defined probiotics as "organisms and substances that contribute to the microbial balance of the intestine."

The concept established by the World Health Organization (WHO) has been widely adopted. According to the WHO, probiotics are "live microorganisms that confer a health benefit on the host when administered in adequate amounts" (FAO/WHO, 2001). The distinctive attributes of probiotics have been recognized as significant enhancers of health (Table 2.1). Recent literature has primarily focused on investigating experimental conditions and the viability of probiotic strains during preparation and storage. This includes their resilience to low pH, gastrointestinal fluids, bile, pancreatic and intestinal secretions, gastric fluids, and even respiratory secretions. Moreover, research delves into aspects such as the adhesion of probiotics to isolated cells or cell cultures and their interactions with other potentially harmful microorganisms.

Table 2.1: List of probiotic bacteria

| S.No. | Probiotic bacteria Genera | Species involved | Reference |
|--------------|--------------------------------------|--|--|
| 1. | <i>Lactobacillus</i> | <i>L. bulgaricus</i> <i>L. paracasei</i> , <i>L. casei</i> , <i>L. crispatus</i> , <i>L. plantarum</i> , <i>L. reuteri</i> , <i>L. Mgasseri</i> <i>L. rhamnosus</i> <i>L. acidophilus</i> | (Ouwehand & Salminen, 2004; Bunesova et al., 2018) |
| 2. | <i>Propionibacterium</i> | <i>P. freudenreichii</i> , <i>P. jensenii</i> | (Bourdichon et al., 2012) |
| 3. | <i>Peptostreptococcus</i> | <i>P. products</i> | (Farrow et al., 1997) |
| 4. | <i>Lactococcus</i> | <i>L. rhamnosus</i> , <i>L. acidophilus</i> , <i>L. lactis</i> <i>L. curvatus</i> , <i>L. plantarum</i> <i>L. reuteri</i> | (Liu et al., 2017) |
| 5. | <i>Pediococcus</i> | <i>P. acidilactici</i> , <i>P. pentosaceus</i> | (Tamang et al., 2016) |
| 6. | <i>Bacillus</i> | <i>B. coagulans</i> , <i>B. subtilis</i> , <i>B. laterosporus</i> | (Khalil et al., 2014) |
| 7. | <i>Enterococcus</i> | <i>E. faecium</i> | (Cai et al., 2017) |
| 8. | <i>Bifidobacterium</i> | <i>B. animalis</i> , <i>B. longum</i> , <i>B. bifidum</i> , <i>B. catenulatum</i> , <i>B. breve</i> , | (Moro et al., 2020) |
| 9. | Akkermansia | <i>A. muciniphila</i> | (Gore et al., 2018) |
| 10. | Bacteroides | <i>B. uniformis</i> | (Sakamoto et al., 2005) |
| 11. | <i>Streptococcus</i> | <i>S. thermophilus</i> , <i>S. sanguis</i> , <i>S. oralis</i> , <i>S. mitis</i> , <i>S. salivarius</i> | (Gänzle, 2015) |
| 12. | <i>Saccharomyces</i> | <i>S. boulardii</i> | (Guevarra et al., 2017) |

2.2.2 Postbiotics

Recent research suggests bacterial compounds may exert comparable effects on signaling pathways and barrier function even without viable bacterial species. The bacterial products discussed in this study are typically classified as postbiotics. They are a group of compounds produced by beneficial microorganisms (such as probiotics) during their metabolic processes. Postbiotics include various substances, such as short-chain fatty acids, vitamins, enzymes, peptides, and organic acids. These postbiotics may possess biological activity like probiotic microorganisms (Żółkiewicz et al., 2020). Postbiotics typically encompass several bacterial metabolic by-products, including bacteriocins, hydrogen peroxide, organic acids, acetaldehydes, alcohol, diacetyl, and other similar compounds. Wegh et al. (2019) have noted evidence suggesting that certain heat-killed probiotics can preserve notable bacterial structures and processes capable of facilitating biological and physiological activities in the host.

The evidence suggests that these metabolic by-products have a broad inhibitory effect on microbial pathogens, making them a viable substitute for antibiotics, according to Aguilar-Toalá et al. (2018). These postbiotic by-products are not poisonous or pathogenic and may withstand human enzyme degradation. They are made from non-viable bacteria or are probiotics' metabolic by-products. Additionally, postbiotics have been observed to enhance the barrier against organisms like *Saccharomyces boulardii* and promote angiogenesis in vitro and in vivo by activating collagen receptors known as 2b1 integrins in epithelial cells (Moradi et al., 2020). Other probiotic species, such as *Bacteroides fragilis*, *B. lactis*, *B. infantis*, *B. breves*, *F. prausnitzii*, *E. coli*, and *Lactobacillus*, have also been reported to possess similar properties (see Table 2.2).

Table 2.2: List of postbiotic bacteria

| S.No. | Bioactive compounds/ postbiotics | Natural Sources | References |
|-------|-------------------------------------|--|--------------------------------|
| 1. | Bacteriocins | <i>Lactobacillus plantarum</i> I- UL4 | (Corsetti & Settanni, 2007) |

| | | | |
|----|-------------------------|-------------------------------------|--------------------------|
| 2. | Soluble mediator | <i>Lactobacillus paracasei</i> | (Mollestad et al., 2019) |
| 3. | Polyphosphate | <i>Lactobacillus brevis</i> | (Bessong et al., 2016) |
| 4. | Butyrate | <i>Faecalibacterium prausnitzii</i> | (Koh et al., 2016) |
| 5. | Heat-killed LG | <i>Lactobacillus rhamnosus</i> | (Cani et al., 2007) |
| 6. | Exopolysaccharides | <i>Lactobacillus pentosus</i> | (Vinderola et al., 2020) |
| 7. | Short-chain fatty acids | <i>Lactobacillus gasser</i> | (Kim et al., 2017) |

2.2.3 Prebiotics

In 1995, Gibson and Roberfroid coined the term "prebiotic," combining "pre" (meaning "before") with "biotic" (meaning "life"). They defined prebiotics as non-digestible food components that beneficially affect the host by selectively promoting the growth and activity of specific gut bacteria, particularly lactobacilli and bifidobacteria. This selective stimulation leads to a healthier composition of the colonic microflora, which can contribute to overall health. Common prebiotics include oligosaccharides like fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), and xylo-oligosaccharides (XOS), which have degrees of polymerization ranging from 2 to 20 monomers.

Prebiotics serve as a nutritional source for gut epithelial cells through the fermentation of carbohydrates by the gut microbiota. Bifidobacteria and other beneficial microorganisms are critical in fermenting these non-digestible oligosaccharides. Prebiotics are naturally found in many plant-based foods, such as fruits, vegetables, and grains. These sources not only provide energy but also deliver significant health benefits. The health advantages of prebiotics include alleviating symptoms of intestinal bowel disorders, reducing the risk of diarrhea, and mitigating inflammation. Prebiotics have also been associated with a lower risk of colon cancer. Additionally, they enhance mineral absorption and bioavailability, improve satiety, and may assist with weight

loss. Prebiotics also show promise in reducing risk factors for cardiovascular disease, further emphasizing their potential in promoting overall well-being.

Table 2.3: List of Prebiotics and their natural sources

| S.No. | Prebiotics | Natural Sources | References |
|-------|-------------------------------|--|-----------------------------------|
| 1. | Fructo-oligosaccharides | Wheat, oats, chicory, Jerusalem artichoke, onion, leek, asparagus, chicory, and garlic | (Roberfroid et al., 2010) |
| 2. | Inulin | Garlic, Elecampane, Burdock Camas, Chicory, Coneflower, Costus, Banana/Plantain, Agave, Banana/Plantain, Jerusalem artichoke etc | (Moro et al., 2018) |
| 3. | Arabinoxylan oligosaccharides | Cereals | (Saha & Cereal Foods World, 2009) |
| 4. | Resistant starch-1,2,3,4 | Beans/legumes, fruits, and vegetables high in starch, including bananas, grainy foods | (Slavin, 2013) |
| 5. | Fructo-oligosaccharides | Banana, artichoke, asparagus, onion, chives, and garlic | (Roberfroid et al., 2010) |
| 6. | Arabinoxylan | Bran of grasses | (Huang et al., 2018) |
| 7. | Xylo-oligosaccharides | Bamboo shoots, Fruits, Vegetables, Milk, Honey | (Zhang et al., 2015) |
| 8. | Galacto-oligosaccharides | Lentil, human milk, green pea, lima bean, chickpea/hummus, and kidney beans | (Mäkelä et al., 2015) |
| 9. | Isomalto-oligosaccharides | Miso, Honey, Sake, Soy sauce, | (Müller et al., 2016) |
| 10. | Lactulose | Skim milk | (Zhou et al., 2017) |

2.2.4 Synbiotics

When a food contains both prebiotics and probiotics, the term "synbiotic" is used. This term refers to products in which the prebiotic component supports the probiotic component in a specific way, as it alludes to synergism. The expectation is that prebiotics will enhance the longevity and growth of probiotics (Adebola et al., 2014). When both probiotics and prebiotics work together in the gastrointestinal environment, their synergistic effects are promoted more effectively. Scientific evidence shows that the synbiotic connection between prebiotics and probiotics contributes significantly to well-being. Due to the recognized benefits for gut health, disease prevention, and treatment, commercial interest in functional foods incorporating synbiotics has gradually increased. Current research and development in this field focus on creating new foods that promote well-being and selecting new cultures, demonstrating an improved ability to colonize the human intestine and metabolize new prebiotics.

Table 2.4 Synbiotics and their bioactive compounds

| S.No. | Bioactive Compounds | Bacteria involved |
|-------|--|--|
| 1. | Isomalto-oligosaccharides | <i>Bifidobacteria, Bacteroides fragilis group</i> |
| 2. | Lactulose | <i>L. rhamnosus, L. acidophilus, L. bulgaricus, Bifidobacteria lactis</i> |
| 3. | Fructo-oligosaccharides | <i>Klebsiella Peptostreptococcaceae, Bifidobacteria, Bacteroides fragilis,</i> |
| 4. | Arabinoxylan and Arabinoxylan oligosaccharides | <i>Bifidobacterium sp.</i> |
| 5. | Inulin | <i>Bifidobacterium animalis, L. paracasei, L. acidophilus,</i> |
| 6. | Resistant starch-1,2,3,4 | <i>Bacteroides, Eubacterium rectal</i> |
| 7. | Galacto-oligosaccharides | <i>Bifidobacterium longum, B. catenulatum</i> |

| | | |
|----|-------------------------|---|
| 8. | Xylo-oligosaccharides | <i>L. plantarum</i> , <i>Bifidobacterium adolescentis</i> |
| 9. | Fructo-oligosaccharides | <i>B. lactis</i> , <i>Bifidobacterium bifidum</i> , |

2.3 Action Mechanism of Probiotics

The specific processes through which probiotics have advantageous effects are still not completely understood. However, several postulated mechanisms offer insight into the favorable effects of these organisms (O'Toole et al., 2008). One of the mechanisms involved in this process entails competing for adhesion sites, whereby probiotics engage in a competitive struggle to secure cellular attachments. Effective colonization of pathogenic organisms often necessitates their interaction with the epithelium of the gastrointestinal (GI) tract. Nevertheless, specific strains of *bifidobacteria* and *lactobacilli* can attach to the epithelial cells and function as "colonization barriers," impeding pathogens' attachment to the mucosal surface. This effect has been empirically validated using strains such as *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG. According to a study conducted in 1999 by Mack et al., both organisms demonstrated the ability to inhibit *Escherichia coli*'s ability to adhere to the cells that make up the human colon.

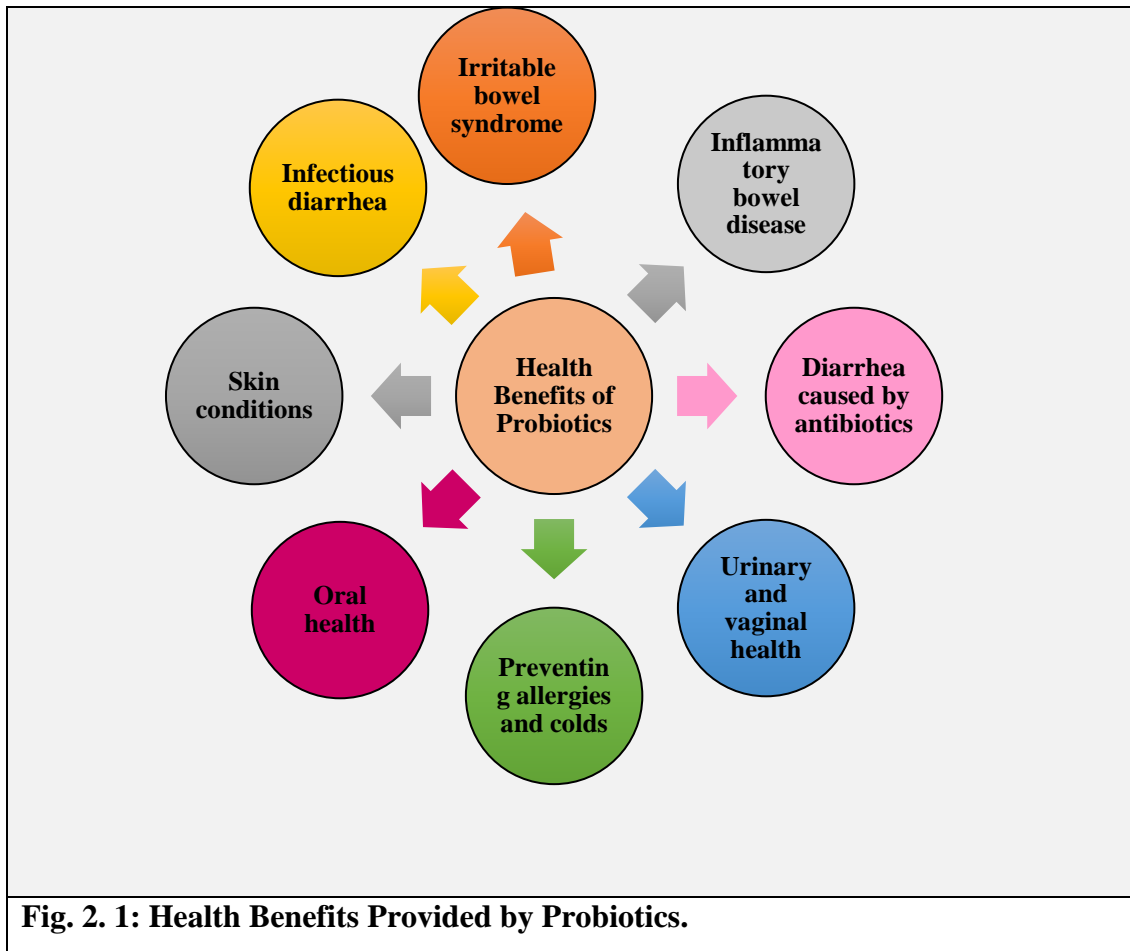
An additional plausible mechanism of action involves the modification of microbial flora through the creation of antibacterial compounds. Lactic acid bacteria frequently generate bioactive substances, including short-chain fatty acids, diacetyl, and hydrogen peroxide. The chemicals produced by probiotic organisms play a role in promoting favorable changes in the composition of microflora. However, it should be noted that not all strains of *lactobacilli* or *bifidobacteria* can produce antimicrobial compounds. Furthermore, these compounds' precise mechanisms and effects are not fully understood, which introduces the potential for both advantageous and detrimental impacts on beneficial and pathogenic bacteria.

Probiotics have also been found to trigger immune responses (Perdigon et al., 1995). These immune responses can manifest as increased secretion of immunoglobulin A (IgA), heightened natural killer cell counts, or improved phagocytic activity by

macrophages (Schiffin et al., 1995). Higher IgA secretion, for instance, can reduce the population of pathogenic species within the gut, consequently enhancing the composition of the microflora. Some researchers speculate that due to these immunomodulatory effects, probiotics may not only combat intestinal and urogenital pathogens but also hold potential therapeutic value for conditions like inflammatory bowel disease (IBD) and pouchitis and even serve as vaccine adjuvants. Additionally, probiotics can engage in nutrient competition, wherein they compete with pathogens for essential nutrients. For example, *Clostridium difficile*, a potentially harmful organism, relies on monosaccharides for growth. In this context, probiotic species can utilize available monosaccharides in sufficient quantities, thereby suppressing *C. difficile* (Vanderhoof et al., 1998).

2.4 Health Benefits Provided by Probiotics

In recent years, researchers have shown increasing interest in studying the impact of probiotics on human health. Okuro et al. (2013) define probiotics as live microbial feed supplements that benefit the host. These benefits include improving the balance of gut microbiota, reducing colon irritation (Holowacz et al., 2016), lowering blood ammonia levels, inhibiting the growth of pathogenic microorganisms, preventing tumor formation, reducing cholesterol levels, promoting vitamin synthesis, and enhancing calcium absorption (Li et al., 2016). Probiotics interact with potentially pathogenic microbes and commensal microorganisms, producing metabolic compounds and other products such as short-chain fatty acids. They communicate with host cells through chemical signaling, colonize the gut, and ultimately inhibit the growth of pathogenic microorganisms (Collado et al., 2007). Some health benefits of probiotics are explained below (Fig. 2.1).



1. Enhance Digestive Health

Probiotics generally provide benefits in the treatment and prevention of gastrointestinal diseases. The specific type of disease and the probiotic species (strain) are crucial factors to consider when using probiotics for these purposes (Drisko et al., 2003). Consuming foods rich in beneficial bacteria and utilizing probiotic supplements can help protect against inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis. The evidence supporting probiotics for improving ulcerative colitis is robust, while the benefits for Crohn's disease may be less significant. Substantial evidence suggests that probiotics effectively combat various forms of diarrhea, including infectious, antibiotic-associated, traveler's, and acute diarrhea. Additionally, probiotics can reduce the pain and severity of symptoms associated with irritable bowel syndrome (IBS) and other related gastrointestinal issues (Gibson, 2004).

2. Aid in Decreasing Antibiotic Resistance

According to the World Health Organization, antibiotic resistance is one of the biggest challenges to food security, development, and global health today. This resistance can arise from misuse, a lack of therapeutic options, and inappropriate application of antibiotics (Amara & Shibl, 2015). Consuming probiotics can help restore the diversity of gut bacteria, which often decreases after antibiotic use, alleviating gut-related issues associated with antibiotics. Furthermore, probiotics from supplements and foods may enhance the effectiveness of antibiotics and help prevent the emergence of antibiotic-resistant bacteria (Ouwehand et al., 2016).

3. Potential Treatment for Mental Illness

Extensive research explores the gut-brain connection, underscoring the gut's role as a "second brain." A review from 2015 highlighted the complex interactions between the gut and brain, particularly in inflammation-related disorders such as obesity, autism, mood disorders, multiple sclerosis, and attention-deficit hyperactivity disorder. Psychobiotics, which influence brain function, are crucial in addressing these conditions, primarily through their anti-inflammatory properties (Flower & Ellingord, 2015). Early studies suggest that probiotic supplementation may alleviate animal anxiety symptoms by reducing inflammation along the gut-brain axis. Investigations in rodents indicate that probiotics could lower stress hormones via the hypothalamic-pituitary-adrenal axis, potentially reducing manic depression. Notably, probiotics may also impact autism symptoms; a 2016 case study demonstrated improvements in a patient with severe autism, as indicated by a better score on the ADOS scale during probiotic treatment for digestive issues (Rogers et al., 2016).

5. Aid in antibiotic-associated Diarrhea

Probiotics are widely recognized as a preventive and therapeutic measure for antibiotic-associated diarrhea (AAD), a common side effect of antibiotic therapy (Barbut & Meynard, 2002). Antibiotics disrupt the gut's normal microbiota, leading to the

overgrowth of harmful bacteria such as *Clostridium difficile*. Probiotics, particularly species from the *Lactobacillus* and *Bifidobacterium* genera, help replenish and restore gut microbiota, thereby maintaining microbial balance and reducing the risk of AAD (McFarland, 2009). The efficacy of probiotics can be attributed to several mechanisms, including competition for nutrients and adhesion sites with pathogens, enhancement of intestinal barrier function, and modulation of the immune response. Clinical studies have produced mixed but promising results; certain strains, such as *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG, have effectively reduced the incidence and severity of AAD in children and adults. However, the effectiveness of probiotics can vary significantly among different strains (Varughese et al., 2013).

6. Infectious Diarrhea

Probiotic microorganisms provide significant health benefits, particularly in treating and preventing infectious diarrhea, such as rotavirus, a worldwide leading cause of infant mortality (Weizman et al., 2010). Proper gut microflora is essential for the host's response to infections, with studies showing that probiotics can reduce antigen absorption in germ-free mice compared to those with normal microbiota. Clinical research highlights the efficacy of probiotics such as *Bifidobacterium animalis* Bb12 and *Lactobacillus rhamnosus* GG in shortening the duration of acute rotavirus diarrhea by enhancing immune responses and inhibiting viral particles (Isolauri et al., 2000; Isolauri et al., 2004). Other probiotics, including *Saccharomyces boulardii*, *Lactobacillus casei*, and *Lactobacillus reuteri*, have also been shown to reduce the duration of acute diarrheal illnesses in children. In adults, probiotics like *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* are effective in preventing traveler's diarrhea; however, findings vary due to differences in study demographics and probiotic formulations (Sazawal et al., 2006). Animal studies also indicate that probiotics can suppress entero-pathogens primarily by producing bacteriocins.

7. Lactose Intolerance

Lactose intolerance is an individual's inability to digest lactose, a sugar in milk and other dairy products. This condition arises from a deficiency of the enzyme lactase, which breaks down lactose into glucose and galactose. Lactose intolerance can be inherited or acquired (Vonk et al., 2012). The genetically determined type occurs when infants do not produce sufficient lactase. At the same time, acquired lactose intolerance can result from conditions such as short bowel syndrome, rotavirus infection, or pelvic radiotherapy, all of which can damage lactase-producing cells or the intestinal lining. When lactose-intolerant individuals consume lactose-containing foods, such as milk or dairy products, undigested lactose enters the large intestine. There, bacteria ferment the lactose, producing gases and other compounds that increase water absorption in the bowel. This process can result in diarrhea, abdominal discomfort, and gas. Interestingly, specific bacterial strains used in traditional yogurt production, like *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* contains higher levels of the enzyme beta-galactosidase, which aids in lactose digestion (Oak & Jha, 2019). Probiotics have also been associated with improved lactose metabolism. Some specific probiotic strains and concentrations have shown promising results in enhancing lactose digestion and alleviating symptoms. Consequently, many individuals have reported positive responses to probiotic supplementation, prompting healthcare professionals to consider it a viable treatment option (de Oliveira et al., 2022).

2.5 What are probiotic or functional beverages...?

In recent years, functional beverages have gained popularity as individuals increasingly seek convenient and effective ways to enhance their health and well-being. These drinks are designed to provide additional benefits beyond essential hydration, such as boosting energy, improving digestion, enhancing mental clarity, or supporting immune function. This article explores the concept of functional beverages, their various types, and the essential components commonly found in them (Saarela, 2009). Functional beverages offer unique health advantages, targeting specific health issues or having effects on the body. They come in various forms, including ready-to-drink (RTD) products, powders, shots, and concentrates.

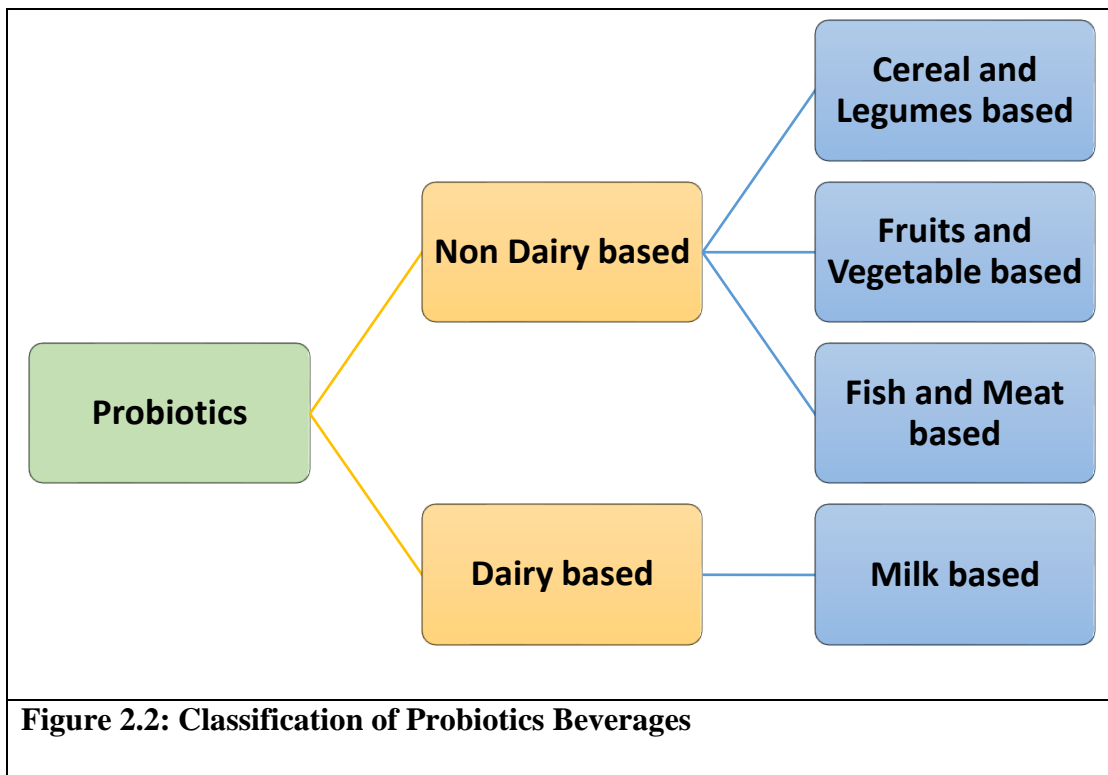
One prominent type of functional beverage is energy drinks, formulated to promote energy and improve mental clarity. These beverages typically contain nutrients such as B vitamins, caffeine, taurine, and herbal extracts like ginseng or guarana. They are popular among individuals seeking a quick pick-me-up after long workdays or intense physical activities. Another category is sports drinks, designed to replenish fluids, electrolytes, and carbohydrates lost during physical exertion (Raman et al., 2019). Sports drinks primarily consist of water, carbohydrates, electrolytes (sodium, potassium, and magnesium), and sometimes vitamins. Athletes and those engaged in prolonged or strenuous workouts often consume them to stay hydrated and enhance performance.

Functional beverages can also target health issues like immune support or weight management. Immune-boosting drinks often include vitamins (like vitamin C or zinc) and botanical extracts known for their immune-supportive properties (such as elderberry or echinacea). In contrast, weight-loss beverages may contain ingredients like green tea extract, fiber, or protein to reduce cravings, increase metabolism, and support lean muscle mass. Recently, there has been considerable focus on the significant contributions of beverages to nutritional intake and overall health. Their variety in size, form, storage, and potential to contain attractive nutrients and bioactive compounds meet market demands. Functional beverages have been critically analyzed as prominent sources of nutraceuticals, addressing categorization, significance, nutritional benefits, food interactions, storage retention, and human delivery. Essential components in functional beverages include vitamins, minerals, essential fatty acids, and amino acids. These drinks can support immune system function, promote gut and cardiovascular health, control weight, and act as adjuvants to counteract aging processes. Functional beverages—such as probiotics, mineral-enriched options, vegetable and fruit drinks, and sports and energy drinks—can be classified into dairy and non-dairy categories (Corbo et al., 2014).

2.6 Classification of Probiotics beverages

Beverage items only provide the required nutrients, avoid or mitigate nutrition-related disease production, and enhance physical and mental well-being. Functional drinks play

an excellent part in this respect. Functional drinks can be defined as drinks or dietary components that, besides the essential role of supplying nutrients, may provide a health benefit (Georgieva et al., 2014). The fastest-growing area of functional beverage processing is probiotic beverages. In certain types of nutrient matrices, probiotic cultures are successfully added. Several beverage products have been used as probiotic distribution mechanisms, including dairy, meat, drinks, cereals, vegetables, and fruit. (Figure 2.2).



2.7 An Indian point of view on probiotic beverages

The Indigenous community has traditionally been manufacturing and maintaining a distinct collection of microorganisms throughout the ages. Examining ethnic fermented/probiotic drinks is a research emphasis to explore novel probiotic microorganisms and biologically active substances (Mishra et al., 2021).

2.7.1 Dairy-based probiotic beverages

The Indian market offers diverse dairy-based probiotic beverages (Table 2.6), including traditional fermented products such as yogurt and other milk items. These beverages, which include curd, chhurpi, mohi, chhu, and lassi, are made from yak, cow, or buffalo milk and are highly valued for their nutritional benefits across different communities. Among these, dahi, often called curd or Indian yogurt, is significant. Its preparation involves fermenting milk with mesophilic *Lactococcus* bacteria, sometimes supplemented with *Leuconostoc*. While *Lactobacillus* strains are predominant in Indian fermented milk products, other species like *Streptococcus* and *Lactococcus* are also present.

Dahi is especially popular in the Darjeeling Hills and Sikkim regions, where Himalayan tribal populations cherish it. Genetic studies have identified various lactic acid bacteria (LAB) strains in dahi, including *Enterococcus faecalis*, *E. faecium*, and *Lc. lactis*. Notably, *Lactobacillus rhamnosus* S1K3 has been found to produce antimicrobial compounds effective against foodborne pathogens. In states like Sikkim and Arunachal Pradesh, naturally fermented milk products such as chhurpi (which is high in protein) and gheu/mar (a fat-rich ingredient) are standard. Chhurpi resembles soft cheese and contains LAB strains like *Lactobacillus lactis*, *Lactobacillus helveticus*, and *Lactobacillus fermentum*, along with acetic acid bacteria such as *Gluconobacter* spp. and *Acetobacter* spp. A 2019 study by Dan et al. indicates that blending starter strains like *Lactobacillus plantarum* and *S. thermophilus* can enhance the flavor and texture of fermented dairy beverages.

Lassi, with roots in ancient Indian texts, has remained a popular choice nationwide. Typically made with sugar, black salt, and cumin seeds, this yogurt-based drink offers versatility in texture and composition, influenced by the type of milk used, preparation methods, and fat content. Celebrated for its low-fat content and rich diversity of beneficial bacteria, lassi is known for its digestive benefits, providing nourishment while helping to alleviate gastrointestinal issues (Banik et al., 2020). Similarly, mohi, a refreshing yogurt-churned beverage, is widely enjoyed in Nepal. Together, these dairy-based options, including lassi, serve as excellent sources of probiotics and offer delightful flavors, making them suitable for individuals with lactose intolerance.

Table 2.6: Dairy-based probiotic beverages and strain involved

| Beverage Name | Probiotic Strain | References |
|----------------------|--|-----------------------------|
| Gheu | <i>Acetobacter spp.</i> , <i>Gluconobacter spp.</i> , <i>Lactococcus lactis</i> , <i>Lactobacillus helveticus</i> , | Shangpliang et al. 2018 |
| Dahi | <i>Lactobacillus rhamnosus</i> , <i>Lactococcus lactis</i> , <i>Enterococcus faecium</i> , <i>Enterococcus faecalis</i> , | Ghosh et al. 2019 |
| Mar | <i>Acetobacter spp.</i> , <i>Gluconobacter spp.</i> , <i>Lactococcus lactis</i> , <i>Lactobacillus helveticus</i> , | Shangpliang et al. 2018 |
| Kefir | <i>Lactobacillus kefir</i> | Kandyliis et al., 2016 |
| Churupi | <i>Lactobacillus helveticus</i> , <i>Lactococcus lactis</i> , <i>Lactobacillus fermentum</i> , <i>Gluconobacter spp.</i> , <i>Acetobacter spp.</i> | Shangpliang et al. (2018) |
| Butter milk | <i>Lactobacillus bulgaricus</i> , <i>Lactococcus lactis</i> | Ranadheera et al., 2017 |
| Yogurt | <i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i> | Sarkar, 2018 |
| Bifidus Milk | <i>Bifidobacterium longum</i> , <i>Bifidobacterium bifidum</i> | Khorshidian et al., 2020 |
| Whey Beverage | <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium lactis</i> | Shori, 2015 |
| Cheddar cheese | <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus helveticus</i> , | Ulpathakumbura et al., 2016 |
| Fermented Skim Milk | <i>Lactobacillus acidophilus</i> <i>Bifidobacterium animalis subsp. Lactis</i> | Ranadheera et al., 2017 |

2.7.2 Cereal-based probiotic beverages

Cereal-based probiotic beverages have the potential to generate fortified functional beverages that provide probiotic advantages (Mishra et al., 2021). An illustrative instance is chhang, a routine fermented beverage produced by indigenous populations residing in Himachal Pradesh in the North-West Himalayas, resembling beer characteristics. This beverage is produced through barley, millet, and rice grains. The process of chhang fermentation encompasses a diverse range of lactic acid bacteria (LAB) strains, such as *Pedicoccus pentosaceus*, *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus casei*, and alongside yeasts including *Pichia kudriavzevii*, *Candida tropicalis*, *Saccharomyces cerevisiae*, and *Saccharomyces fibuligera*, (Thakur et al., 2015). Chhang and sura are notable examples of traditional cereal-based fermented alcoholic beverages commonly drunk by tribal and rural inhabitants in Himachal Pradesh. Chhang is produced from rice, while sura is derived from millet. The preparation of these inoculums, known as "daheli" and "phab" in traditional practices, has been documented by Thakur et al. (2015). The microbial investigation of sura revealed the presence of prominent bacteria such as *Saccharomyces cerevisiae*, several *Candida* species, *Leuconostoc*, and *Lactobacillus*. A recent study by Chavan et al. (2018) developed a novel probiotic beverage using non-germinated and germinated moth finger millet, bean seeds, and barley. This beverage was then inoculated with *Lactobacillus acidophilus*. Utilizing this starting culture in the fermentation process led to notable enhancements in the functional characteristics of the beverage.

A traditional fermented beverage named Bhaati Janar originates from the Himalayan region, is produced from rice, and is commonly consumed as a staple component of the diet. This beverage is characterized by a subtle alcoholic content and a tangy flavor profile, making it a popular choice among postpartum women and elderly residents in rural communities. In an independent study, Giri, Sen, et al. (2018) investigated the utilization of *Lactobacillus plantarum* L7 in the fermentation process of Bhaati Janar. In another study in 2019, Sharma and colleagues successfully isolated potentially advantageous lactic acid bacteria (LAB) strains from a wheat flour dough called "babroo" that undergoes traditional fermentation. The identification of three specific probiotic bacteria, namely *Lactobacillus fermentum* L42, *Lactobacillus plantarum* P27, and *Lactobacillus fermentum* M21, by phylogenetic analysis, is associated with the

babroo fermentation process. In a study by Ca´ceres et al. in 2019, the impact of traditional fermentation on the health and nutritional characteristics of germinated brown rice was examined. The researchers aimed to develop a yogurt-like product by enriching rice with 0.5% gelatin, 5% glucose, and 7% sucrose. The utilization of a commercial starter culture containing LAB strains at a temperature of 42°C resulted in the fermentation process, producing a beverage with significant phenolic content γ -aminobutyric acid (GABA) antioxidant activity and angiotensin I-converting enzyme (ACE) inhibition properties. The researchers also investigated the utilization of *Lactobacillus plantarum* M-13, a probiotic strain, in producing a fermented oat flour beverage. This beverage has benefits that are good for consumers. (Gupta and Bajaj 2017). Additionally, they used honey as a component aimed at boosting the health benefits of the beverage. The enhancement of factors such as the concentration of oat flour (8.0% w/v), the concentration of honey (3.0% w/v), and the duration of incubation (48 hours) resulted in a notable improvement in the viability of probiotics over four weeks.

Table 2.7: Cereal-based probiotic beverage and strain involved

| Cereal and Legume | Probiotic Microorganism | References |
|--------------------------|--|-------------------------------|
| Barley, Moth Beans | <i>L. acidophilus</i> | Chavan et al., 2018 |
| Germinated Brown Rice | <i>Lactic acid bacteria</i> | Ca´ceres et al., 2019 |
| Fermented Wheat | <i>L. fermentum K75, L. fermentum K78 and L. plantarum K90</i> | Giri et al., 2018 |
| Fermented Rice | <i>L. plantarum</i> | Sharma et al., 2010 |
| Wheat Flour | <i>Leuconostoc mesenteroides, Leuconostoc reffinolactis, L. coprophilus, L. plantarum, L. fermentum, L. acidophilus.</i> | Ravindran and RadhaiSri, 2020 |

| | | |
|-----------------|---|-------------------------------|
| Maize Flour | <i>Saccharomyces cerevisiae, Lactobacillus brevis, Candida glabrata, Candida tropicalis, Geotrichum candidum, Geotrichum penicillatum</i> | Ten Berge et al., 2019 |
| oat flour | <i>L. plantarum, Bifidobacterium lactis</i> | Asadzadeh et al., 2015 |
| Buckwheat flour | <i>L. acidophilus</i> | Vasile et al., 2016 |
| Sorghum flour | <i>Leuconostoc, Enterococcus and L. brevis</i> | Kumari et al., 2018 |
| Soymilk | <i>L. kefir, L. brevis, L. acidophilus, Bifidobacterium animalis, Kluyveromyces lactis.</i> | Sridharan and Das, 2019 |
| Peanut soymilk | <i>L. acidophilus, L. lactis, L. rhamnosus, Pediococcus acidilactici</i> | do Amaral Santos et al., 2014 |

2.7.3 Fruit-based probiotic beverages

The demand for probiotic drinks manufactured from fruits has increased in recent years. Juice and beverage manufacturers have difficulty adding probiotic microorganisms to fruit-based products while retaining viability (Table 2.8). Researchers are working hard to develop new, inventive items to increase the variety of functional beverages on the market. Research on the appropriateness of certain probiotic cultures as supplements or additions to fruit drinks was undertaken by Sheehan et al. in 2007. Based on their investigation, several lactic acid bacteria (LAB) strains, including *Lactobacillus paracasei* NFBC 43338, *Lactobacillus rhamnosus* strain GG, and *Lactobacillus casei* DN-114001, have demonstrated potential for incorporation into fruit juices due to their ability to withstand acidic environments. In their study, Mousavi et al. (2011) examined the production of probiotic pomegranate juice through the fermentation of pomegranate juice using four LAB strains, namely *L. delbrueckii*, *L. plantarum*, *L. acidophilus*, and *L. paracasei*. The fermentation lasted for 72 hours, during which the temperature was maintained at 30 °C in a microaerophilic atmosphere. During a four-week storage

period, these LAB cultures ingested citric acid and exhibited maximum viability and growth in pomegranate juice.

Probiotic beverages infused with mango fruit offer customary health benefits. In the study by Reddy et al. (2015), mango juice was fermented at 30°C for 72 hours. Throughout the fermentation process, microaerophilic conditions were carefully maintained. A selection of probiotics, namely *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus acidophilus*, was introduced to the fermentation process. The completed juice received a positive reception due to its vibrant hue, delightful taste, and various sensory attributes. Ranjitha et al. (2018) found that incorporating *Lactobacillus helveticus* MTCC 5463 into mango beverages exhibited promising outcomes in developing probiotic mango drinks. The pH of the solution decreased to 3.2 because of the fermentation process. Dimitrovski et al. (2015) have reported that apple juice is a suitable fermented substrate when utilizing the probiotic bacteria *Lactobacillus plantarum* strain PCS 26. The outcome is a functional beverage that potentially exhibits an extended shelf life and is well-received in terms of sensory appeal.

Table 2.8: Fruit juice-based probiotic beverages and strains involved

| Fruit juice | Probiotic Microorganism | References |
|--------------------|---|---|
| Mango Juice | <i>L. delbrueckii</i> MTCC 911, <i>L. acidophilus</i> MTCC 10307, <i>L. plantarum</i> MTCC 9511 and <i>L. casei</i> | Reddy et al., 2015 |
| Pomegranate juice | <i>L. plantarum</i> , <i>L. delbrueckii</i> , <i>L. paracasei</i> , <i>L. acidophilus</i> | Mousavi et al., 2011 |
| Pineapple Juice | <i>L. casei</i> NRRL B442, <i>L. rhamnosus</i> , <i>L. casei</i> , <i>L. paracasei</i> | Costa et al., 2013 |
| Apple Juice | <i>L. paracasei</i> , <i>L. paracasei</i> <i>L. plantarum</i> , <i>L. rhamnosus</i> | Lilio-Perez et al., 2021; Adebayo Tayo et al., 2016 |

| | | |
|-------------------|--|-----------------------|
| Grape Juice | <i>Pediococcus pentosaceus</i> | Kumar et al., 2017 |
| Raspberry juice | <i>L. casei</i> | Olivares et al., 2019 |
| Orange Juice | <i>L. acidophilus, Bifidobacterium bifidum</i> | Alves et al., 2017 |
| Amla Juice | <i>L. paracasei</i> | Peerajan et al., 2016 |
| Peach Juice | <i>L. casei, L. delbrueckii</i> | Pakbin et al., 2014 |
| Sweet Lemon Juice | <i>L. Plantarum</i> | Hashemi et al., 2017 |
| Sugarcane Juice | <i>L. casei</i> | Amanda et al., 2018 |
| Watermelon Juice | <i>L. Plantarum</i> | Santos et al., 2019 |
| Mosambi Juice | <i>Saccharomyces cerevisiae, Wickerhamomyces anomalous, Pichia barker, Yarrowia lipolytica</i> | Suvarna et al., 2018 |

2.7.4 Vegetables-based probiotic beverage

Probiotic beverages manufactured from vegetables, including carrot root, peanuts, cabbage, onion, tomato, and ginger, have been recognized for their health-enhancing properties (Monlet et al., 2014). These beverages are commonly produced through lactic acid fermentation, employing probiotic LAB strains (Table 2.9). They serve as a dairy-free alternative suitable for individuals with milk protein intolerance, appealing particularly to vegetarians seeking a wholesome beverage option (Prado et al., 2002). Yoon et al. (2004) conducted a study utilizing tomato juice as a substrate for developing a probiotic juice using four distinct LAB strains. The resultant probiotic juice exhibited a reduced pH of 3.5, increased acidity of 1.67%, and achieved cell viability of 108 CFU/mL after a 72-hour fermentation at 30°C. In the Northeastern states of India and the Himalayan region, a fermented cucumber-based probiotic drink called Khalpi is consumed. This beverage undergoes fermentation through hetero-fermentative LAB strains.

In their study, Jaiswal et al. (2013) researched the manufacturing process of a probiotic beverage made from cabbage juice. This involved using various lactic acid bacteria (LAB) strains, including *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*. The lactic acid (LA) production was a significant result of the fermentation process of cabbage juice. Additionally, the fermented cabbage juice demonstrated a high retention rate of its phytochemicals, including total phenolic and total flavonoid content and associated bioactivity, with over 75% preservation. According to the findings of Vanajakshi et al. (2015), a probiotic beverage with desirable characteristics can be obtained by fermenting a mixture of moringa leaf and beetroot in a ratio of 1:2. This fermentation process involves the participation of *Lactobacillus plantarum* and *Enterococcus hirae*. The fermented beverage showed promising commercial viability as a rejuvenating and beneficial choice.

In another study, Panghal et al. (2017) investigated a probiotic beverage made from beetroot, prepared under an ideal pH of 6.5 and a temperature of 37°C. The probiotic strains utilized in the beverage formulation were *L. delbrueckii*, *L. rhamnosus*, and *L. plantarum*. The evaluation of the probiotic capacity of beetroot juice focused on the survivability of lactic acid bacteria. The beet juice containing probiotics exhibited a steady decline in pH and sugar content as time progressed. Additionally, there was a simultaneous increase in phenols, flavonoids, and antioxidants compared to the original fresh juice sample. Vegetable juices are well regarded for their nutritional value and rejuvenating properties, rendering them ideal carriers of functional health components such as crude fiber, vitamins, minerals, dietary fiber, and bioactive substances, including carotenoids, choline, glucosinolates, phytoestrogens, and phenolic compounds.

Table 2.9: Vegetable juice-based probiotic beverages and strains involved

| Vegetables | Probiotic microorganisms | References |
|-------------------|---------------------------------|--------------------------|
| Pumpkin | <i>Lactobacillus plantarum</i> | Genevois et al., 2019 |
| Mushroom | <i>Lactobacillus casei</i> | Garbetta et al., 2018 |
| Cabbage Juice | <i>Lactobacillus casei</i> | Dimitrovski et al., 2016 |

| | | |
|----------|---|--------------------------------|
| Broccoli | <i>Streptococcus thermophilus</i> , <i>Bifidobacterium bifidum</i> , <i>Lactobacillus bulgaricus</i> , <i>Lactobacillus acidophilus</i> , | Maryati et al., 2017 |
| Carrot | <i>Lactobacillus fermentum</i> , <i>Lactobacillus Paracasei</i> , <i>Lactobacillus plantarum</i> | Ghaempanah et al., 2020 |
| Tomato | <i>Lactobacillus acidophilus</i> <i>Lactobacillus plantarum</i> C3, <i>Lactobacillus casei</i> A4, <i>Lactobacillus delbrueckii</i> D7 | Yoon et al., 2004 |
| Beetroot | <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus delbrueckii</i> | Panghal et al., 2017 |
| Spinach | <i>Lactobacillus brevis</i> , <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus plantarum</i> C3, <i>Lactobacillus casei</i> A4 and <i>Lactobacillus delbrueckii</i> D7 | Jaiswal and Abu-Ghannam., 2013 |
| Cucumber | <i>Lactobacillus acidophilus</i> NCDC 11, <i>Lactobacillus plantarum</i> NCDC 414, | Sharma and Mishra, 2013 |
| Lettuce | <i>Lactobacillus plantarum</i> | Panghal et al., 2018 |

2.8 Probiotic Bacteria and Cultures

Microbial communities are increasingly being studied for their potential to treat human and animal diseases. Lilley and Stillwell (1965) introduced the term "probiotics" to describe beneficial bacteria, highlighting these novel applications. According to Fuller (1992), a probiotic is a live microbial feed supplement that improves the microbial balance of the host. The definition of "probiotics" has evolved to refer to a single or mixed culture of living microorganisms that, when administered to humans or animals—either as dried cells or in fermented products—positively influence the host

by enhancing the characteristics of the native microflora. Investigating the probiotic effects of nutrients in diets containing living microorganisms is an ancient practice.

A good probiotic should be non-pathogenic, non-toxic, and beneficial to the host animal. Most bacteria recommended by Fuller (1992) as probiotics belong to the *Lactobacillus* species, which appears to be the most prevalent. Examples of such bacteria include *L. rhamnosus* GG, *L. plantarum*, *L. casei*, *Streptococcus lactis*, and *L. acidophilus*, among others.

Literature suggests several potential clinical and nutritional benefits associated with probiotic diets. For instance, bacterial enzymatic hydrolysis can enhance the bioavailability of proteins and fats (Friend et al., 1984). Bacterial proteases may increase the availability of free amino acids, improving the host's nutritional status, mainly if the host is deficient in endogenous protease production. Additionally, it has been established that lactic acid bacteria boost the vitamin B complex content in fermented foods (Tamine & Deeth, 1980). Probiotic fermentation has also been shown to improve the digestibility of proteins and starches, reduce anti-nutrient levels, and enhance mineral bioavailability.

The probiotic potential of a microorganism is strain-dependent. A strain is classified as a probiotic only after its health effects on the host have been adequately demonstrated. Certain plants harbor *Lactobacillus* species from which specific strains have been isolated, characterized, and recommended as probiotics. Notable strains include *B. animalis*, *B. longum*, *B. bifidum*, *B. lactis*, *B. adolescentis*, and *Lb. Acidophilus*, *Lb. gasseri*, *Lb. casei*, *Lb. paracasei*, *Lb. crispatus*, *Lb. rhamnosus*, *Lb. Plantarum*, *Lb. reuteri*, *Lb. fermentum*, *Lb. johnsonii*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Enterococcus faecium*. Lactic acid bacteria (LAB) include various genera such as *Lactococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus*. Historically, a diverse array of food-grade microorganisms has been utilized in food preservation. *Saccharomyces boulardii* is the only non-pathogenic yeast recognized for its probiotic properties.

The positive effects of probiotic therapy can include reduced pathogen susceptibility and shortened infection duration (Antoine, 2010). Probiotics also play a role in

regulating the immune system at both local and systemic levels. Metchnikoff noted that certain probiotics, such as *Bifidobacterium longum*, do not colonize the intestine to provide beneficial health effects. In contrast, other probiotics, like *Lactobacillus casei*, serve a transitory role by preserving and maintaining the balance of microbial flora in the gut.

Probiotics can be derived from a variety of unique microorganisms. The following explanation may help clarify how microorganisms are identified, named, and classified (Figure 2.3). The genus of a bacterium serves as its scientific name, such as *Lactobacillus*. This classification represents a broad grouping of species based on shared characteristics, including physical attributes, biochemical needs, and metabolic pathways. The second name of a bacterium indicates its species (for example, *acidophilus*), which provides a more specific classification that distinguishes it from other organisms based on shared traits. The term "strain" refers to an even more specific classification that divides individuals within the same genus into subgroups based on distinct characteristics that differentiate these bacteria from other members of the genus (for example, the LA5 strain).

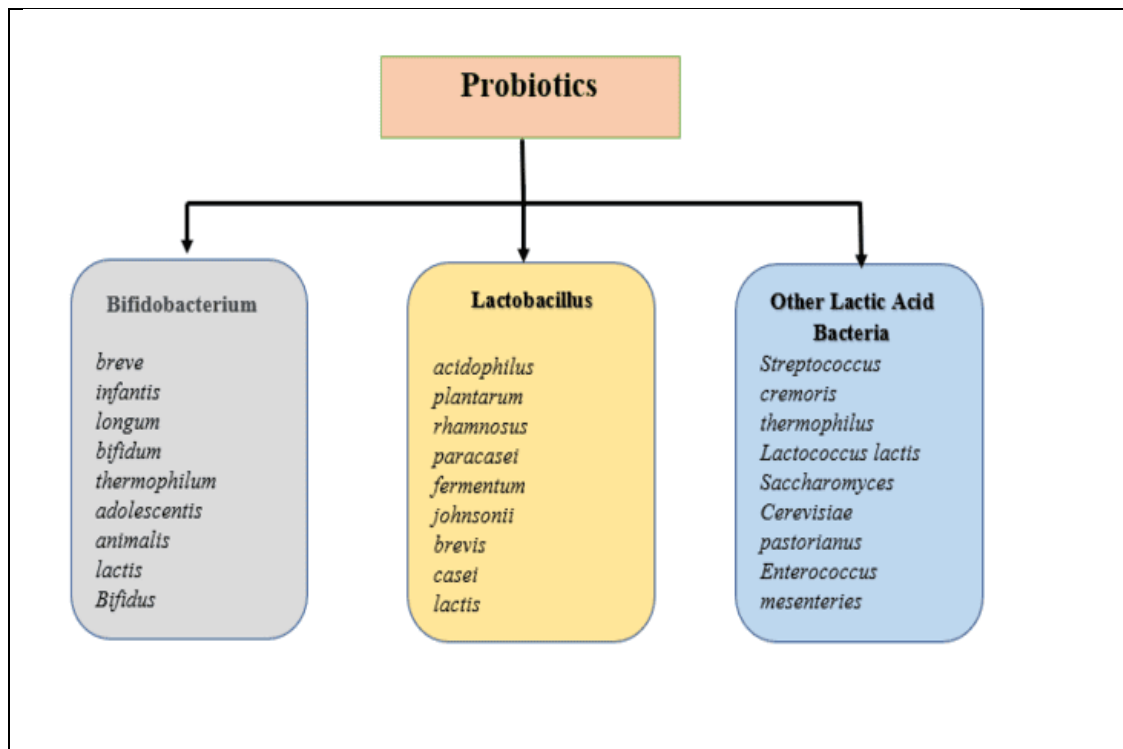


Figure 2.3: Classification of Probiotic Bacteria

2.8.1 *Lactobacillus* Species

According to Fujisawa et al. (1992), gram-positive rods known as *Lactobacillus* are required, and optional anaerobes are naturally present in humans' gastrointestinal and genitourinary tracts. Vanderhoof and Young (2004) explain that "lactobacillus" refers to bacteria capable of producing lactic acid rather than lactose. Unlike antibiotics, lactobacilli are utilized therapeutically as probiotics. They provide nutritional benefits, such as promoting growth factors and enhancing the biocompatibility of minerals, to recolonize certain body parts; therefore, they are often referred to as "friendly" bacteria (Madsen et al., 1999). *Lactobacilli* decreases intestinal permeability and stabilizes the mucosal barrier (Shornikova et al., 1997).

Alterations in the natural flora can lead to the colonization of pathogenic species, which may produce symptoms such as diarrhea, cramps, and, less frequently, *C. difficile*-induced *pseudomembranous colitis* (PMC). The underlying principle is that using lactobacillus probiotics during antibiotic therapy can prevent or reduce the loss of natural flora and harmful bacterial invasion. This assertion is supported by substantial evidence (Alander et al., 1999; Sullivan et al., 2003). The presence of hydrogen peroxide-producing lactobacilli has been associated with reduced incidences of bacterial vaginosis and trichomoniasis, as these bacteria are bacteriostatic against the vaginal pathogen *Gardnerella vaginalis* (Maggi et al., 2000). Additionally, lactic acid produced by lactobacilli lowers vaginal pH, which may inhibit the growth of pathogenic organisms. *Lactobacillus* is also used in animal models to bind food carcinogen particles, thereby reducing colon tumor development associated with carcinogenic exposure (Goldin et al., 1996). Furthermore, early research suggests that lactobacilli, particularly *L. plantarum*, may help lessen the severity of antibiotic-induced intestinal colitis (Mao et al., 1996).

Studies indicate that *L. sporogenes* and *L. bulgaricus* may have both hypolipidemic and anti-atherosclerotic effects. Limited clinical data suggests reducing LDL cholesterol levels without affecting HDL levels (Doncheva et al., 2002). Milk and fermented products, such as yogurt and acidophilus milk, often positively impact cholesterol levels. Bile acids can bind to lactobacilli and other probiotic bacteria, influencing

cholesterol metabolism. Additionally, these probiotics may increase the formation of fatty acids in the gut, which helps lower fatty acid content in the blood by either preventing the generation of hepatic cholesterol or facilitating the transfer of plasma cholesterol to the liver (Losada et al., 2002).

2.8.2 *Bifidobacterium* Species

Bifidobacterium *Bifidobacterium* is classified as a pleomorphic, rod-shaped bacterium that is anaerobic, gram-positive, and non-sporulating. This genus produces lactic acid and acetic acid as metabolic by-products during glucose metabolism. According to secondary literature, the BB536 strain of probiotic bacteria was initially isolated from the gastrointestinal tract of healthy newborns (Cremonini et al., 2001). The concurrent administration of *bifidobacteria*, *Lactobacillus* species, and *Saccharomyces boulardii*, a probiotic yeast, appears to mitigate the negative consequences of *Helicobacter* treatment; however, it does not seem to enhance adherence to the prescribed regimen. Furthermore, when used in combination with *Bifidobacterium infantis* and *Lactobacillus acidophilus*, there has been a noted decrease in the occurrence of necrotizing enterocolitis (NEC) and associated mortality in severely ill infants (Hoyos, 1999).

2.8.3 *Saccharomyces* Species

S. Boulardii, also known as *Saccharomyces cerevisiae*, is a non-pathogenic strain of yeast used to treat and prevent multi-eye diarrhea. *S. Boulardii* was isolated from tropical fruit skins contained in Indochina. These fruit skins have long been used by the indigenous people of Indochina to avoid and cure diarrhea (Buts, 2005). *S. Boulardii* is formulated by lyophilizing living yeast cells (freeze-drying) and performing lactose encapsulation in the formulation. It is challenging to differentiate *S. boulardii* from other *S. Cerevisiae* strains identified by phenotypic criteria, so molecular typing is necessary to classify these infections. Molecular comparison analyses indicate that *S. Boulardii* is very similar or almost equivalent to *S. cerevisiae* in genetic terms. The findings show that the YKL139w and YLR177w gene microsatellite polymorphism analysis and the Ty917 hybridization analysis are the most valuable Equipment for the proper recognition of *S. boulardii* (Fietto et al.,2004). Physiologically and

metabolically, nonetheless, *S. Boulardii* exhibits conduct that is somewhat distinct from *S. Cerevisiae*, particularly concerning growth yield temperature tolerance and acidic stresses, which are essential characteristics for the probiotic usage of a microorganism. The monograph of the German Committee E-Lists *S. boulardii* as *S. Cerevisiae* CBS 5926 by Hansen.

2.8.4 Bacillus Species

Bacillus coagulans is a Gram-positive, rod-shaped bacterium known for its ability to make lactic acid. Due to this characteristic, it is occasionally misclassified as a lactic acid bacteria group member, such as *Lactobacillus*. Specific commercial products incorporating *B. Coagulans* are marketed as "spore-forming lactic acid bacterium" or *Lactobacillus sporogenes*. The formation of spores is a crucial characteristic for identifying these bacteria. The therapeutic utilization of coagulants has similarities to other probiotic strains. However, it must be noted that *B. Coagulans* does not naturally occur in the human flora. For probiotics to effectively maintain the natural flora and prevent pathogenic colonization, they must possess the ability to survive and establish themselves within the intestinal mucosa. The fate of Bacillus spores following human consumption is a topic of ongoing inquiry. The ability of Bacillus spores to undergo germination within the digestive tract and establish colonization remains unclear, as noted by Duc et al. (2004). *Bacillus coagulans* can reduce the colonization of harmful germs in multiple ways. *B. Coagulans* are known to possess coagulin and lactic acid, exhibiting antibacterial properties. These components have been found to effectively inhibit the growth of harmful bacteria (Hyronimus et al., 1998). A research investigation conducted on an animal model has demonstrated that consuming bacillus spores enhances the immune response (48). According to proponents, *Bacillus coagulans*, a type of probiotic, offers advantages compared to other species like *Lactobacillus* due to its ability to be preserved indefinitely in desiccated forms. *Bacillus* spores have notable resistance to both acidic conditions and elevated temperatures.

2.9 Characteristics of an ideal Probiotics

Ideal probiotics have several key characteristics that make them effective in promoting health and well-being (Figure 2.4). These characteristics include:

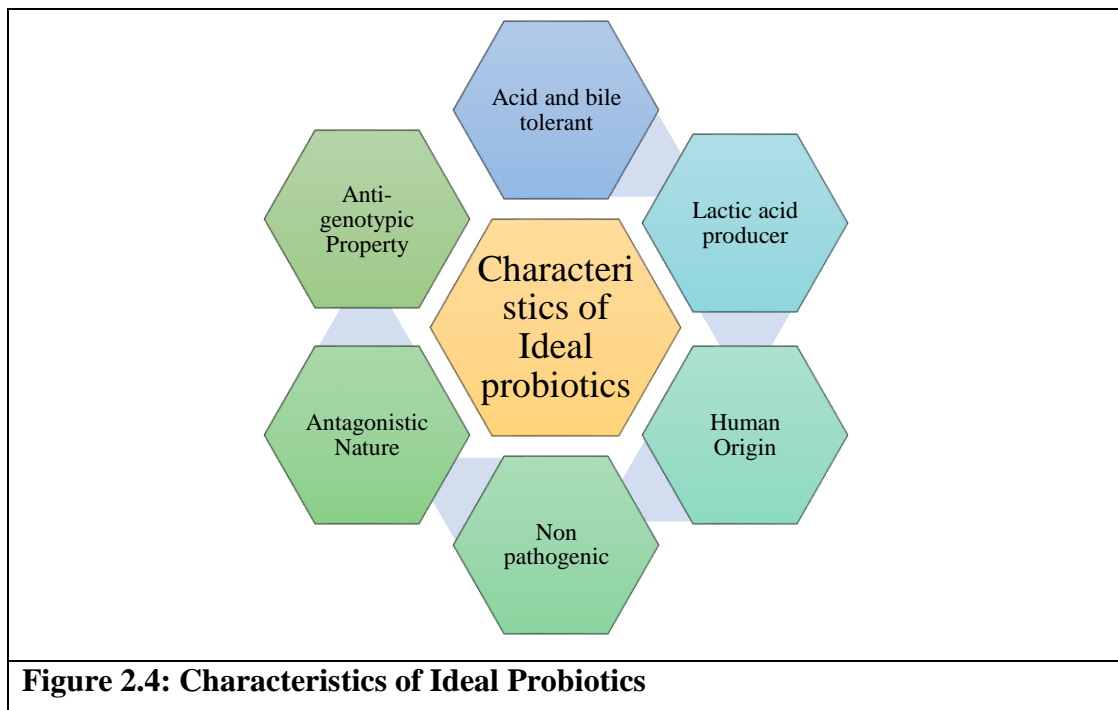


Figure 2.4: Characteristics of Ideal Probiotics

1. **Bile and Acid Tolerant:** Acid and bile tolerance are essential characteristics of probiotics as they allow the beneficial bacteria to be able to withstand the highly acidic environment of the stomach as well as the presence of bile salts in the lumen of the small intestine. This enables the probiotics to reach the intestines alive, where they can exert their health benefits. While not all probiotic strains possess inherent acid and bile tolerance, specific strains have been identified for their ability to survive in these conditions. Here are a few examples of acid- and bile-tolerant probiotics: *Lactobacillus reuteri*, *Bifidobacterium longum*, *Lactobacillus plantarum*, and *Bacillus coagulans*. It is worth noting that the acid and bile tolerance can vary among different strains within the same species. The survival and efficacy of probiotics can also depend on other factors, such as the formulation, delivery method, and viability of the probiotic product. When selecting acid- and bile-tolerant probiotics, it is advisable to look for products tested explicitly for these characteristics that have demonstrated their ability to survive and function in the gastrointestinal tract.
2. **Lactic acid Producer:** Lactic acid-producing bacteria, commonly called lactic acid bacteria (LAB), are a group of bacteria known for their ability to convert sugars into

lactic acid through fermentation. They play a significant role in various food and beverage fermentations and are also used as probiotics for potential health benefits. Some well-known lactic acid producers include *Lactobacillus* species, *Streptococcus* species, *Lactococcus lactis*, and *Pediococcus* species. These lactic acid-producing bacteria contribute to fermented foods' preservation, flavor, and texture while providing potential health benefits. As probiotics, they may help maintain a healthy balance of gut bacteria, support digestion, enhance the immune system, and improve overall gut health.

3. **Antimicrobial compounds production:** Probiotics, in addition to their ability to confer health benefits through their presence and activity in the gut, can also produce antimicrobial compounds. These compounds are substances produced by probiotic bacteria that can inhibit the growth or kill harmful microorganisms. Some examples of antimicrobial compounds from probiotics are lactic acid, bacteriocins, hydrogen peroxide, and short-chain fatty acids (SCFAs). These antimicrobial compounds produced by probiotics contribute to the overall balance and health of the gut microbiota by inhibiting pathogenic bacteria growth and promoting beneficial bacteria growth. It is important to note that the production and effectiveness of these compounds can vary depending on the specific probiotic strain and its interaction with the surrounding environment.

4. **Human Origin:** Human-originated probiotics refer to probiotic strains that naturally occur in the human body or can be derived from human sources. These strains are typically isolated from the human gut or other body parts and utilized for their potential health benefits. A few examples of human-originated probiotics are *Lactobacillus* and *Bifidobacterium* strains, *Saccharomyces boulardii*, *Streptococcus thermophilus*, and *Propionibacterium freudenreichii*. It is important to note that not all probiotics derived from human sources are suitable for every individual or health condition. The specific strains and their effectiveness can vary, so it is crucial to consult with healthcare professionals or follow product guidelines when considering human-originated probiotics.

5. **Adhesion to cell wall:** A critical characteristic of probiotics is their ability to adhere to the intestinal epithelium, the inner lining of the intestinal wall. Adhesion to the intestinal wall allows probiotics to colonize the gut and exert their beneficial effects. While probiotics can adhere to the intestinal wall through various mechanisms, the cell wall of probiotic bacteria plays a crucial role in this process. The cell wall is the outermost layer of bacterial cells and consists of complex structures, including proteins, polysaccharides, and lipids. Here are a few ways in which cell wall components contribute to the adhesion of probiotics: surface proteins, polysaccharides, lipoteichoic acids, and Surface charge adhesion of probiotics is strain-specific, meaning different strains of probiotic bacteria may have different adhesion properties. Additionally, the ability to adhere can depend on factors such as intestinal epithelium's health and competing microorganisms' presence.

6. **Non-Pathogenic:** A non-pathogenic probiotic refers to a type of probiotic microorganism that does not cause disease or harm to the host. Probiotics comprise living yeasts or bacteria that, when taken in sufficient quantities, confer health advantages on the host organism, which is most commonly the body of humans. Probiotics are sometimes referred to as "friendly bacteria." Some people, especially those with compromised immune systems or other health disorders, may be at a greater risk of suffering unpleasant effects when taking probiotics, even though probiotics are usually believed to be safe for the majority of people. Therefore, it is essential to choose probiotic strains that have been extensively studied and are known to be non-pathogenic. Some commonly used non-pathogenic probiotic strains include *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus rhamnosus*, and *Saccharomyces boulardii*. The safety and efficacy of probiotics can vary depending on the specific strain and the individual's health condition. If you are considering taking probiotics, it is advisable to consult with a healthcare professional who can recommend the most suitable strains for your needs.

7. **Longer Survival rate:** Several probiotic strains have been studied for their ability to survive and colonize the gastrointestinal tract. Probiotics must be alive and capable of surviving the harsh conditions of the gastrointestinal tract to reach the intestines, where

they exert their beneficial effects. Ideal probiotics have high viability, meaning they can survive exposure to stomach acid and bile salts and maintain their potency until they reach the target site. While individual survival rates can vary depending on factors such as the specific strain, delivery method, and the individual's gut environment, here are a few probiotic strains that have been found to have relatively higher survival rates are *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG and *Streptococcus thermophilus*. Remember that survival rates and effectiveness can vary depending on the formulation, delivery method (capsules, liquid, etc.), and individual factors.

2.10 The probiotic and gut microbiota System

The large intestine, more especially the digestive system, contains the biggest community of microbes in the form of human microbiota (Matamoros et al., 2013). The gastrointestinal or gut microbiome is the name given to this particular microbial population. The vast use of molecular biology techniques for bulk amplification of DNA Sequences of complete bacterial populations over the last decade has led to the awareness of the role of intestinal microbiota in health and disease (Toh & Allen-Vercoe, 2015), as only about 10% of intestinal bacteria can be cultivated on conventional agar plates (Eckburg et al., 2005). Rapidly growing evidence suggests that the early acquisition, growth, and protection of unique bacterial populations within the gut is crucial to human health, and excellent opportunities for action are created by a better understanding of them (Saavedra & Dattilo, 2012). The gut microbiome executes essential functions throughout the period, such as metabolic reactions, trophic effects, barrier function, and the development of innate immunity and adaptive immunity in the host. Increased and essential microbial abundance, diversity, and morphology changes accompany infant gut microbiome composition and development. Health, cultural and environmental influences, transmission mode, diet, family climate, illnesses, and treatments used to impact these modifications (Matamoros et al., 2013). At birth, the human baby's intestine is sterile, and the formation of the gut microbiome in babies is relatively easy but becomes more complex in grown-ups.

It is assumed that the gut microbiome develops from the microbiome of its mother (breastmilk of the mother, skin, and vagina) as well as from the condition in which the

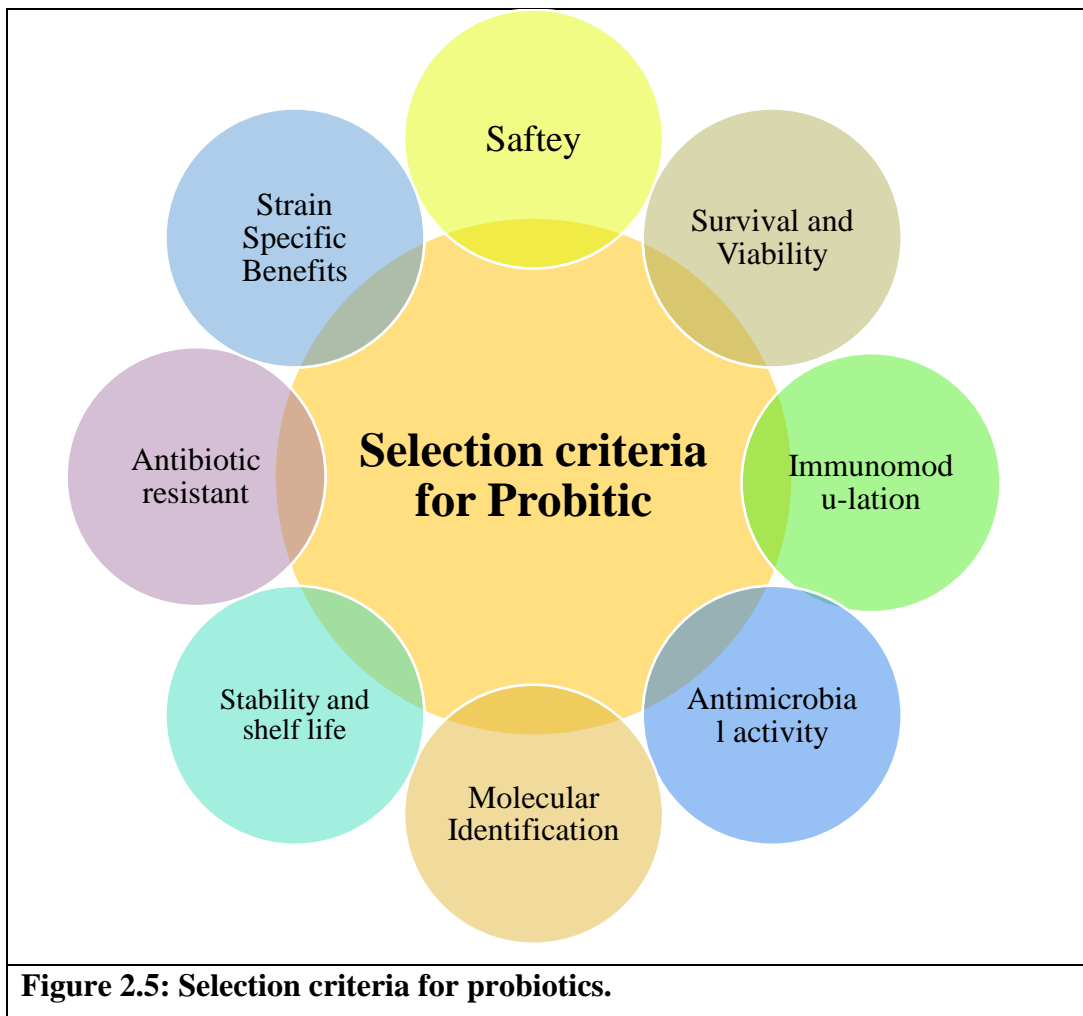
newborn is delivered. There seems to be a high degree of heterogeneity among individuals concerning microbial populations, including those living under the same environmental conditions. The diversity of microbial communities in the human body is influenced by various factors, including age, eating habits, immune status, and other factors that are not yet fully understood (Isolauri et al., 2004). Vaginal medication delivery, breastfeeding, and the child's interaction with the environment have been identified as factors that support the appropriate colonization of the intestinal microbial community and the development of the intestinal immune system. On the other hand, delivery via C-section, premature birth, antibiotic use, infant formula feeding, indoor living, excessive sanitation, and synthetic food preservation have been associated with evidence suggesting a disruption of natural colonization processes. The overall sizeable intestinal population was estimated to contain approximately 10^{11} - 10^{12} CFU / g of intestinal substance, to contain approximately 500-1000 different bacterial species in a given person (Zhu et al., 2010), and to represent approximately 10^{12} cells / g of dry-weight feces. Then, the intestinal microbiota conducts much metabolic activity to facilitate consistent removal by feces. The abundance of the viral microbiome, also known as the virome, is more substantial than the bacterial load in the gut. Specifically, the bulk of the viral entities in the gut are bacteriophages, viruses that infect bacteria and Archaea. It is widely acknowledged that the colonic microbiota mainly comprises a limited proportion of yeasts and other eukaryotic microorganisms. (e.g., protists) and *Lactobacillus* (Schulze and Sonnenborn, 2009; Reyes et al., 2010; Mills et al., 2013; Parfrey et al., 2014). The Common genera or species that are found inside the gut intestinal microbiota include *Clostridium*, *Bifidobacterium*, *Eubacterium*, *Bacteroides* and *Ruminococcus* as subdominant microbiota, *Streptococcus*, *Escherichia coli*, *Lactobacillus*, *Staphylococcus*, *Veillonella* and *Proteus* (Tannock, 2003; Krych et al., 2013).

Despite this, one would expect that a probiotic candidate isolated from the microbiota of human intestines might perform better in humans than a strain isolated from another source (food, for example) or another species (for example, pigs). This is because it might be expected that it would be ecologically better to use the species-specific criteria, which are frequently and traditionally employed during the investigation of

new probiotic species. Both the strains collected from the environment of the intestinal tract and those derived from fermented foods have been found to contain probiotic qualities. For instance, in the often harsh acidic conditions encountered in fermented milk, various commercial probiotic strains of gastrointestinal provenance performed efficiently (Viljoen, 2001; Vinderola et al., 2002). These studies were published in the journals Viljoen and Vinderola. Important metabolic and functional properties can be seen both in gut-derived *lactobacilli* and in strains of *lactobacilli* that have been isolated from fruit, according to research by Ren et al. (2014). The immunological response of the mucous membrane of the intestine in mice can be stimulated by commensal and non-commensal strains of *L. fermentum* and *L. acidophilus* (Dogi & Perdigón, 2006). This function is of concern to probiotic bacteria. The criterion for the origin of humans is still applicable, but this time for "historical reasons" and "common sense" rather than for reasons that are supported by good biology, which are not yet readily apparent (Morelli, 2000).

2.11 Selection criteria for probiotic lactic acid bacteria

LAB involves a set of considerations and characteristics that help determine their suitability for use as probiotics. Probiotics are live microorganisms that confer health benefits to the host when administered adequately. Here are some critical selection criteria for probiotic LAB (Figure 2.5):



- 1) **Safety:** Safety is paramount when selecting probiotic LAB strains. They should have a long history of safe use in humans. They should not cause adverse effects or infections, especially in vulnerable populations such as infants, the elderly, or immune-compromised individuals. Here are some key points about the safety of probiotic bacteria:
 - Generally Recognized as Safe (GRAS): Many strains of probiotic bacteria have been classified as GRAS by regulatory authorities, such as the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA).
 - Safety in Healthy Individuals: Probiotics are typically well-tolerated by healthy individuals and considered safe for most people, including children and pregnant women.

- **Possible Side Effects:** Some people may experience mild side effects when starting probiotics, such as bloating, gas, or digestive discomfort. These symptoms are usually temporary and subside as the body adjusts to the new bacteria.
- **Risk for Immunocompromised Individuals:** While probiotics are generally safe, individuals with compromised immune systems, such as those undergoing chemotherapy or organ transplant recipients, may be at a higher risk of developing infections from certain strains of probiotic bacteria. Immunocompromised individuals must consult their healthcare provider before taking probiotic supplements.
- **Quality and Source:** The safety of probiotics can vary depending on the quality and source of the product. It is essential to choose probiotic supplements from reputable manufacturers that provide detailed information about the strains, potency, and expiration dates.
- **Specific Strains and Conditions:** Different strains of probiotic bacteria have varying effects and may be more appropriate for specific health conditions. Research is ongoing to determine which strains are most beneficial for different health concerns. **Individual Variations:** Each person's response to probiotics may vary. What works well for one person may not have the same effect on another

2) **Survival and viability:** Probiotic LAB strains need to survive the harsh conditions of the gastrointestinal tract, including exposure to stomach acid and bile salts, to reach the intestines alive. They should have a high tolerance to low pH and bile acids to ensure their viability and functionality. When used in food systems, probiotic bacteria must survive the journey from the mouth to the lower intestinal tract. Therefore, these bacteria must resist enzymes like lysozyme found in the oral cavity. As they pass through the stomach and enter the upper intestinal tract, which contains bile, they should be able to resist the digestive process. Probiotics must endure the adverse conditions encountered in the host's upper gastrointestinal tract to be effective in the lower digestive tract (Ding & Shah, 2007). They must be able to withstand the harsh conditions of the stomach, with its low pH levels of 1.5-3.0, as well as the presence of bile in the upper intestine (Chou & Weimer, 1999). In order to exhibit probiotic

properties, these strains need to reach the lower intestinal tract and establish themselves there.

In another type of research, a vast collection of lactic acid bacteria, including over 200 strains of *Lactobacillus* and *Bifidobacterium*, was analyzed to determine which bacteria are most suited for usage as probiotics. As part of the selection process, we determined each candidate's ability to withstand bile and acid. It was decided to select four strains, three from dairy sources and one from human origin. *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* LA-1, available in the marketplace as probiotic strains, were used to compare these bacteria. According to Prasad et al. (1998), the isolated strains were subjected to a variety of pH levels, ranging from 1 to 3, as well as tests to determine their tolerance to bile at concentrations of 0%, 0.5%, and 1%-per-volume (w/v). In a different piece of research, twenty-nine strains of *Lactobacillus* with a dairy background were evaluated in vitro to determine the probiotic potential of each strain. Within the pH range of 1.0 to 3.0, these bacteria's resistance to the antibiotic was evaluated.

The ability to tolerate bile salt was assessed using 0.3% ox gall. All strains tested demonstrated resistance to pH 3.0 for 3 hours. However, they lost their viability within 1 hour when exposed to pH 1.0. Additionally, all strains tolerated a 0.3% bile salt concentration for 4 hours. Three *Lactobacillus* species obtained from human milk were investigated in a separate experiment to determine their potential as probiotic strains. These species were identified as *Lactobacillus gasseri*, with one of them being *Lactobacillus fermentum*. Their survival in a low pH environment and gastrointestinal conditions was compared to commercially available probiotic strains such as *L. rhamnosus* GG, *L. casei*, and *L. johnsonii* La1. The results indicated that the strains, particularly *L. gasseri*, exhibited promising potential as probiotic strains (Martin et al., 2004).

- 3) **Adhesion to host cells:** The adhesion of probiotics to host cells is crucial in their colonization and beneficial interaction with the human body. When probiotics adhere to the surface of host cells, they can exert their beneficial effects by modulating the host's immune response, inhibiting the growth of harmful bacteria, and promoting

overall gut health. Here are some key points regarding the adhesion of probiotics to host cells:

- i. **Adhesion Mechanisms:** Probiotics employ various mechanisms to adhere to host cells. These mechanisms include:
 - a. **Receptor-Ligand Interactions:** Probiotics possess specific adhesins or surface molecules that can recognize and bind to specific receptors on the surface of host cells. These adhesins can be proteins, glycoproteins, or carbohydrates
 - b. **Mucus-Binding:** The mucus layer that lines the gastrointestinal tract acts as a protective barrier and contains various glycoproteins and mucins. Some probiotics have mucus-binding proteins that enable them to adhere to the mucus layer, allowing them to colonize and interact with the underlying epithelial cells.
 - c. **Biofilm Formation:** Probiotics can form biofilms, complex structures of microbial cells embedded in a matrix of extracellular polymeric substances. Biofilms can facilitate probiotics' adhesion to host cells and increase their stability and protection.
- ii. **Host Cell Factors:** Host cells also play a role in adhesion. The surface characteristics of host cells, such as the presence of specific receptors or molecules, can influence the adhesion of probiotics. Host cell factors, including mucus composition, glycocalyx structure, and surface charge, can affect the ability of probiotics to adhere to the intestinal epithelium.
- iii. **Strain-Specificity:** Adhesion abilities can vary among different strains and species of probiotics. Some strains may have enhanced adhesion properties due to specific surface molecules or adhesins they possess. It is important to note that not all probiotics have the same adhesion capabilities, and strain selection is a critical factor in determining their potential efficacy.
- iv. **Competitive Exclusion:** Probiotics' adhesion to host cells can provide a competitive advantage by preventing harmful bacteria from attaching to the same sites. This phenomenon, known as competitive exclusion, helps maintain a balanced gut microbiota and can contribute to overall gut health.

Understanding the mechanisms and factors involved in probiotics' adhesion to host cells is essential for selecting and developing effective strains. It allows researchers

to target specific health benefits and optimize probiotic interventions for various conditions.

4) **Immunomodulatory properties:** Probiotic LAB should possess immunomodulatory capabilities, which can modulate the host's immune response. They should be able to stimulate the production of beneficial cytokines and promote a balanced immune system, helping to regulate inflammation and support immune function. While they are primarily known for their positive effects on the gut microbiota and digestive health, research has also shown that probiotics can exert immunomodulatory properties. Here are some of the key ways in which probiotics can influence the immune system:

- **Regulation of immune responses:** Probiotics have been found to regulate immune responses by modulating the production and activity of various immune cells and molecules. They can enhance the activity of specific immune cells, such as natural killer (NK) cells, macrophages, and dendritic cells, which are essential for immune defense against pathogens. Additionally, probiotics can regulate the balance between pro-inflammatory and anti-inflammatory cytokines, thereby influencing immune responses.
- **Enhancement of gut barrier function:** The gut barrier plays a crucial role in preventing the entry of harmful substances and pathogens into the bloodstream. Probiotics can strengthen the gut barrier by increasing mucus production, enhancing tight junction integrity between intestinal cells, and promoting the secretion of antimicrobial peptides. Probiotics help prevent excessive immune activation and inflammation and maintain a healthy gut barrier.
- **Regulation of systemic immune responses:** Probiotics can also modulate systemic immune responses beyond the gut. They can influence the activity of immune cells in the blood, lymph nodes, and other organs, affecting immune function throughout the body. This modulation of systemic immunity can benefit various conditions, including allergies, autoimmune diseases, and respiratory infections.
- **Anti-inflammatory effects:** Many probiotic strains have been found to possess anti-inflammatory properties. They can inhibit the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-

alpha (TNF-alpha), while promoting the secretion of anti-inflammatory cytokines, such as interleukin-10 (IL-10). This anti-inflammatory action helps to regulate immune responses and reduce chronic inflammation.

- Interaction with the gut microbiota: Probiotics can influence the composition and function of the gut microbiota, which has extensive crosstalk with the immune system. By promoting the growth of beneficial bacteria and inhibiting the growth of pathogenic bacteria, probiotics contribute to a balanced gut microbiota, which is essential for optimal immune function.

It is important to note that the immunomodulatory effects of probiotics can vary depending on the specific strain, dosage, and individual characteristics. While probiotics have shown promise in modulating immune responses and supporting immune health, further research is still needed to fully understand their mechanisms of action and identify the most effective strains and formulations for specific immune-related conditions.

5) **Antimicrobial activity:** Probiotic LAB can produce antimicrobial substances such as organic acids, bacteriocins, and hydrogen peroxide, which can inhibit the growth of pathogenic bacteria in the gut. Probiotic bacteria are known for their beneficial effects on human health, particularly in maintaining a healthy balance of the gut microbiota. While their primary role is not antimicrobial activity, specific probiotic strains have been found to exhibit antimicrobial properties against various pathogens. Here are some key points regarding the antimicrobial activity of probiotic bacteria:

- Production of antimicrobial substances: Probiotic bacteria can produce a range of antimicrobial substances, including organic acids (such as lactic acid and acetic acid), hydrogen peroxide, bacteriocins (small antimicrobial peptides), and enzymes (such as lysozyme). These substances can inhibit the growth of pathogenic bacteria by altering the pH, creating oxidative stress, or directly damaging their cellular structures.
- Immune modulation: Some LAB strains can modulate the host immune response, enhancing the production of antimicrobial peptides and improving the defense mechanisms against pathogens.

- Nutritional competition: LAB can utilize available nutrients more efficiently than many other bacteria, reducing the availability of resources for pathogenic microorganisms and limiting their growth.
 - Specific strains and pathogens: The antimicrobial activity of probiotic bacteria can vary depending on the specific strains and the targeted pathogens. Different probiotic strains may have different mechanisms of action and varying effectiveness against specific pathogens. For example, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Bifidobacterium bifidum* are commonly studied probiotic strains with antimicrobial properties.
 - Clinical applications: The antimicrobial activity of probiotic bacteria has potential clinical applications. Probiotics have been investigated for their role in preventing and treating various infections, such as gastrointestinal infections, urinary tract infections, respiratory tract infections, and vaginal infections. However, it is essential to note that probiotics should not be considered as a replacement for conventional antimicrobial therapies but rather as a complementary approach.
- 6) **Resistance to antibiotics:** It is essential to ensure that probiotic LAB strains do not carry antibiotic resistance genes or are not prone to acquiring antibiotic resistance. This precaution helps prevent the transfer of resistance genes to other bacteria in the gut and reduces the risk of compromising the effectiveness of antibiotics in the host. Certain antibiotics exhibit resistance against a wide range of bacteria, including LAB (lactic acid) and probiotic bacteria. This resistance can be attributed to genes found on chromosomes, transposons, or plasmids (Adams, 1995). However, the available evidence regarding the circumstances under which these genetic elements can be transferred is inconclusive, and it remains uncertain whether this condition will pose a clinical concern. Using probiotic bacteria containing specific medications raises concerns about the genetic traits associated with crop resistance. Probiotic bacteria possess transferable genes for drug resistance but cannot be utilized for human consumption. Consequently, it is urgently necessary to develop a systematic approach to assess drug resistance profiles in *lactobacilli* and *bifidobacteria* (Reid et al., 2001). Due to the significance of this matter, it has been proposed that further research is required to determine the antibiotic resistance of both these bacteria. It is recommended

that when working with probiotic strains, precautions should be taken to ensure that they do not carry transferable genes that encode resistance against commonly used medications. Moreover, investigations into the antibiotic tolerance of *Lactobacilli* and *Bifidobacteria* and the potential transferability of genetic elements to other intestinal and foodborne bacteria are also warranted. For example, while certain strains of *Enterococcus* exhibit probiotic properties, it has been established that *Enterococcus* is a significant source of hospital-acquired infections, with isolates increasingly demonstrating resistance to vancomycin. Furthermore, *Enterococcus* is not approved for human use as a probiotic (Reid et al., 2001).

Antibiotic resistance occurs when bacteria develop the ability to survive and grow in the presence of antibiotics that generally kill them. This resistance can arise through genetic mutations or other bacteria' acquisition of resistance genes. While antibiotic resistance is a significant concern for pathogens that cause infections, it is generally not a major issue with probiotic strains. Most probiotic bacteria have not shown significant resistance to antibiotics commonly used in clinical practice. However, it is worth noting that some strains of bacteria used in probiotic products may possess intrinsic resistance to certain antibiotics. This means they naturally have mechanisms to resist the effects of those specific antibiotics. In most cases, these resistance mechanisms do not extend to other antibiotics. Choosing probiotic products that have undergone rigorous quality control and testing is crucial to ensure their safety and effectiveness. When using antibiotics, it is generally recommended to continue taking probiotics as long as they are not contraindicated by the specific antibiotic being used.

7) **Strain-specific health benefits:** Different LAB strains may possess unique properties and health benefits. Specific strains might be more effective in addressing certain conditions, such as diarrhea, lactose intolerance, or inflammatory bowel diseases. Therefore, selecting LAB strains with documented efficacy for the intended health benefit is crucial. While the general benefits of probiotics apply to many strains, certain strains have been studied for their specific health benefits. Here are some examples:

- *Lactobacillus rhamnosus* GG: This strain has been extensively studied and is known for its ability to support gastrointestinal health. It has been shown to

alleviate diarrhea symptoms, including those caused by antibiotics and viral infections. *L. rhamnosus* GG may also help reduce the risk of respiratory tract infections in children.

- *Bifidobacterium lactis* BB-12: This strain has been associated with improved gut health and immune function. It has been studied for its potential to reduce the symptoms of irritable bowel syndrome (IBS) and alleviate constipation. *B. lactis* BB-12 may also enhance immune responses, particularly in infants and the elderly.
- *Saccharomyces boulardii*: Though technically a yeast and not a bacterium, *S. boulardii* is a probiotic due to its beneficial effects on gut health. It has been extensively studied for its ability to prevent and treat antibiotic-associated diarrhea and *Clostridium difficile* infection. *S. boulardii* may also help reduce inflammatory bowel disease (IBD) symptoms and traveler's diarrhea.
- *Streptococcus thermophilus*: This strain is commonly used to produce yogurt and other fermented dairy products. It has been associated with lactose digestion and may help improve lactose intolerance symptoms. *S. thermophilus* may also support immune function and contribute to a healthy gut environment.

8) **Stability and shelf-life:** Probiotic LAB strains should have good stability during production, storage, and distribution. They should remain viable and maintain their beneficial properties throughout their shelf-life to ensure efficacy when consumed. The stability and shelf-life of probiotics can vary depending on several factors, including the specific strain of probiotics, the formulation, and the storage conditions. Here are some general considerations:

- **Strain selection:** Different probiotic strains have varying levels of stability. Some strains are more robust and can better withstand harsh conditions, such as exposure to moisture, heat, or acidity. It is essential to choose probiotic strains known for their stability and resilience.
- **Formulation and packaging:** The formulation of the probiotic product, such as capsules, tablets, powders, or liquids, can affect its stability. Proper packaging is essential to protect the probiotics from moisture, light, and oxygen, which can

degrade their viability. Many probiotics require refrigeration to maintain potency, while others can be stored at room temperature.

- **Manufacturing processes:** The manufacturing processes used to produce probiotics can influence their stability. Factors such as temperature, moisture control, and quality control measures during production can impact the viability of the probiotic organisms.
- **Storage conditions:** Proper storage conditions are crucial for maintaining the viability of probiotics. Refrigeration is often recommended for probiotic products, as low temperatures can slow down the degradation of the organisms. However, some probiotics are stable at room temperature for a certain period, as indicated on the product label. It is essential to follow the storage instructions provided by the manufacturer.
- **Shelf-life determination:** The shelf-life of a probiotic product is determined through stability testing. This involves assessing the viability and potency of the probiotic organisms over a specified period under different storage conditions. The manufacturer typically establishes a shelf-life based on the results of these tests.

9) **Genomic and phenotypic characterization:** Detailed genomic and phenotypic characterization of probiotic LAB strains is necessary to identify their taxonomic classification, genetic stability, metabolic activities, and potential virulence factors. This characterization helps assess the safety and functional attributes of the strains. Here is an overview of the process:

- **Genomic Characterization:** Genomic characterization involves sequencing and analyzing the genetic material of probiotic strains. This is typically done using next-generation sequencing technologies, which provide detailed information about the organism's DNA sequence.
 - a. **Genome Sequencing:** The first step is to isolate the DNA from the probiotic strain and sequence its entire genome. This blueprints the organism's genetic content, including its genes, regulatory regions, and other non-coding regions.
 - b. **Gene Annotation:** Once the genome is sequenced, bioinformatics tools are used to identify and annotate the genes present in the probiotic strain. This process

helps identify potential functions of the genes and provides insights into the organism's metabolic capabilities.

- c. **Comparative Genomics:** Researchers can identify common genes and unique genetic features by comparing the genomes of different probiotic strains. Comparative genomics helps understand the genetic diversity among probiotics and provides clues about their functional differences.
 - d. **Functional Genomics:** Studies aim to understand how genes contribute to the probiotic's phenotype. Techniques such as gene expression profiling (transcriptomics) and protein analysis (proteomics) can provide insights into the active genes and pathways under specific conditions.
- **Phenotypic Characterization:** Phenotypic characterization involves studying probiotic strains' observable traits and behaviors, including their growth patterns, metabolic activities, and host-environment interactions.
 - a. **Growth Characteristics:** Researchers examine the growth rate and requirements of probiotic strains under different conditions, such as temperature, pH, and nutrient availability. This information helps optimize their production and storage.
 - b. **Metabolic Activities:** Probiotics can produce various metabolites, such as short-chain fatty acids, antimicrobial compounds, and vitamins. Phenotypic characterization investigates the metabolic capabilities of probiotic strains, shedding light on their potential health benefits.
 - c. **Adhesion and Colonization:** Probiotics must adhere to the gut epithelium to exert their effects. Phenotypic studies evaluate probiotics' adhesion and colonization abilities to understand better their mechanisms of action and persistence in the gut.
 - d. **Immune Interactions:** Probiotics interact with the host immune system, modulating immune responses. Phenotypic characterization investigates the immunomodulatory properties of probiotics, including their ability to enhance immune function or suppress inflammation.

- e. **Safety and Stability:** Phenotypic studies assess the safety and stability of probiotic strains, including their resistance to antimicrobial agents and ability to survive the gastrointestinal tract.

Researchers can comprehensively understand probiotics' genetic potential, metabolic capabilities, and health-related properties by combining genomic and phenotypic characterization. This knowledge contributes to developing targeted probiotic interventions and improves our understanding of how these microorganisms interact with the host.

10) **Human clinical studies:** Lastly, well-designed human clinical studies should support the effectiveness and safety of probiotic LAB strains. Clinical trials provide scientific evidence of the strains' health benefits and aid in establishing dosage recommendations and indications for use. It includes:

- **Safety and Tolerability Trials:** These trials aim to assess the safety and tolerability of a probiotic strain or formulation in healthy individuals or specific patient populations. They typically involve a small number of participants and focus on monitoring adverse events and potential side effects.
- **Efficacy Trials:** Efficacy trials investigate the effectiveness of probiotics in preventing or treating specific health conditions. These trials often have a larger sample size and involve randomized, controlled designs. Participants are divided into groups, one receiving probiotic intervention and another receiving a placebo or a different treatment. The study assesses various outcomes to determine whether the probiotic positively impacts the condition of interest.
- **Mechanistic Trials:** Mechanistic trials aim to understand how probiotics work at a biological level. These trials often involve fewer participants and focus on exploring specific mechanisms, such as changes in gut microbiota composition or immune system markers in response to probiotic treatment.
- **Dose-Finding Trials:** Dose-finding trials help determine the optimal dosage of a probiotic intervention. These trials involve administering different doses of the probiotic to participants and monitoring the response to identify the most effective and safe dose.

- **Long-Term Trials:** Long-term trials assess the effects of probiotic interventions over extended periods, usually several months or years. These trials aim to evaluate the sustainability of probiotic benefits and investigate any potential long-term effects or changes in health outcomes

It is important to note that specific clinical trial data availability may vary, and new studies are constantly being conducted. To access the most up-to-date information on human clinical trials of probiotics, I recommend consulting reputable scientific databases and clinical trial registries or contacting healthcare professionals and researchers specialized in the field.

2.12 Research related to millet-based products

For people worldwide, millet grains are a significant source of nutrients like vitamins, minerals, proteins, carbohydrates, and fiber. These nutrients can be used as sources of non-digestible carbohydrates that, in addition to promoting several positive health effects, can also preferentially speed up the development of *Lactobacilli* and *Bifidobacteria* in the intestine, serving as prebiotics (Andersson et al., 2001). Although several fermented millet products without milk have been made for human consumption in the past, only the probiotic qualities of the microorganisms found in conventional fermented millet-based products have been recognized.

In their study, Kumar, Vijayendra, and Reddy (2015) examined trends in dairy and non-dairy probiotic products. Most recently, Bansal et al. (2016) released a review on non-dairy probiotic meals. Vasudha and Mishra (2013) highlighted research on probiotic beverages derived from non-dairy sources. These reviews highlight the importance of non-dairy foods in delivering probiotic bacterial strains.

Probiotic drinks Launched in India in 2019-2020

| S.No. | Year | Probiotic Beverages | Main Ingredient | Company |
|-------|----------|---------------------|-----------------|---------------|
| 1. | Mar-2019 | Plentiful | Saffron | Lifeway Foods |

| | | | | |
|----|----------|---------------------------------|----------------------------------|---------------------|
| 2. | May-2019 | Wellness drink | Lactose | Fonterra NZMP |
| 3. | Jun-2019 | Materna Opti-Lac | Milk | Nestle |
| 4. | Jan-2020 | Harmless Harvest Drinks | Coconut | Harmless Harvest |
| 5. | Mar-2020 | Kombucha Apple Cider Vinegar | | KeVita |
| 6. | Apr-2020 | NesQuino | Fruits and Vegetable Syrup | Nestle |
| 7. | Jul-2020 | Low fat Yogurt | Milk | Danone |
| 8. | Oct-2020 | Function Beverage | | Bio-K Plus |

A few millet-based beverages are discussed below:

- Madua, the most widely consumed beverage made from finger millet, is made in Arunachal Pradesh. The millet is roasted for 30 minutes, then chilled and simmered till tender. A perforated basket containing Ekam leaves is filled with the cooked grains and starter culture, and the mixture is incubated at room temperature for 4 to 7 days. After fermentation, hot water is removed from the surface and put into a tank for storage. Madua is the name given to the obtained substance. A high-quality media pleasing to the eye is golden yellow. In Arunachal Pradesh, India, various finger millet-based beverages were created and enjoyed (Shrivastava et al., 2012).
- Boza is consumed in Bulgaria, Turkey, Romania, and Albania, particularly in the Balkans, the Middle East, and Central Asia. It is a homogenous mixture of cereals like millet, maize, wheat, and barley, blended with sugar or saccharin, from light to dark beige, soft, mildly mild to mildly sour. The fermentation process involves adding yeast or a starter culture to the cooked grains and allowing them to ferment for a certain period. This fermentation process gives boza its characteristic tangy

and slightly sour taste. Sometimes, additional ingredients such as sugar, water, and spices like cinnamon or vanilla may be added to enhance the flavor. Boza is known for its thick, viscous texture, similar to a smoothie or milkshake. It is often consumed as a refreshing beverage, especially during the hot summer months. Boza is also considered a traditional winter drink in some cultures, as it is believed to provide warmth and energy. Microflora characterization of Bulgarian boza reveals that it comprises primarily yeasts and lactic acid bacteria at an average ratio of 2.4 LAB/yeast. The isolated lactic acid bacteria *Leuconostoc reffinolactis*, *Leuconostoc mesenteroides*, *Lactobacillus Acidophilus*, *Lactobacillus coprophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis*. (Blandino et al., 2003). Aside from its taste, boza is also appreciated for its nutritional value. It is rich in carbohydrates, proteins, vitamins, and minerals, making it a good energy source. However, it is essential to note that boza is also relatively high in calories and can contain alcohol, depending on the fermentation process and recipe used.

- Bushera is a traditional fermented beverage consumed in some parts of East Africa, particularly in Uganda, Rwanda, and Burundi. It is made from mashed grains, typically sorghum or millet, soaked in water and left to ferment for some time. The fermentation process of bushera involves natural yeast and bacteria in the environment, which convert the starches in the grains into sugars and alcohol. The resulting drink is typically low in alcohol content, ranging from 1% to 5%, and has a slightly sour taste. After this step, sprouted millet or sorghum flour is added, and the mixture is left to ferment at room temperature for one to six days. The Bushera obtained lactic acid bacteria belonging to five different taxa. These bacteria include *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*. *Lactobacillus Brevis* was often segregated from other animals (Muianja et al., 2003). Bushera is often enjoyed as a refreshing and nutritious beverage. It is commonly consumed as a traditional staple during meals or as a social drink during gatherings and celebrations. It is also known for its probiotic properties, as fermentation produces beneficial bacteria that can promote digestive health.

- Kefir is a fermented milk drink with a slightly sour taste and a creamy texture. It is made by adding kefir grains to milk and allowing it to ferment for a certain period. Kefir grains are not actual grains but rather a combination of bacteria and yeast that form a symbiotic culture. These grains contain a variety of beneficial microorganisms, including lactic acid bacteria, yeasts, and acetic acid bacteria. During fermentation, the microorganisms in kefir grains metabolize the lactose in milk, converting it into lactic acid. This fermentation process gives kefir its unique flavor and texture. Kefir is known for its probiotic properties, as it contains a diverse range of beneficial bacteria and yeasts that can positively affect gut health when consumed. Kefir is believed to have originated in the Caucasus Mountains of Eastern Europe. It has been consumed for centuries in that region and has gained popularity worldwide due to its potential health benefits. In addition to its probiotic properties, kefir is a good source of calcium, protein, and various vitamins and minerals. Kefir can be made from different types of milk, such as cow's milk, goat's milk, or plant-based alternatives like coconut or soy milk. The flavor and consistency of kefir may vary depending on the type of milk used and the fermentation process. Kefir can be consumed independently or used in various recipes, such as smoothies, dressings, or baked goods. It is commonly available in grocery stores, or you can make it at home using kefir grains and milk. Fermentation requires specific conditions and time, so achieving the desired results may take some practice.
- Kavass is a traditional fermented beverage in Eastern Europe, particularly Russia and Ukraine. It is a non-alcoholic drink made from fermented grains, most commonly rye bread, and is often enjoyed during hot summer. Making kvass involves soaking stale or leftover rye bread in water and adding sugar, yeast, and sometimes fruits or herbs for flavor. The mixture is left to ferment for a couple of days, during which the yeast converts the sugars into alcohol and carbon dioxide. However, fermentation is usually brief, resulting in a beverage with a shallow alcohol content, typically less than 1%. Kvass is tangy and slightly sour, similar to a light beer or mildly effervescent soda. It is typically dark brown and can be cloudy in appearance. Traditional recipes may vary in ingredients and flavors, with

some variations using beets, fruits, or honey. In its home countries, kvass is readily available commercially, often sold in bottles or poured from large barrels at street markets. It is also a popular homemade beverage, with families making their batches using traditional recipes. Kvass is known for its refreshing qualities and is often consumed as a thirst-quenching beverage, especially during hot weather. It can be enjoyed plain or with ice and is sometimes used as a base for cocktails or mixed with other beverages.

COMPARISON BETWEEN MILK, FRUIT, AND MILLET-BASED BEVERAGES

| Nutritional Components | Milk-based Probiotic (Yakult) https://www.yakult.co.in/whyyakult.php | Fruit Based Probiotic (Goodbelly) https://goodbelly.com/ | Millet Based Probiotic (Togwa) (Ndabikunze, B. K, 2001) |
|-------------------------------|---|---|--|
| Protein | 1g | 1g | 9.1g |
| Fat | 0g | 0g | 1.85g |
| Carbohydrates | 12g | 23g | 72g |
| Iron | 0mg | 2g | 15.3g |
| Sodium | 15mg | 10mg | 13.3g |
| Potassium | 50mg | 120mg | 167 |
| Calcium | 40mg | 0mg | 3.95mg |
| Sugar | 14g | 19g | 2g |
| Benefits | It increases the number of beneficial bacteria in the digestive system and makes it difficult for the harmful ones to take over. | Improve Gut Health. | Multiple Health benefits, rich in antioxidants, controls blood sugar levels. |

| | | | |
|------------------|--|--|-----------------|
| Drawbacks | Added Sugar is not suitable for people with lactose intolerance. | High concentrations of fructose are not suitable for people with fructose intolerance. | Less shelf life |
|------------------|--|--|-----------------|

2.13 Probiotics market trends

The probiotics market has been experiencing robust growth, driven by several key factors. First and foremost is increasing consumer awareness regarding the importance of maintaining gut health. This heightened awareness is backed by scientific research demonstrating the significant impact of gut microbiota on digestion, immunity, and even mental health. Consequently, consumers actively seek products promoting gut health, leading to a surge in demand for probiotics. Additionally, the market has been buoyed by the rising popularity of functional foods and beverages as consumers look for products that offer health benefits beyond essential nutrition. Probiotics, known for supporting digestive health, have become a key ingredient in these functional products. Moreover, expanding probiotic applications beyond traditional categories like yogurt and supplements has further fueled market growth. From dairy alternatives to skincare products, probiotics are incorporated into diverse products, appealing to a broader consumer base. Furthermore, the COVID-19 pandemic has underscored the importance of immune health, increasing interest in products that support immunity, including probiotics. Amidst these trends, ongoing research and development efforts continue to drive innovation in the market, with manufacturers developing new strains and delivery formats to meet evolving consumer demands. As probiotic products become increasingly accessible through various retail channels and online platforms, the market is poised for continued expansion. However, manufacturers must navigate complex regulatory environments and address challenges such as heightened competition and the need for transparency in labeling and marketing practices. Despite these challenges, the outlook for the probiotics market remains positive, with sustained growth expected as consumers prioritize health and wellness in their purchasing decisions.

2.13.1 Market potential for probiotics all over the world

Probiotics are an increasing market category, utilizing bacterial culture as dietary and medicinal additives. According to the consumer report, Europe ranks first in product numbers and is the fastest-growing probiotic industry, while Japan ranks second in the probiotics marketing sector.

The practice of consumption of natural probiotic items such as fermented milk is relatively strong in **European** countries. European customers also ingest probiotics in a variety of dietary additives and vitamins. Inside Europe, the dairy industry is the market's most established segment of probiotic yogurt and fermented milk, which customers use as a regular dosage. Probiotic dietary supplements are slowly gaining popularity, but new products are continually developing. Unsurprisingly, the demand for fortified dairy foods with probiotic bacteria has successfully generated benefits. The fastest-growing item group is yogurt-type or fermented milk-related beverages, although the variety of applications related to probiotic food is not restricted to milk-dependent items. Other items on the market are also offered, including probiotic fruit drinks, berry soups, and fermented foods focused on soy and cereals. Western Europe's overall demand for probiotic food is 11.4 billion Euros. The main segment includes yogurt and sweets, with revenues of € 1 billion and the most significant share of the probiotic milk industry. Annual increase in sales over the next five years is expected at about 7–8 percent.

Japan is considered the origin of various functional foods, and it is a strong and well-developed sector of functional foods and probiotic-supported products. Between 1988 and 1998, over 1700 items under the functional food group were released in Japan, with an approximate turnover of about 14 US\$ billion in 1999. The industry was reported to be US\$ 5 billion in 2003 and about 5.73 US\$ billion in 2006, although more than 500 goods were available internationally and sold under the FOSHU name in 2005 (Side 2006).

The **USA** market is also rising increasingly due to the US population's greater tolerance and attraction to probiotic goods coupled with the idea of health care prevention. The United States already owns the world's highest and fastest-growing practical food and nutraceutical market (Side, 2006). In 2016, probiotics reached a market value of \$21.3

billion. This funds oversized shipments from Japan, North and South Korea, China, India, Brazil, the EU, Australia, New Zealand, and other countries. The probiotics industry was estimated to be valued at USD 58.17 billion in 2021. From 2021 to 2030, it is anticipated to expand at a CAGR (compound annual growth rate) of 7.5%. The growing demand of consumers for preventative healthcare and the creation of potent probiotic strains are the main factors driving the market's expansion. When consumed in sufficient quantities, probiotics benefit the body, including improved gut health and decreased inflammation in the intestines. Probiotics are vital in preventive healthcare as they strengthen the immune system, thereby preventing diseases. Consequently, the market is anticipated to grow significantly during the forecast period due to the growing awareness about preventive healthcare.

Canada's nutraceutical and functional food industry is relatively small and gradually growing. Canadian trade in nutraceuticals and natural food-aided goods accounted for 3 percent of the global demand over the US (35 percent) and EU (32 percent) throughout 2013. Statistical evidence indicates that much of Canada's usable food and nutraceutical shipments were to the United States, Japan, and the EU in 2002 (Fem, 2007).

China is growing as the world's rising manufacturer of dietary supplements, and it is hard for imported goods to compensate for demand alone. Manufacturers aiming to manufacture nutraceuticals look forward more to developing manufacturing facilities in China to serve the Chinese market or sell to other countries markets. More than 3,000 local dietary supplement producers are on the market in China, responsible for about 559 international labels and over 10,000 health food items. Average annual growth in the market value of health foods in China between 2010 and 2015 was recorded from RMB 133 to RMB 450, respectively (Fem, 2007).

Japan, which for the first time brought functional products to the world in the 1980s, forms the Asia-Pacific market's most significant portion. However, in the next few years, China has seen a rapid rise in the sales of practical food goods. To expand, several businesses consider it rational to use dairy goods as a "delivery system" for usable ingredients, which has almost doubled the dairy probiotic demand in China in the last

four years. Probiotics and other functional ingredient sales pursue China's remarkable dairy product growth (Basu et al., 2007).

2.13.2 Status of probiotics in India

The food sector is one of India's hotspots for investment. Following enormous increases in awareness about probiotics and their health benefits among the Indian community, the demand for probiotic foods has gained substantial popularity. Indian and global companies have come into the picture since they entered the Indian food business in 2007. As recorded in 2010, the Indian probiotic market was valued at \$2 million. According to statistics reported by USA-based research company Frost and Sullivan, probiotic products in India in August 2012 produced sales of about US\$ 310 million in 2011. The market valuation was projected to rise by 2018 to US \$522.8 million. Milk and fermented milk goods collectively account for 62 percent of the probiotic drug market share (Indian Customer Study, 2010).

The probiotics market in India is anticipated to increase steadily throughout the anticipated period at a pace of 13.56% annually, reaching a value of US\$961.856 million in 2025 from US\$448.456 million in 2019. With the help of solid institutions and trade alliances, India has emerged as the second-fastest-growing major economy in the world. It is predicted to rank among the top three economic powers by 2025. India is the second most populous nation in the world, with 1.37 billion people, and 62% of them are under 35. The nation's Real GDP growth is still driven by population increase, and roughly 60% of the GDP comes from private spending. The food processors, importers, distributors, retailers, and food service providers make up India's expanding agriculture industry. India produces much food and has seen a 10% growth in its imports of bulk, intermediate, consumer, and agricultural goods from \$22 billion in 2013 to \$25 billion in 2018.

The demand for imported consumer foods in India has experienced a significant increase of 68% in the six years leading up to 2019. This growth has been sustained by a consistent annual growth rate of 9%. The expansion of the market is supported by the emergence of professional importers who prioritize brand-oriented products and the proliferation of modern retail outlets and hotels that offer imported goods. Despite these

encouraging improvements, several issues restrict the Indian market for imported goods. High tariffs, persistent import restrictions, and severe domestic industry competition constrain its potential. However, there has been a considerable increase in the availability of foreign packaged and consumer-ready goods at upscale supermarkets, specialty sections of bigger chains, and countless small local stores. Along with the variety of customer tastes, India also has a rising population of consumers concerned about their health. This shows the general public is moving toward more deliberate and healthful dietary choices.

2.14 Why is this millet-juice-based beverage better than other cereal and fruit-based beverages...?

Drinks based on finger millet are considered good nutritious drinks, which can be replaced by rice or wheat-based drinks. It is seen as a storehouse of protein-rich nutrients, amino acids, minerals, and vitamins (Table 2.10). It is a good laxative with its rich fiber content and helps to prevent constipation. Because of its strong calcium content, finger millet drink is excellent for children, older adults, and pregnant mothers. It is also very good for women who lactate, as it helps to produce enough breast milk. It is a very healthy diet for people with diabetes; it helps slow digestion and release blood glucose. It helps treat various diseases and disorders, including high blood pressure, heart difficulties, and asthma. It assists in the production of more hemoglobin and the battle against malnutrition and degenerative disorders. In 2017, according to O.S.K. Reddy, the body's metabolism was not disrupted by the foxtail millet's continued glucose production slowly and steadily. People who drink traditional beverages made from foxtail millet have a lower risk of developing diabetes, and this drink is also known as a healthy option for the cardiovascular system due to its high magnesium content (O.S.K. Reddy, 2017).

On the other hand, cereal-based beverages like rice or wheat-based drinks do not provide this many health benefits. They only offer advantages when probiotic bacteria are included in them. PH is one of the most critical parameters impacting the microbial viability of fruit-based probiotic drinks. Naturally, Fruit juices contain many organic acids and a low pH, raising the concentration of undissociated forms. Probiotic bacteria

are thought to be impacted by the interaction between an acidic environment and the inherent antibacterial activity of accumulated organic acids. The pH of millet is nearly 6.2 in such cases when these fruit juices are combined with millet-based beverages then, the pH of the drink will become favorable for the viability and stability of Probiotic bacteria, and millet works as a suitable matrix with lots of health benefits for the transportation of Probiotic bacteria into the gut. The millet is richer in nutrients than rice or wheat, and fruits are wealthy sources of nutrients and sugar. When the millet solution and fruit juices are blended, the beverage formed is highly nutritious and naturally sweet, and the probiotic bacteria can survive easily.

Table 2.10: Nutritional composition of millets compared to major cereal (g/100g)

| | Moisture | Protein | Fat | Dietary Fiber | Carbo- Hydrates | Minerals (mg) | Iron (mg) |
|----------------------------|----------|---------|-----|------------------|--------------------|------------------|--------------|
| Rice | 13.7 | 6.8 | 0.5 | 1.5 | 76.9 | 10 | 07 |
| Wheat | 12.8 | 11.8 | 1.5 | 12.9 | 71.2 | 41 | 53 |
| Maize | 14.9 | 11.1 | 3.6 | 10.5 | 66.2 | 10 | 23 |
| Pearl Millet | 12.4 | 11.6 | 5.0 | 12.0 | 67.5 | 42 | 80 |
| Finger Millet | 13.1 | 7.3 | 1.3 | 19.8 | 66.8 | 344 | 50 |
| Foxtail Millet | 1.2 | 12.3 | 4.3 | 14.0 | 60.9 | 31 | 28 |
| Little Millet | 11.5 | 7.7 | 4.7 | 12.2 | 67.0 | 17 | 93 |
| Barnyard Millet | 11.1 | 6.2 | 2.2 | 13.2 | 65.5 | 20 | 50 |

2.14.1 Foxtail Millet Production and Nutritional Composition

Production

Foxtail millet (*Setaria italica* (L.) P. Beauv.) has been categorized as a significant millet in global production, ranking as the sixth most productive crop. According to the study

by Saleh et al. (2013), The millet in question is a cereal grain that falls under the *Poaceae* family and subfamily *Panicoideae* of the *Setaria* genus. It is known for its convenient cultivation. According to Austin (2006), foxtail millet, found in archaeological sites in northern China, is considered one of the most ancient, cultivated cereals globally. Its cultivation can be traced back to approximately 7400-7935 years. Fossils from Europe have been found dating back to a minimum of 4000 years ago. According to the study by Lu et al. (2009), Foxtail millet is cultivated in 26 nations and holds the second position in global millet output. Bala et al. (2004) say foxtail millet is the fourth most productive variety. Its ability to yield well is attributed to the utilization of conventional production procedures that do not involve the use of pesticides. This characteristic and its classification as a commodity derived from organic farming facilitate its accessibility and availability (Sheahan, 2014). The grain in question exhibits a height ranging from 2 to 5 feet. It possesses the unique characteristic of being cultivable in regions characterized by aridity and low temperatures, distinguishing it from other millet varieties. This plant species can yield 1 metric ton of feedstock in sandy and loamy soils with 2.5 inches of moisture. Notably, this plant exhibits a lower moisture need than maize, necessitating around one-third less moisture for its growth. The plant exhibits the ability to thrive in both high-altitude environments, reaching around 1.5 kilometers above sea level, as well as in lower-lying areas located near sea level. Culturing crops in the semi-arid tropics is of significant economic importance, as they can be harvested within 75-90 days. Under saline circumstances, it is also feasible to cultivate. The cultivation of this millet species is widespread in several regions, including Africa, China, Russia, India, the United States, and Europe. According to the study directed by Pawar et al. in 2006, Similar to most other millet cultivars, this particular crop exhibits exceptional drought resistance, making it well-suited for areas characterized by erratic precipitation patterns.

Phylogenetically, foxtail millet grains also have husk and bran layers like other millet grains. The kernel's husk forms 13.5 percent (w / w), and only 1.5-2 percent (w / w) is the bran and germ. (Dharamraj et.al., 2016) It has a small propagation period; from planting to flowering, it takes 5-8 weeks; from flowering to seed maturity, it takes 8-15 weeks, and therefore, it can develop hundreds of seeds per inflorescence. The genome

of the foxtail millet was sequenced by the Department of Energy, Joint Genome Institute, United States, in 2012; as a model crop, it has been used to study the genetic makeup and genomics of other millets varieties, non-millet cereals, and other grains. (Muthuamilarasan et. al., 2016) It is also interesting to understand the plant architectural features, genome diversity, and physiology or morphology of different bioenergy grasses as research material (Kamara et al., 2010). A sufficient number of nutritional elements, mainly starch, protein, vitamins, and minerals, are found in Foxtail millet.

The 30-year leading to 1960 saw the Green Revolution, wherein, because of their perceived higher yields and the imminent effect they could have on counteracting food scarcity, wheat, and rice were prioritized as the main staple food grain crops for global consumption. This worldwide emphasis led to a significant development of irrigation infrastructures, improvement and modernization of management strategies, hybridized seed growth, synthetically manufactured fertilizers, and specialized pesticides, all for improving the productivity of rice and wheat (Zhang et al., 2015) The favorable treatment for major cereal grains such as rice and wheat was in comparison to any other global cereals which will almost certainly not survive, corner-lined millets such as foxtail millet to small-scale cultivation, where such crops can flourish under comparatively little agricultural inputs (Padulosi et al., 2015) These variables have led to the primary consumption of foxtail millet seeds by the poorer populations, with the more affluent populations only harvesting these seeds for poultry feed and forage. (Chandrasekara et.al., 2012) Hence, underinvestment and perception as a feed ingredient have thwarted the commercial exploitation and production of foxtail millet. It is now understood that millet, such as foxtail millet, will help mitigate hunger inexpensively. In several countries in Asia and Africa, the processing of foxtail millet into value-added food goods may also contribute significantly to economic development.

Nutritional Composition

Foxtail millet grains possess a remarkable nutritional profile, boasting 14-16% protein content, 6-8% of crude fat, and significant amounts of zinc, calcium, and iron (Zhang et al., 2007). In addition to its superior biological value in terms of digestible protein compared to rice and wheat, foxtail millet outshines them by containing higher levels

of seven out of eight essential amino acids that are vital for the human body and cannot be synthesized internally. Furthermore, foxtail millet stands out as a valuable source of dietary fiber, with approximately 2.5 times the amount found in rice. This characteristic makes it a promising contributor to digestive health, benefiting the intestines and stomach (Liang et al., 2010). The bran of foxtail millet is particularly noteworthy, containing 8-10% crude oil and being rich in linoleic acid, which is nearly 66.5%, and oleic acid is about 13.0% (Liang et al., 2010). Throughout its extended history of cultivation and utilization as a principal constituent of the daily diet, various culinary applications have been developed using the seeds of foxtail millet. In India and China, foxtail millet flour is utilized to make snacks, chapattis, pancakes, and bread (Diao et al., 2014). Northern China has embraced the prominence of steamed bread made from a composite flour that includes soybean, wheat, and foxtail millet, showcasing different consumption methods. However, in some nations, foxtail millet is primarily grown for fodder, birdseed, silage, and hay.

Foxtail millet is gaining popularity worldwide due to its nutritional composition and various health benefits. Here is an explanation of its nutritional composition:

- **Carbohydrates:** Foxtail millet is rich in complex carbohydrates, the primary energy source. It contains both starch and dietary fiber. The carbohydrate content varies, but it is, on average, around 60-65% of the total weight.
- **Dietary Fiber:** Foxtail millet is an excellent source of dietary fiber, including soluble and insoluble fiber. Fiber helps maintain a healthy digestive system, regulate blood sugar levels, and promote satiety. It also aids in preventing constipation and reducing the risk of various gastrointestinal disorders.
- **Protein:** Foxtail millet contains a moderate amount of protein, essential for the growth, repair, and maintenance of body tissues. The protein content is approximately 12-15% of its weight. It is a good source of plant-based protein, making it suitable for vegetarians and vegans.
- **Fats:** Foxtail millet is low in fat, with less than 5% fat content. Its fat is primarily unsaturated fats, including essential fatty acids such as omega-3 and omega-6. These fats are beneficial for heart health and overall well-being.
- **Vitamins:** Foxtail millet is a good source of various vitamins, including niacin (vitamin B3), thiamine (vitamin B1), riboflavin (vitamin B2), and vitamin A. These

vitamins are crucial in energy metabolism, maintaining healthy skin, supporting the nervous system, and promoting good vision.

- **Minerals:** Foxtail millet is rich in minerals such as iron, magnesium, phosphorus, and calcium. Iron is essential for oxygen transport and prevents iron deficiency anemia. Magnesium is vital for bone health, muscle function, and energy production. Phosphorus maintains bone health; calcium is vital for strong teeth and bones.
- **Antioxidants:** Foxtail millet contains various antioxidants, including phenolic compounds and flavonoids. These antioxidants help neutralize harmful free radicals and protect the body against oxidative stress and inflammation.

Recent nutritional and medicinal research has shed light on the health benefits of millet, including foxtail millet. This versatile crop is an essential energy source for pregnant and lactating women and individuals who are sick or have diabetes. Notably, foxtail millet has shown the potential to reduce blood sugar levels in diabetic women. It holds significant value for type-II diabetes patients by reducing blood glucose concentration, glycosylated hemoglobin, and serum lipids (Thathola et al., 2010). In China, it has even been employed in the treatment of rheumatism. All these factors collectively establish foxtail millet as a versatile crop with immense potential and numerous health benefits.

2.14.2 Finger millet Production and Nutritional Composition

Production

The term "mille" originates from the Greek "mille," meaning "thousand," denoting the abundance of grains in millet (Shahidi & Chandrasekara, 2013). Finger millet (*Eleusine coracana*) is a diminutive-seeded cereal crop categorized under the millet family. Its nomenclature derives from the finger-like appearance of its panicles (Ramashia et al., 2018). Originating from Ethiopia, it has various cultivars distinguished by color, with dark varieties used in traditional beer and porridge, while white cultivars are preferred for bread. Finger millet is resilient to diverse agroclimatic conditions, thriving in semi-arid regions with limited rainfall (Kumar et al., 2016). Cultivation spans from February to August, with harvesting between June and January. Its cultivation extends from South Carolina to the Himalayas, primarily in Africa and India, where it contributes significantly to global production, with India being the leading producer (Shobana et

al., 2013). Known as the "dancing grain" in India, it ranks fourth most significant among cereals in semi-arid regions. Finger millet grains are spherical, 1.0 to 1.5 mm in diameter, primarily brown (Gull et al., 2014). They exhibit a unique utricle-like structure, contributing to elevated dietary fiber content. The grains comprise a pericarp, germ, and endosperm, with the pericarp removed before processing due to its inedibility (Mathur, 2012). The endosperm, attached to the seed coat, is used for flour production. Despite brown being the predominant cultivar, white and red varieties exist but are less popular. Finger millet's unique characteristics make it a vital crop, particularly in regions prone to aridity, providing sustenance and economic viability for millions worldwide. (Adhikari,2012; Jideani 2012).

Nutritional Composition

Finger millet grains are rich in essential minerals such as calcium (Ca) and phosphorus (P), with the highest calcium content among millet varieties, ranging from 162.0 to 358.0mg/100 (Manjula et al., 2015). Calcium is crucial in various populations, including newborns, pregnant women, the elderly, and individuals with obesity, diabetes, and malnutrition. Incorporating finger millet into the diet can alleviate calcium deficiency. Phosphorus, another mineral in finger millet (Jayasinghe et al., 2013), promotes tissue development and energy metabolism, with a content ranging from 130.0 to 283.0 mg/g (Chappalwar et al., 2013). Additionally, finger millet contains iron (3-20%), magnesium related with blood pressure reduction and heart health, and superior nutritional qualities compared to other millets (Ramashia et al., 2018). Despite its nutritional value, finger millet is often neglected and underutilized.

Regarding vitamins, finger millet is high in vitamin A and B complexes but lacks vitamin C in its dried form (Rajiv et al., 2011; Shukla & Srivastava, 2014). Essential amino acids such as methionine, cysteine, tryptophan, leucine, lysine, isoleucine, phenylalanine, and threonine are present, aiding in lowering cholesterol levels and reducing cancer and obesity risks (Devi et al., 2014; Dlamini & Siwela, 2015). Finger millet also contains essential fatty acids crucial for brain and neural tissue development, with a low-fat content (1-2%), contributing to better storage properties and weight maintenance Verma & Patel (2013). Finger millet has the highest methionine levels (194 mg/g) compared to other millets. Its low-fat content, dietary fiber, and non-starchy

polysaccharides provide nutritional and physiological benefits such as hypocholesterolemic and hypoglycemic effects (Roopa and Premavalli (2008).

Inhibitors like trypsin inhibitors, tannins, phytic acid, phytate, and flavonoids found in finger millet may reduce mineral bioavailability (Ottaway, 2008). However, significant polyphenols like phenolic acids and tannins act as antioxidants, enhancing immune system function (Singh & Raghuvanshi, 2012). Tannins also serve as barriers against fungal invasion but can hinder food digestibility and mineral absorption, affecting pancreatic and thyroid gland activity (Sood et al., 2017). Recent research suggests that production technologies like malting, fermentation, and soaking can enhance nutrient bioavailability (Mathanghi & Sudha, 2012; Thapliyal & Singh, 2015). Finger millet grains offer a wealth of essential nutrients crucial for overall health. Their high mineral content, amino acids, and fatty acids benefit various populations, especially those vulnerable to nutritional deficiencies (Palanisamy et al., 2012). While inhibitors like tannins may reduce bioavailability, innovative production methods can mitigate their effects (Udeh et al., 2017). Finger millet's nutritional and functional qualities make it a valuable addition to diets worldwide, deserving more attention and utilization to combat malnutrition and promote overall well-being (Shibairo et al., 2014).

2.14.3 Barnyard Millet production and Nutritional Composition

Production

Barnyard millet, belonging to the *Echinochloa* genus, is a long-standing agricultural crop known for its robust growth in warm and temperate regions worldwide. It holds significant agricultural importance across Asia, particularly in India, China, Japan, and Korea, where it ranks as the fourth most cultivated minor millet, contributing to food security for various populations in Germany (De Wet et al., 1983). India leads global barnyard millet production, boasting a substantial cultivation area and output volume. This millet species, scientifically termed *Echinochloa frumentacea*, shares evolutionary processes with its African counterpart and is cultivated across diverse geographical locations, including India, the Central African Republic, Tanzania, and Malawi (Doggett, 1989).

The domestication of barnyard millet species is a subject of historical interest, with *Echinochloa frumentacea* primarily farmed in tropical regions and *Echinochloa esculenta* in temperate climates (Sood et al., 2015). Archaeological evidence traces the cultivation of *E. esculenta* in Japan back to the Yayoi period, around 4 to 5 millennia ago, with the oldest documented domestication dating to the Jomon Dynasty, approximately 2000 B.C. Genetic diversity analysis suggests that *E. esculenta* originated from a subset of the *E. crus-galli* population, with distinct categorization between the two species Yabuno (1975). Investigations into domestication effects on barnyard millet species have revealed significant modifications compared to their wild counterparts, including reduced vegetative branching, compact growth, larger inflorescence size, decreased seed dispersal, and enlarged seed dimensions (Nesbitt, 2005). Human-driven selection during domestication prioritized traits such as reduced seed breakage, elimination of dormancy, sturdy culms, broad leaves, and spherical spikelets, aligning with domestication syndromes observed in related millet species.

Archaeological data indicate a notable increase in seed size during Japanese barnyard millet domestication over millennia, suggesting a persistent human preference for larger seeds. The research findings indicate that there was a notable increase of around 20% in the dimensions of *Echinochloa* caryopses between the Middle Jomon period (3470 B.C.E.–2420 B.C.E.) and the preceding Early Jomon period (5000 B.C.E.–3470 B.C.E.). Cross-compatibility studies between domesticated and ancestral barnyard millet varieties offer insights into domestication mechanisms and genetic foundations of domestication traits in this crop (Takase, 2009). Barnyard millet is a vital global agricultural resource, cultivated extensively across diverse climates to ensure food security. Understanding its domestication history and genetic adaptations provides valuable insights into human agricultural practices and sheds light on the evolutionary processes shaping this vital crop.

Nutrient Composition

Barnyard millet (*Echinochloa frumentacea*) is a small-seeded cereal grain belonging to the millet family. It is commonly grown in Asia, particularly India, China, and Japan. Overall, barnyard millet is a nutritious grain that offers a range of macronutrients,

including carbohydrates, protein, fiber, and essential micronutrients. It can be a valuable part of a balanced diet, particularly for those seeking gluten-free and nutrient-dense alternatives to other grains.

The composition of barnyard millet can vary slightly depending on factors such as growing conditions and processing methods.

Barnyard millet is known for its nutritional composition, which includes various essential nutrients. Here is the approximate nutritional composition of barnyard millet per 100 grams of barnyard millet Carbohydrates 73g, Dietary fiber 5g, Fat 3.6 g, Protein 11g, Thiamine 0.2mg, Riboflavin 0.1mg, Niacin 2.8mg, Pyridoxine mg, Folate 44mg, Calcium 12mg, Iron 6.2mg, Magnesium 137mg, Phosphorus 290mg, Potassium 195mg, Zinc 2.1mg. Barnyard millet is a good source of dietary fiber, providing about 5 grams per 100 grams. It is also relatively low in fat and calories, making it suitable for those seeking a nutritious, low-calorie grain option. The millet contains several essential minerals, including iron, magnesium, phosphorus, and potassium. Additionally, it contains various B vitamins, essential for energy production and the functioning of the nervous system.

2.15 Some Major Advantages and Health Benefits of Millet-based Probiotic Beverages

People are now well conscious of their safety and health after a few days, and the demand and desire for fermented products are growing in various encouraging ways owing to the capacity of the human digestive system. This specific involvement is recognized as a probiotic influence (Sahlin, 1999). Metchinkoff previously addressed the usage of fermented milk in diet to avoid such gastrointestinal diseases and to encourage health benefits. It is known that the human gut's microflora weighs around 102 grams and can comprise 10^6 - 10^7 CFU, more than 500 strains (Gustafsson, 1983). Fermented products have multiple safety benefits such as flatulence mitigating, anti-cholesterolemic benefits, transit period impact, bowel function and glycemic index, anti-carcinogenic effect, and immune-active results. The quantity of oligosaccharides that are essential for inducing flatulence dramatically decreased. Bean flour inoculated with *Lactobacillus* and fermented with moisture levels of 20 percent shows a drop in stachyose production (Duszkiewicz-Reinhard et al., 1994).

Earlier research found hypercholesterolemic dietary intake results for one week in human samples. The capacity of twenty-three LAB strains extracted from various fermented milk items shows no cholesterol was present within the cells as cholesterol-binding bacterial cells (Taranto et al., 1997). The function of LAB was identified within the cells in rising cholesterol concentration. Fermented products containing possible *lactobacilli* have been reported in the food canal to remove or decrease procarcinogens and carcinogens (Mitall & Garg, 1995).

Oral dosing of *L.rhamnosus GC* was considered helpful in reducing the fecal production of *b-glucuronidase* in humans, which suggested a decline in the transformation of procarcinogens to carcinogens fed probiotic (Sabikhi & Mathur, 2004). The milk fermented with *Lactobacillus acidophilus LA-2* reversed the fecal mutagenicity in the human bowel. Earlier experiments have shown that using LAB mixed cultures has a greater spectrum of action toward mutagens than the standard Sample strains. However, there is also conflicting evidence that suggests the theory that probiotic bacteria thrive in preventing and treating cancer. LAB is reported to have multiple therapeutic effects on the host's immune system after colonization in the gut, which are present in fermented milk products. *Lactobacillus casei*, during oral administration, enhanced the peritoneal macrophage role and increased IgA development (Sato et al., 1988). Millet-based probiotic beverages can offer several health benefits due to the combination of millet's nutritional profile and beneficial probiotic bacteria. Here are some potential health benefits:

- Probiotic benefits: Probiotics are beneficial bacteria supporting gut health and overall well-being. Millet-based probiotic beverages can contain specific strains of probiotics, such as *Lactobacillus* or *Bifidobacterium*, which aid in maintaining a healthy balance of gut bacteria. These probiotics help improve digestion, boost the immune system, and positively affect mental health.
- Nutritional value: Millet is a highly nutritious grain rich in essential nutrients such as fiber, vitamins (B-complex vitamins, vitamin E), minerals (iron, calcium, phosphorus, magnesium), and antioxidants. Incorporating millet into a probiotic beverage can enhance its nutritional value and promote a balanced diet.

- Digestive health: Probiotics are live bacteria that can help promote a healthy balance of gut bacteria, supporting digestion and reducing digestive issues like bloating, gas, and constipation. Consuming millet-based probiotic beverages can introduce these beneficial bacteria into the digestive system, aiding in overall digestive health.
- Improved nutrient absorption: Probiotics can enhance nutrient absorption by improving the gut's ability to break down and absorb nutrients from food. Like finger millet (ragi), millets contain essential vitamins, minerals, and antioxidants. By incorporating probiotic beverages into your diet, you may enhance the absorption of these nutrients.
- Enhanced immune function: A significant portion of the immune system resides in the gut. Probiotics help maintain a healthy gut microbiome, which can support immune function. Regular consumption of millet-based probiotic beverages may contribute to balanced gut microbiota and potentially strengthen the immune system.
- Reduced inflammation: Imbalances in gut bacteria can contribute to chronic inflammation linked to various health problems. Probiotics have shown promise in reducing inflammation markers in the body. Millets, rich in antioxidants and anti-inflammatory compounds, combined with probiotics, may help reduce inflammation and promote overall well-being.
- Potential weight management: Some studies suggest that probiotics may play a role in weight management. They can affect the gut's energy regulation, fat storage, and appetite control. Millets have a favorable nutrient composition, including dietary fiber, which can help promote satiety and support weight management combined with a healthy diet and lifestyle.
- Gluten-free option: Millets are naturally gluten-free, making them an excellent alternative for individuals with gluten sensitivities or celiac disease. Millet-based probiotic beverages offer a safe and nutritious choice for those who need to avoid gluten-containing grains like wheat, barley, and rye.
- Blood sugar management: Millets have a low glycemic index, meaning they slowly release sugar into the bloodstream, preventing rapid spikes in blood sugar levels.

This characteristic makes millet-based probiotic beverages suitable for individuals with diabetes or those aiming to effectively manage their blood sugar levels.

- Versatility and taste: Millets can be used to create a variety of flavorful beverages, including fermented probiotic drinks. Depending on the specific variety used, the taste profile of millets ranges from mild and nutty to slightly sweet. Millet-based probiotic beverages offer a unique and refreshing taste that can be enjoyed as a healthy alternative to sugary or artificially flavored drinks.

Objectives of the study

This study was designed to produce novel millet-based probiotic beverages using probiotic lactic acid bacteria isolated from various sources. This product provides several health benefits to consumers.

The aim of the study was achieved through the following objectives:

1. Isolation, characterization, and identification of probiotic lactic acid bacterial strains from different sources.
2. To analyze cereal varieties for various physio-chemical properties.
3. To develop, evaluate, and study a cereal-based probiotic beverage using a probiotic lactic acid bacterial strain and to study the product's nutritional value.
4. To study product storage stability in different packages and storage conditions.

Research Gap:

Despite the growing interest in functional foods and the health benefits associated with probiotics, there is a noticeable gap in developing novel probiotic beverages that combine millet and fruit juices as substrates. While probiotic lactic acid bacterial (LAB) strains are well-studied for their health benefits, particularly in dairy-based products, limited research explores their efficacy and viability in non-dairy mediums like millet and fruit juice blends. Current research predominantly focuses on using cereals or fruits as substrates for probiotic fermentation. However, the combination of these two, especially with nutrient-rich and gluten-free millets, remains underexplored. Additionally, while the nutritional benefits of millets and fruits are individually well-documented, there is a lack of comprehensive studies that analyze the synergistic effects of these ingredients in a probiotic beverage on both nutrient content and probiotic viability. Moreover, such a novel product's sensory attributes, consumer acceptability, and shelf-life stability need further investigation. This research aims to fill these gaps by exploring the potential of millet and fruit juice-based probiotic beverages, optimizing their formulation, and conducting a thorough nutrient and viability analysis.

HYPOTHESIS

In brief, this hypothesis suggests that integrating probiotic bacteria into millet and fruit juice-based beverages will enhance their nutritional composition, prolong their shelf life, and improve their sensory attributes, yielding novel and health-enhancing beverages for consumers.

The present study aims to investigate the potential enhancement of nutritional content in millet and fruit juice-based beverages by introducing probiotic lactic acid bacteria. LAB, including species such as *Lactobacillus* and *Bifidobacterium*, have been observed to synthesize vitamins, raise the bioavailability of minerals, and improve the digestion of nutrients. As a result, drinking these beverages may offer supplementary health advantages beyond those often associated with fruit juices or millet-based beverages.

Incorporating probiotic LAB is expected to improve the preservation of these innovative beverages, extending their shelf life. Lactic acid produced during fermentation serves as an inherent means of preservation due to its ability to reduce the pH level and impede the proliferation of bacteria that cause deterioration. This is expected to lead to an extended product viability duration, mitigating food wastage and enhancing consumer access to these items.

The anticipated outcome of the probiotic fermentation process is an enhancement in the sensory qualities of the beverages, specifically in terms of flavor, aroma, and texture. Lactic acid bacteria can generate flavor compounds and metabolites that possess distinct and desirable sensory attributes, which can enhance the quality of the products. Furthermore, the inherent acidity produced during the fermentation process can contribute to a desirable tanginess, augmenting the overall sensory satisfaction for consumers.

Chapter 3

Materials & Methods

3. Methodology

3.1 Collection of Samples

For the isolation of lactic acid bacterial Strains, Samples are collected from different sources, i.e., Milk sample, Curd sample, pickle sample, Oral Cavity Sample, idli sample, and Dosa sample. All the collected samples Were stored under refrigerated conditions. MRS media is used to isolate *Lactobacillus* Strain because it contains Sodium acetate, a significant component that suppresses the growth of other bacteria (Zhang et al., 2020).

3.2 Isolation of Probiotic Lactic Acid Bacteria

The isolation is carried out by inoculating serially diluted (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) pickle, curd, milk, idli, and dosa batter samples in sterile peptone water on the MRS agar plates by spread plate method. The Petri plates were then incubated at 37°C for 24 hours. To calculate the viable count per ml of a given formulation, the colonies are first counted using a colony counter, and then that number is multiplied by the dilution factor. After that, the individual colonies are taken up and streaked once more on MRS agar to complete the purification process. After the plates have been streaked, they are stored for incubation at 37°C for three days in an atmosphere free of oxygen. In addition, the purified colonies are labeled with information regarding the source of isolation and stored in glycerol stock (Al-Dhabi et al., 2020).

3.3 Characterization of isolated bacteria:

3.3.1 Phenotypic characterization

Phenotypic characterization of bacteria encompasses a suite of methodologies aimed at elucidating observable traits and behaviors of bacterial cells. At its core lies examining physical, biochemical, metabolic, and structural features to discern bacterial species' identity, physiology, and potential capabilities. Initial assessments often involve microscopic morphological observations, where bacterial cells' shape, size, and arrangement provide vital clues. Colony morphology on agar plates further aids in distinguishing characteristics such as size, shape, color, texture, and elevation. Biochemical tests are pivotal in assessing metabolic capacities through substrate utilization and enzyme production, including fermentation patterns

and enzymatic activities. Physiological traits like temperature and oxygen requirements provide additional insights into bacterial growth preferences and environmental adaptations. Antibiotic susceptibility testing gauges the response to antimicrobial agents, guiding treatment strategies and revealing resistance profiles. Serological assays detect specific antigens or antibodies associated with bacterial cells, aiding in typing and strain identification. While traditional methods dominate, molecular techniques like PCR and DNA sequencing offer increasingly detailed insights into bacterial genetics and relatedness.

Preliminary morphological identification

The purified isolates were then screened for various characteristics, such as colony morphology, staining, cellular morphology, and biochemical features, and compared to Bergey's Manual of Systematic Bacteriology (Kandler & Weiss, 1986) for preliminary confirmation.

3.3.2 Colony morphology

Colony morphology refers to the visual characteristics and physical appearance of bacterial or fungal colonies grown on agar plates. It is an essential aspect of microbiological identification and characterization. By observing colony morphology, scientists can gather information about the growth patterns, size, shape, color, texture, and other features of individual colonies, which can help identify and classify the microorganisms present. (Holt et al., 1994).

3.3.3 Gram Staining

The Gram staining procedure involved the application of a thin and uniform layer of bacteria onto a microscope slide, followed by air drying. The slide was subsequently subjected to a heat-fixation process, wherein it was carefully exposed to a flame or a mild heat source, facilitating the adherence of the bacteria to the slide. The slide was immersed in a primary stain known as crystal violet, resulting in the cells being stained a purple color. Crystal violet is classified as a cationic dye that exhibits basic properties, enabling it to form strong electrostatic interactions with the anionic constituents present within the bacterial cell. Iodine was utilized as a mordant agent to augment crystal violet's affinity towards bacterial cells. The formation of a complex between iodine and crystal violet generates a more significant molecular entity, which then becomes entrapped within the

bacterial cell. Subsequently, the slide underwent a rinsing process utilizing a decolorizing solution, alcohol, or acetone. This step involves the differentiation of bacteria by their cell wall structure. In order to visualize Gram-negative bacteria, a counterstain, such as safranin, was employed. The use of safranin results in the pink or red staining of cells that have been decolorized, hence facilitating their differentiation from the Gram-positive bacteria that exhibit a purple coloration. Ultimately, the slide was subjected to microscopic analysis utilizing oil immersion. According to Holt et al. (1994), Gram-positive bacteria are observed as clusters or chains with a purple coloration. In contrast, Gram-negative bacteria are observed as solitary cells with a pink or red coloration.

3.3.4 Motility Test

The motility test was carried out to ascertain the organism's motility. Bacterial cultures were stabbed into the HI media for the motility test and incubated at 37°C for 48 hours. In contrast to clear vision with development along the stab line, turbidity and growth outside the stab line suggested an excellent reaction (Holt et al., 1994).

3.3.5 Endospore Test

Endospores are dormant structures formed by certain bacteria as a survival mechanism in unfavorable conditions. The test involves preparing a heat-fixed smear of the bacterial sample on a glass slide. The primary stain, usually malachite green, was applied to the slide to cover the bacteria. The heat was applied to help the stain penetrate the endospores without affecting vegetative cells. The slide was rinsed with water to remove excess stains. A counterstain, like safranin or basic fuchsin, is applied to stain any vegetative cells present. The stained slide was mounted with a coverslip using a mounting medium. The slide was examined under a microscope. Endospores appear as green structures within or near bacterial cells, while vegetative cells appear pink or red (Holt et al., 1994).

3.3.6 Catalase Test

The catalase test is a typical biochemical test used in microbiology to identify the presence of the catalase enzyme in bacteria. It involves the breakdown of H₂O₂ into H₂O and O₂ by the action of catalase enzyme. A test distinguishes between bacteria that generate catalase and those that do not. A small amount of the bacterial culture is taken

and put on a glass slide or in a test tube to perform the catalase test. 3% Hydrogen peroxide was added (a few drops at a time) to the culture. If the bacteria produce catalase, they will degrade the hydrogen peroxide, causing the release of oxygen and water and forming bubbles or effervescence. The effervescence is a good sign because it shows that catalase is present. Conversely, there will not be any effervescence, and the hydrogen peroxide will remain intact if the bacteria do not make catalase. (Holt et al., 1994).

3.4 Biochemical tests

In order to ascertain the different biochemical features of each isolate and also to confirm the identity of LAB cultures as per Bergey's manual, another number of tests were carried out as given below:

3.4.1 Methyl Red Test

The Methyl Red test involves inoculating a tube with a pure culture of the bacteria into a medium made of glucose called MR-VP broth. The tube was incubated at an appropriate temperature (generally 37°C) for a predetermined time, usually 48 to 72 hours. Methyl red was used as an indicator, and a few drops were added to the broth after incubation. The pH will be below 4.4, and the methyl red indicator will remain red if the organism creates enough acid during glucose fermentation. The pH will rise over 6.2 if the organism does not create enough acid, which will cause a yellow color change (Holt et al., 1994).

3.4.2 Voges Proskauer Test

A biochemical test known as the Voges-Proskauer (VP) test is used to identify the presence of acetoin, a metabolic byproduct of specific bacteria. The test is based on the bacteria's capacity to convert glucose into the neutral byproducts 2,3-butanediol and acetoin. Several reagents can be used to identify acetoin production. The procedure for the VP test is as follows: Inoculated a tube of peptone broth with the bacteria to be tested and incubated at 37°C for 24 to 48 hours and transferred 0.6 mL of the culture to a washed and dried test tube. 0.2 mL of alpha-naphthol reagent was added to the test tube, and the mixture was mixed well. The alpha-naphthol reagent serves as a catalyst. 0.2 mL of 40% potassium hydroxide (KOH) solution was added to the test tube, and the mixture

was mixed gently and incubated in the tube at room temperature (37°C) for approximately 24 hours. Observed the color change in the tube. The development of a pink-to-red color will be indicated as a positive reaction within the specified period (Holt et al., 1994).

3.4.3 Indole Test

The indole test is based on tryptophanase, an enzyme that converts tryptophan into indole, pyruvate, and ammonia. The test involved inoculating the organism into a solution known as tryptone broth, which only contains tryptophan as a nitrogen source, and incubating it for 24 hours at 37°C. After incubation, the broth was treated with a few drops of Kovac's reagent, typically p-dimethylaminobenzaldehyde. A red layer appears on top of the broth if the organism can produce indole, which causes the formation of the red chemical indole-3-pyruvic acid. A negative result does not necessarily suggest that the organism does not belong to the Enterobacteriaceae family; it is crucial to remember that not all bacteria can make indole (Holt et al., 1994).

3.4.4 Carbohydrate fermentation test

Various isolates obtained from milk, curd, and pickles were analyzed for their fermentation capabilities using 11 different carbohydrates. The carbohydrates used for testing included starch, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, rhamnose, ribose, and sucrose. 10 ml test tubes were used for the carbohydrate fermentation test. The isolates were first activated in 10 ml MRS broth by incubating them at 37°C for 24 hours. Phenol red carbohydrate media, was prepared and sterilized, and different sugars were added aseptically. To detect gas production, sterile Durham tubes were inserted into the broth, and phenol red dye was added to the broth to detect acid production. The broth was inoculated with a test sample and incubated for 24- 48 hours at 37°C. After incubation, a change in color for acid production and bubble formation in Durham tubes for gas production or fermentation was observed. (Lim and Im, 2009).

3.4.5 Citrate Utilization Test

The procedure for the citrate test: Inoculated a citrate utilization medium, such as Simmons citrate agar, through the process of streaking or inoculating the surface of the medium with a loopful culture of the organism being tested. We incubated the plate at the appropriate temperature. The standard incubation temperature is 37°C. We observed the plate after 24 to 48 hours of incubation. If the organism can utilize citrate, it will grow, and the medium will change from green to blue due to the alkalization of the medium (Holt et al., 1994).

3.4.6 Nitrate Reduction Test

The ability of an organism to reduce nitrate (NO₃⁻) to nitrite (NO₂⁻) or further to nitrogenous gases like nitric oxide (NO), nitrous oxide (N₂O), or nitrogen gas (N₂) was assessed using the nitrate reduction test. This test assists in identifying and classifying bacteria according to their capacity for nitrate reduction. The first step of the test was inoculating the target microorganism into a tube containing a nitrate broth medium. As a nitrogen source, potassium nitrate was typically included in the medium. The inoculated nitrate broth tube is incubated at an appropriate temperature (often 37°C) for a certain amount, usually 24 to 48 hours. After incubation, a small amount of reagent A (sulfanilic acid) and reagent B (-naphthylamine) is added to determine whether nitrite (NO₂⁻) is present. A red color will appear if nitrite is present, signifying a successful outcome. This demonstrates the organism's capacity to convert nitrate to nitrite (Holt et al., 1994).

3.5 Characterization of isolates for probiotic properties

3.5.1 Casein Hydrolysis Activity

This test was done to check the protease activity of the test organism. Casein agar plate or 15 skim milk plates with additional agar were prepared for this test. These plates were used to check the development of bacteria and only included casein as a nitrogen source. The test organisms were then streaked onto the surface of the casein agar plate using a sterile inoculating loop. They incubated the plates at 37°C for 24 to 48 hours. They examined the plates after incubation to determine if the bacterial growth had a clear zone around it. A clear zone means that the organism has produced caseinase, hydrolyzed the

casein protein, and created smaller peptide fragments. If there is no clearance, casein hydrolysis by the organism does not occur. (Chandok et al., 2014).

3.5.2 Kligler's Iron Agar (KIA) Test

Sefcova et al. (2021) treated them to the Kligler's Iron Agar (KIA) test to determine how each purified isolate consumes lactose and glucose. The butt was stabbed and streaked to inoculate a freshly prepared culture. After 24 hours of incubation at 37°C, the findings were recorded. According to the test results (Hatami et al., 2022), the slant and butt's colors changed, and H₂S gas or other gases were produced. If glucose were the only component that was fermented, the butt would be alkaline, while the slant would be acidic. Both the butt and the slant turned out to be acidic when both lactose and glucose were fermented, whereas they both turned out to be alkaline when neither sugar was fermented. (Hatami et al., 2022).

3.5.3 Hemolytic activity

The hemolytic activity test for *Lactobacillus* can be performed using blood agar plates to detect hemolytic activity based on the lysis of red blood cells by LAB. The blood agar plates were prepared and allowed to cool at room temperature for the hemolytic activity test. Bacterial culture was streaked on the blood agar plate with a sterile inoculation loop or swab and then incubated in the incubator with the agar side up and at the ideal temperature of 37C for 24 to 48 hours. The Plates were checked for any indications of hemolysis after 24 hours of incubation. The three categories of hemolysis are alpha-hemolysis and partial hemolysis, characterized by a greenish tinge surrounding the bacterial development. Beta hemolysis is a complete hemolysis denoted by a clear zone enclosing the bacterial growth, and in gamma-hemolysis, there was no hemolysis, as seen by the blood agar around the bacterial growth remaining unchanged (Wei et al., 2022).

3.5.4 Arginine hydrolysis

The arginine hydrolysis test was used to determine the ability of microorganisms, such as bacteria, to break down arginine, an amino acid. For the arginine hydrolysis test for *Lactobacillus*, Arginine dihydrolase broth was prepared and inoculated with the test

organism. The broth was then incubated in the medium at the temperature, usually around 37°C, for about 24 to 48 hours to gather preliminary results. In order for microorganisms to be able to utilize the readily available glucose, the color of the solution must first shift from violet to yellow. As the media gets more acidic, the arginine dihydrolase enzyme becomes active. For 24 hours, the culture was kept at 37°C to give the microbes enough period to consume the arginine. The tube was inspected after 48 hours of incubation for the final findings. The arginine dihydrolase test succeeds when the color changes from yellow to purple (Soccol et al., 2010).

3.6 Evaluation of Probiotic Attributes of Microorganisms

3.6.1 Acid tolerance test

An acid tolerance test for *Lactobacillus* was commonly performed to assess the ability of this bacterial species to survive in acidic conditions, such as those encountered in the human gastrointestinal tract. The test involves exposing the *Lactobacillus* strains to different pH levels and monitoring their viability. To check the acid tolerance of *Lactobacillus*, an MRS growth medium was prepared and allowed to cool at room temperature. The broth was inoculated with the test culture and incubated overnight at 37°C. Acidified media was prepared with varying pH levels from 1-4. The pH of the MRS broth was adjusted using hydrochloric acid or other suitable acids. Colonies were transferred into the acidified media tubes and incubated at 30-37°C. After the incubation of 18- 24 hours, the viability of the cultures was analyzed by observing growth or performing viable plate counts or by taking O.D. at 560nm (Chen et al., 2022).

3.6.2 Bile Tolerance Test

The bile tolerance test was a method used to assess the ability of microorganisms to survive in the presence of bile salts. Bile salts are naturally produced in the liver and stored in the gallbladder, and they play an essential role in digestion and the absorption of fats. For this test, an MRS growth medium was prepared and allowed to cool at room temperature. Then, different bile concentrations ranging from 0.5%-2% were adjusted in the MRS broth using Ox bile salt. The colonies were transferred into the tubes and incubated at 30-37°C. After 24-48 hours of incubation, assess the viability of the

cultures by observing growth or performing viable plate counts or by taking O.D. at 560nm (Chen et al., 2022).

3.6.3 NaCl Tolerance Test

The NaCl (sodium chloride) tolerance test was a standard method to assess the salt tolerance of microorganisms, including lactobacillus species. MRS broth was prepared with different NaCl concentrations ranging from 2%-8% for this test. The media was then allowed to cool at room temperature. After that, the broth was inoculated with the test cultures and incubated at 30-37°C. After 24-48 hours of incubation, assess the viability of the cultures by observing growth or performing viable plate count or by taking O.D. at 560nm. (Chen et al., 2022).

3.6.4 Temperature Tolerance Test

The temperature tolerance test was a standard method used to assess the ability of microorganisms to grow at different temperatures. MRS broth was prepared, cooled, and inoculated with the test organism for this test. Then, the broth was Incubated at different temperatures, ranging from 10°C to 45°C. After 24-48 hours of incubation, the viability of the cultures was assessed by observing growth, performing viable plate counts, or taking O.D. at 560nm (Chen et al., 2022).

3.6.5 Anti-Microbial Activity

In order to assess the antibacterial efficacy of Lactobacillus, wells were made on the surface of Mueller Hinton agar plates using a sterilized cork borer that had been heated with a flame. These wells serve as receptacles for the inclusion of the test samples. Several pathogenic bacteria, such as *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Enterococcus faecalis*, and *Escherichia coli*, were sampled by swabbing them onto agar plates, ensuring complete coverage of the entire surface. Approximately 0.1 ml of cell-free extract derived from the test organism was aliquoted and introduced into the wells on the plates. The cell-free extract comprises antimicrobial chemicals that are generated by the isolates. Subsequently, the plates were subjected to incubation at a temperature of 37°C for 24 hours. After the designated incubation period, the plates were carefully scrutinized to detect any alterations in the

growth patterns of the bacterial pathogens surrounding the wells. The presence of inhibition zones or decreased development in the surrounding area of the wells would suggest possible antibacterial activity (Chen et al., 2022).

3.6.6 Antibiotic Susceptibility Test

The antibiotic susceptibility of the isolates was determined using the standard disc diffusion method on MHA (Mueller-Hinton agar) plates. Muller Hilton agar plates were prepared and allowed to solidify at room temperature. Test culture was spread on the plates, and antibiotic discs were added. Plates were then incubated at 37°C for 24 hours. The diameter of the zone of inhibition around each disc was measured millimeters after incubation. The measured zone diameters were then compared to the standards provided by the M02-A12 Clinical and Laboratory Standard Institute guidelines (CLSI) to determine the susceptibility of the isolates to each antibiotic (Mishra & Ghosh, 2018).

3.6.7 Lactic acid production

Lactic acid production is determined by titrating the sample against a known concentration of sodium hydroxide (NaOH). Sodium hydroxide is a strong base that reacts with lactic acid to form sodium lactate. A 0.1N sodium hydroxide (NaOH) titration method was used to check lactic acid production. The titration process was performed in triplicates for each sample to ensure accuracy. The volume of NaOH required to neutralize the lactic acid was recorded (Mishra & Ghosh, 2018).

3.6.8 Salt Aggregation Test

The salt aggregation test is a method used to assess the hydrophobicity of bacterial surfaces. It involves observing the aggregation of bacteria in the presence of salt solutions. Prepared a bacterial suspension by growing the bacteria in a suitable culture medium and harvesting the cells. The bacterial cells were washed with a buffer solution to remove residual media components. Then, cells were resuspended in a buffer to create a standardized cell suspension. A series of salt solutions with increasing concentrations (e.g., 0.5 M, 0.25 M, 0.125 M, 0.05 M, 0.01 M, etc.) were prepared. Equal aliquots of the bacterial suspension were added to each salt solution. Then, gently

mix the bacterial suspension and salt solution and incubate for a specific period, usually around 30 minutes. After the incubation, the suspension was observed for visible bacterial aggregates or clumping. Higher aggregation or clumping of bacteria indicates higher hydrophobicity of the bacterial surface (Mishra & Ghosh, 2018).

3.6.9 B-galactosidase Activity

The assessment of beta-galactosidase activity is a widely employed technique for quantifying the enzymatic activity of the beta-galactosidase enzyme. In this experiment, a single ONPG disc was introduced into sterile tubes, and 1 ml of sterile normal saline was added to each tube. The tubes were subjected to gentle agitation and employed promptly for the experimental analysis. The isolated test organisms were introduced into the tubes using a sterile loop. The tubes were subjected to incubation at a temperature of 37°C for 24 hours. The emergence of a yellow hue indicated a favorable outcome, while the absence of any color alteration denoted a negative outcome (Mishra & Ghosh, 2018).

3.7 Genotypic Characterization of Isolates

Genomic DNA isolation: DNA was isolated from Microbial samples using the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee Stores Pvt Ltd.

3.7.1 Lysis/homogenization:

- Cultured cells were grown in a single layer and broken open by sterilely suspending 1-3 colonies. This mixture was combined with 500 µl of lysis buffer in a small tube, and cell lysis was achieved through repetitive pipetting.
- An additional 4 µl of RNase and 500 µl of neutralization buffer were introduced to the lysed solution. The contents were mixed thoroughly, and the tubes were kept in a water bath at 65°C for 30 minutes. The DNA solutions were gently mixed to prevent excessive DNA fragmentation by turning the tubes upside down.
- After 10-minute centrifugation at 10,000 RPM, the resulting thick liquid at the top was carefully transferred to a new tube, avoiding any disturbance to the pellet at the bottom.

- 600 μ l of a mixture of Chloroform and Isoamyl Alcohol was added to this liquid, followed by vigorous manual mixing.
- Another 10-minute centrifugation at 10,000 rpm was performed, after which 600 μ l of the watery layer was cautiously moved to a fresh small tube.

3.7.2 Binding:

- After adding 600 μ L of binding buffer, the mixture was wholly combined using pipetting. Then, it was let to sit at room temperature for 5 minutes.
- 600 μ L of the produced mixture was transferred to a specialized filter column in a collecting tube.
- Executed a 2-minute, 10,000 rpm centrifugation, removing the liquid that made it past the filter.
- Transferred 600 μ L of the lysate solution and reassembled the filter column with the collecting tube.
- After another 2-minute centrifugation at 10,000 rpm, the assembly was discarded with liquid passing through the filter.

3.7.3 Washing:

- Introduced 500 μ L of washing buffer I to the filter column and carried out a 2-minute centrifugation at 10,000 rpm. The liquid that passed through was then removed.
- Reconstructed the filter column setup and supplemented it with 500 μ l of washing buffer II. A 2-minute centrifugation at 10,000 rpm was performed, and the liquid that flowed through was discarded.
- The assembled column was subjected to a 5-minute spin-drying process at 10,000 rpm.
- Transferred the filter column into a sterile 1.5-ml microcentrifuge tube.

3.7.4 Elution:

- I dispensed 100 μ l of Elution buffer into the center of the filter column, ensuring that it did not contact the filtered liquid.

- Allowed the tubes to be incubated for 2 minutes at room temperature and then subjected to a 2-minute centrifugation at 10,000 rpm. The DNA was now present in the buffer within the microcentrifuge tube.
- The DNA concentrations were assessed using either the Qubit fluorometer 3.0 or a 1% Agarose Gel Electrophoresis.

3.7.5 PCR Protocol

The Polymerase Chain Reaction (PCR) technique utilizes primers to amplify specific DNA sequences from cloned sources or found within genomes. This amplification process relies on a highly distinct enzyme. Within PCR, the DNA polymerase enzyme is employed to guide the creation of new DNA strands from building blocks called deoxynucleotide substrates, utilizing a single-stranded DNA template. When paired with a longer single-stranded DNA template, the DNA polymerase enzyme appends nucleotides to the 3' end of a custom-designed oligonucleotide primer. Therefore, if a synthetic oligonucleotide primer is paired with a single-stranded template DNA containing a matching region, the DNA polymerase can use it as a starting point and lengthen its 3' end. This extension leads to the formation of an elongated segment of double-stranded DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM MgCl₂ and
- 0.02% bromophenol blue.

PRIMER DETAILS

| Primer Name | Sequence Details | Number of Bases |
|-------------|-----------------------------|-----------------|
| 27F | 5' AGAGTTTGATCTGGCTCAG 3' | 20 |
| 1492R | 5' TACGGTACCTTGTTACGACTT 3' | 20 |

Added 5 μL of isolated DNA to a PCR reaction solution containing 1.5 μL of both Forward and Reverse Primers, 5 μL of deionized water, and 12 μL of Taq Master Mix. PCR was carried out using the subsequent thermal cycling parameters:

- 1. Denaturation:** The DNA template underwent thermal exposure at 95°C, resulting in the breakage of the relatively weak hydrogen bonds that maintain the structural integrity of the DNA strands within the double helix configuration. The segregation above resulted in the emergence of DNA molecules consisting of a single strand.
- 2. Annealing:** The mixture underwent a cooling process, often reaching a temperature of approximately 55°C. The process described enabled the attachment of primers to their corresponding nucleotides on a DNA template.
- 3. Extension:** The reaction temperature was increased to 72°C, considered the ideal temperature for the enzymatic activity of DNA polymerase. The enzyme facilitated primer elongation through consecutive addition of nucleotides, employing the target DNA as a template.

PCR Conditions

| STAGES | TEMPERATURE | TIME | 25 Cycles |
|----------------------|-------------|----------|--------------|
| Initial Denaturation | 95°C | 2 min | |
| Denaturation | 95°C | 30 sec | |
| Annealing | 50°C | 30 sec | |
| Extension | 72°C | 2 min | |
| Final extension | 72°C | 10 min | |
| Hold | 4°C | ∞ | |

3.7.6 Purification of PCR Production

They eliminated unused PCR primers and dNTPs from the PCR products utilizing Millipore's Montage PCR Clean-Up kit. The resulting PCR products were subjected to sequencing, employing the primers. ABI PRISM® BigDye™ Terminator Cycle

Sequencing Kits, along with AmpliTaq® DNA polymerase (FS enzyme) from Applied Biosystems, were employed for the sequencing process.

3.7.7 Sequencing protocol

Each template underwent a single-pass sequencing procedure utilizing the specified 16S rRNA universal primers. The resulting fragments, labeled with fluorescence, were purified using an ethanol precipitation method to remove unused terminators. The purified samples were then dissolved in distilled water and subjected to electrophoresis using an ABI 3730xl sequencer from Applied Biosystems.

3.7.8 Alignment

After obtaining the sequencing and alignment data, the nucleotide sequences were converted into FASTA format. Each sequence was given a unique identifier (e.g., >sequence1. (AATTAGGAGTTAGGA) Moreover, it is saved in a notepad file. These sequences were then searched in the public nucleotide database to identify any homology using the BLAST (Basic et al.) program. The BLAST tool, provided by the NCBI (National Center for Biotechnology Information) and accessible at <https://blast.ncbi.nlm.nih.gov/>, was used for this purpose. By analyzing the BLAST results, the 16S rRNA gene sequence that exhibited the highest similarity to the query sequence was assigned a name corresponding to the given genus and species. This process enabled the identification of the isolates.

3.7.9 Phylogenetic Tree

In molecular phylogenetic analysis, the obtained sequences were compared to the five most closely related entries of 16S rDNA found through a BLASTN analysis. These six sequences were then aligned using the CLUSTALW multiple sequence alignment program, and the resulting alignment was saved as a . DND file along with the distance matrix data. The DND file was subsequently analyzed for phylogenetic relationships using MEGA 5, a software for predicting phylogenetic evolution. A phylogram was constructed based on the analysis, which provided insights into evolutionary relationships and confirmed the identity of the query entry up to the genus and species level. The multiple distance matrix obtained from the analysis was utilized with the

neighbor-joining (NJ) method to construct the phylogenetic trees, as described by Yu et al. in 2012.

3.8 Statistical optimizing Physical and chemical factors for the Maximum Growth of Microorganisms Using Plackett-Burman Design and Response Surface Methodology

3.8.1 Plackett-Burman Design

Plackett-Burman's (P-B) design analyzes significant variables for the growth of microorganisms. After a preliminary study of bacteria, 11 physical and chemical factors were selected for PB design to identify the significant factors that have a critical role in the growth of LAB. A strategy for organizing and carrying out experiments that produce the most data with the fewest analyses is known as statistical experimental design (SED), also known as design of experiments (DOE). When dealing with many possible causal factors that might affect one or more exciting responses, the most crucial components are identified using a "screening design" experimental technique. It gets simpler to evaluate fewer variables in subsequent trials by doing this. Prior screening studies enable the elimination of irrelevant elements, saving time and materials that would otherwise be needed for more implicated research. The orthogonal arrays developed by Plackett and Burman (P.B.) are particularly helpful for screening since they precisely estimate the direct impacts of the minor design. P.B. is made for two-level factorial screening, with high and low levels at each level (Karamad et al., 2020). For screening 'n' components, a run P.B. design technique with a 'n + 1' is used. The sample size is maintained as a multiple of fours rather than a power of two, represented as 4k observations, where k is a number between 1 and n. This distinctive quality defines the design. The design is most suited when utilizing a n x 4 method (8, 12, 16, 20, etc.) for n-1 variables in trials with more than seven variables (7, 11, 15, 19, etc.). However, three requirements must be met for a two-level factorial design:

- 1) Factor Selection: The factor should have the best response possible while being computed.

2) Range selection: The ranges of the factors should be sufficient to compute the effect.

3) Prevent range combinations: The ranges chosen for each factor should not be combined with those of other factors, as this might result in the experiment and factor setup failing.

Using the PB design, LAB biomass production with an influence on 11 factors was chosen. A 95% relative significance threshold was used for the experimental trials. The fundamental values of the independent variables were coded using PBD into High (+1) and Low (-1) levels, shown in Table 3.1.

Table 3.1 Factors Chosen for PB Design

| Sr. no. | Factors | Low level (-1) | High level (+1) |
|---------|--------------------|----------------|-----------------|
| 1 | pH | 2 | 8 |
| 2 | Temperature | 25 °C | 45 °C |
| 3 | Sodium Chloride | 5% | 8% |
| 4 | Bile Salt | 0.5% | 2% |
| 5 | Inoculum size | 0.5% | 3% |
| 6 | Incubation Period | 24h | 96h |
| 7 | Ascorbic acid | 0/1% | 0.5% |
| 8 | Ammonium Citrate | 0.05% | 0.1% |
| 9 | Magnesium sulphate | 0.1% | 0.5% |
| 10 | Manganese sulphate | 0.1% | 0.5% |
| 11 | Calcium Carbonate | 0.1% | 0.5% |

3.8.2 Response Surface methodology

Optimizing the medium using the Response Surface Method (RSM) involved utilizing central composite design (CCD) within Design Expert version 12 statistical software. This allowed for the determination of optimal experimental conditions, the creation of response surface visualizations, and the statistical assessment of collected data (Polak-Berecka et al., 2010). The central composite design of RSM was adopted for the experiment, employing pH, temperature, NaCl concentration, and incubation period as

independent variables (3.2). These factors were chosen to optimize various physical and chemical conditions.

Table 3.2: The central composite design of RSM

| Std | Run | pH | Temperature | Nacl% | Incubation Period (Hours) | Expected Values | Observed Values |
|-----|-----|-----|-------------|-------|---------------------------|-----------------|-----------------|
| 22 | 1 | 2.5 | 35 | 5 | 60 | | |
| 12 | 2 | 4 | 45 | 2 | 96 | | |
| 15 | 3 | 1 | 45 | 4 | 96 | | |
| 27 | 4 | 2.5 | 35 | 3 | 60 | | |
| 1 | 5 | 1 | 25 | 2 | 24 | | |
| 25 | 6 | 2.5 | 35 | 3 | 60 | | |
| 4 | 7 | 4 | 45 | 2 | 24 | | |
| 7 | 8 | 1 | 45 | 4 | 24 | | |
| 3 | 9 | 1 | 45 | 2 | 24 | | |
| 29 | 10 | 2.5 | 35 | 3 | 60 | | |
| 8 | 11 | 4 | 45 | 4 | 24 | | |
| 18 | 12 | 5.5 | 35 | 3 | 60 | | |
| 14 | 13 | 4 | 25 | 4 | 96 | | |
| 20 | 14 | 2.5 | 55 | 3 | 60 | | |
| 28 | 15 | 2.5 | 35 | 3 | 60 | | |
| 23 | 16 | 2.5 | 35 | 3 | 24 | | |
| 16 | 17 | 4 | 45 | 4 | 96 | | |
| 10 | 18 | 4 | 25 | 2 | 96 | | |

| | | | | | | | |
|----|----|-----|----|---|----|--|--|
| 21 | 19 | 2.5 | 35 | 1 | 60 | | |
| 11 | 20 | 1 | 45 | 2 | 96 | | |
| 9 | 21 | 1 | 25 | 2 | 96 | | |
| 17 | 22 | 0.5 | 35 | 3 | 60 | | |
| 6 | 23 | 4 | 25 | 4 | 24 | | |
| 5 | 24 | 1 | 25 | 4 | 24 | | |
| 30 | 25 | 2.5 | 35 | 3 | 60 | | |
| 2 | 26 | 4 | 25 | 2 | 24 | | |
| 13 | 27 | 1 | 25 | 4 | 96 | | |
| 26 | 28 | 2.5 | 35 | 3 | 60 | | |
| 19 | 29 | 2.5 | 15 | 3 | 60 | | |
| 24 | 30 | 2.5 | 35 | 3 | 32 | | |

3.9 Collection of Millet Grains

All three millet varieties, finger millet, foxtail millet, and barnyard millet, were collected from Punjab Agriculture University, Ludhiana. Apple and pineapple were procured from the local Jalandhar market, and juice was extracted from the fruits from the juice extractor.

3.10 Analyze the millet varieties for various physicochemical properties

3.10.1 Physical analysis of millet grains

3.10.1.1 Seed weight: 1000 grains from all varieties were randomly selected. Each variety was weighed separately in triplicate using an electronic balance with a minimum sensitivity of 0.01 mg. The measurements were recorded in grams (A.O.A.C., 2000).

3.10.1.2 Seed Density: A precise measurement of 50 grams of seeds was obtained and transferred into a measuring cylinder to determine the seed density. Subsequently, 50 ml of distilled water was added to the seeds. The seed volume was determined by subtracting the initial (50 ml) from the total volume. Finally, the seed density was calculated using the appropriate formula (A.O.A.C., 2000).

3.10.1.3 Seed Volume: In triplicate, fifty seeds of each millet variety were placed into separate 50 ml measuring cylinders. Subsequently, 25 ml of demineralized water was added to each cylinder. The change in total volume (25/50) from adding the seeds was recorded (A.O.A.C., 2000).

3.10.1.4 Bulk Density: A designated amount of the sample was placed into a pre-weighed 5 ml measuring cylinder (W1). The measuring cylinder was tapped gently to remove air gaps between the flour, and the sample volume was recorded (W2). The updated mass of the sample and the measuring cylinder was then measured (A.O.A.C., 2000). Bulk density was computed as:

Bulk Density = $(W2 - W1) / \text{Volume of seed}$

3.10.1.5 Hydration Capacity: About 50g of seeds were weighed, counted, and placed into a measuring cylinder for the hydration capacity test. Then, 150 ml of water was added to the seeds. The cylinder was covered with aluminum foil and left undisturbed overnight at room temperature (A.O.A.C., 2000). The following day, the seeds were drained, excess water was removed using filter paper, and the swollen seeds were reweighed.

3.10.1.6 Hydration index: Hydration index was calculated using the following formula:

Hydration index = $\text{Hydration capacity per seed} / \text{Weight (g) of one seed}$

3.10.1.7 Swelling Capacity: In the swelling capacity test, 50g of seeds were weighed and counted, and their initial volume was measured. The seeds were then

soaked overnight. The volume of the soaked seeds was recorded using a graduated cylinder (A.O.A.C., 2000).

Swelling capacity = Volume after soaking – Volume before soaking/ 50

3.10.1.8 Swelling index: Swelling index was calculated using the following formula:

Swelling index = Swelling capacity per seed/ Volume (ml) of one seed

3.10.1.9 Germination Percentage: To calculate the germination percentage, a random selection of seeds was soaked overnight and placed on wet filter paper inside Petri plates. All the samples were then incubated at 37°C for at least 48 hours. Throughout the incubation period, the filter paper was kept moist by sprinkling water (A.O.A.C., 2000). Finally, the germination percentage was determined for each variety of millet:

Percent germination = Seeds germinated/ Total seeds x 100

3.10.2 Chemical analysis of millet grains

3.10.2.1 Moisture Content

About 5 grams of millet seeds were placed in a pre-weighed petri dish in an oven set at 105°C for 6 hours. After cooling in a desiccator, the recorded weight was utilized to calculate the moisture percentage following the formula from AOAC (Association of Official Agricultural Chemists) in 2000:

Moisture (%) = Loss in weight (g) /100 Weight of sample (g) X 100

3.10.2.2 Total Ash Content

A 5-gram sample was weighed in a crucible and heated over a low Bunsen burner flame with the lid half open until the fumes disappeared. The crucible and lid were placed in a furnace and heated at 550°C overnight without covering the lid. After the complete

burning process, the lid was put back on to prevent the loss of fluffy ash, and the sample was allowed to cool down. Once the sample had turned grey, the ash was weighed along with the crucible and lid. The percent ash content was calculated using the following formula, based on the AOAC (Association of Official Agricultural Chemists) method from 2000:

$$\text{Percent Ash (\%)} = (\text{Loss of Weight (g)} / \text{Sample Weight (g)}) \times 100$$

3.10.2.3 Total Fat Content

The moisture-free flour sample was weighed into moisture-free thimbles, and the crude fat was extracted by refluxing with petroleum ether using a Soxhlet apparatus, following the provided AOAC method from 2000. The percentage fat content was determined using the following formula:

$$\text{Fat (\%)} = (\text{Loss of weight (g)} / \text{Sample weight (g)}) \times 100$$

3.10.2.4 Total Protein Content

The protein content in millet seeds and beverages was assessed by determining the nitrogen content through the Kjeldahl method, as outlined in the AOAC 2000 procedure. To calculate the crude protein content, the following formula was utilized:

$$\text{Crude Protein (\%)} = (0.00014 \times V \times (S - B) \times 100) / (V1 \times W \times F \times 6.25)$$

In this formula:

V represents the volume of the titrant used.

S indicates the sample titration value.

B denotes the blank titration value.

V1 stands for the volume (in milliliters) of the sample used.

W represents the weight (in grams) of the sample.

F is the factor for any dilution applied during the analysis.

3.10.2.5 Crude Fibre Content

The fiber percentage in millet seeds and probiotic beverages was determined using the method outlined in AOAC 2000. The procedure involved obtaining dried residues from moisture and fat-free samples through digestion with acid and alkali in a crucible, followed by weighing. The discrepancy in the crucible's weight before and after ashing the digested residues was employed to compute the fiber percentage using the provided formula:

$$\text{Crude Fiber (\%)} = ((W2 - W3) / W1) \times 100$$

In this formula

W1 represents the weight of the sample (in grams).

W2 is the weight of insoluble matter (weight of crucible + insoluble matter - weight of crucible).

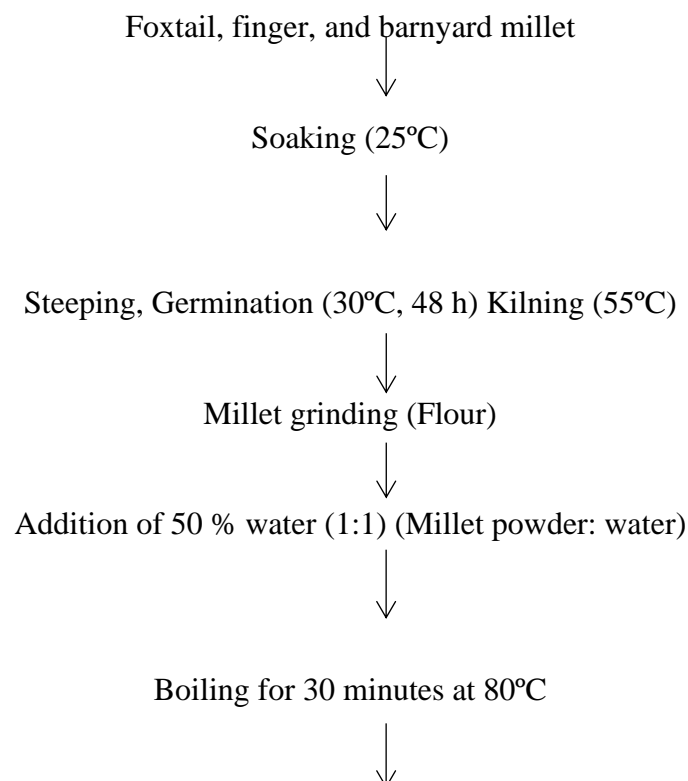
W3 stands for the weight of ash (weight of crucible + weight of ash - weight of crucible).

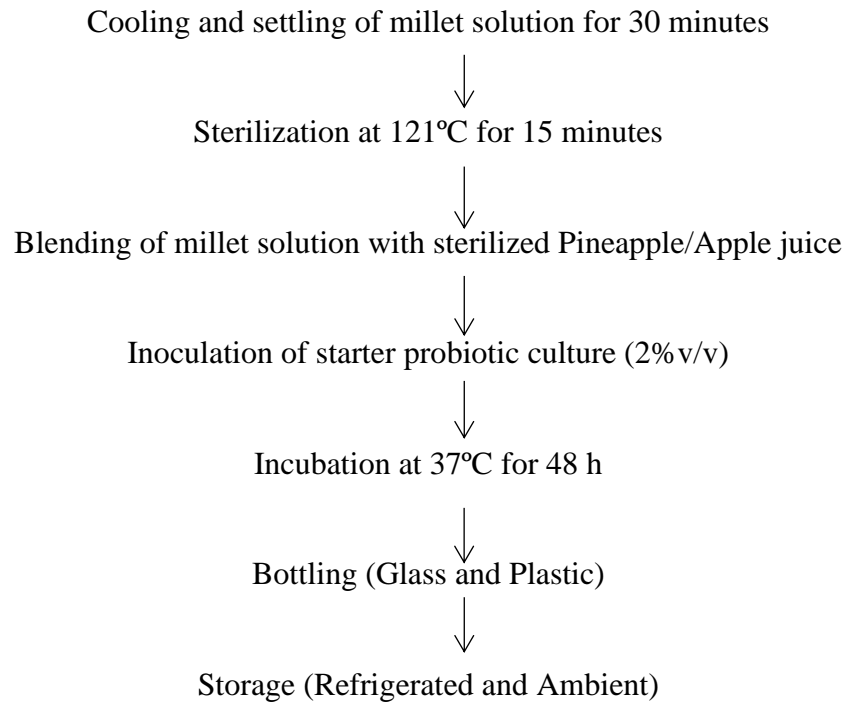
3.11 Production of Probiotic millet beverages

The obtained foxtail, finger, and barnyard millet will be cleaned and rinsed with water, after which they will be used for flour production. The millet seeds will be soaked in a water solution containing 0.1% formaldehyde, in a ratio of 1:2, for 24 hours at room temperature (approximately 25°C). To prevent gas accumulation around the seeds, they will be periodically stirred, and the soaking water will be changed every six hours. Once soaked, excess water will be drained through a mesh, and the grains will be placed between layers of muslin cloth and filter paper on trays. These trays will germinate at 25°C to 30°C for 48 hours, with intermittent sprinkling to maintain moisture levels. Following germination, the sprouted grains will be carefully separated from non-germinated grains, and the sprouted seeds will be dried at 55±2°C until their moisture content reaches a maximum of 12%. The rootlets and dried seeds will be manually removed through scrubbing, and the grains will be thoroughly rubbed to separate any vegetative parts. The separated vegetative parts will then be eliminated through winnowing. The germinated millet grains will be ground into fine flour using a grinder.

The powdered flour will be further homogenized by sieving through a 50-mesh sieve, resulting in flour particles with a size of approximately 270 microns or smaller. To prepare the beverage, millet flour will be added to sterile water in a 1:1 concentration and boiled at 80°C for 30 minutes. The prepared mixture will be sterilized by autoclaving at 121°C for 15 minutes and then cooled. Different apple and pineapple juice concentrations will be added to the sterilized millet solution to create beverages with varying ratios. In the second step, different treatments will be prepared by combining millet solution with apple juice with a concentration of 50:50 (v/v) in the specified volumes. Each treatment will then be inoculated with a selected starter probiotic culture at a concentration of 2% v/v for lactic acid fermentation. The fermentation process will occur at a temperature of 30°C for 48 hours, during which the titrable acidity (as percent lactic acid), pH, strain viability, and other chemical characteristics will be monitored. After 48 hours, the treatments will be packaged in glass and plastic bottles and stored under ambient and refrigerated conditions.

Flow chart





3.12 Microbiological analysis of product

Microbiological analysis was conducted on various beverages made from millet, and the standard plate count (SPC) was measured at different storage intervals. To perform the analysis, 1 ml samples were transferred to 9 ml sterilized dilution blanks, shaken well, and further diluted. Duplicate plates were prepared using nutrient agar as the growth medium and incubated at 37°C for 48 hours. The colony-forming units (CFU) were counted using a digital colony counter. The results were reported as log CFU x dilution/ml of the solution (Bottari et al., 2015).

3.13 Detection of Foodborne Pathogens

Foodborne pathogens were detected after 15 days of storage at 4°C by selective enumeration on specific media. *Salmonella* spp. was detected using Salmonella.

Shigella Agar, *Staphylococcus aureus* using Mannitol Salt Agar, *E. coli* using EMB Agar, and *Listeria monocytogenes* and *Yersinia enterocolitica* using Sheep Blood Agar plates, following the method described by Doyle (2001).

3.14 Sensory Analysis of Product

For the sensory analysis, the product was sent to FSSAI. A sensory test was conducted on the different treatments using a panel of semi-trained judges. The judges were instructed to rinse their mouths before or between tasting the samples. Various quality attributes, such as color, flavor, consistency, taste, and overall acceptability, were evaluated and recorded using a standardized form.

Chapter – 4

Results and Discussion

4. Results and discussion

4.1. Sample Collection

Probiotics are found in fermented and non-fermented foods, with several species of *Lactobacilli* being the primary source. To conduct the study, test samples were collected from various regions, specifically Amritsar, Muktsar, and Jalandhar in Punjab, as well as Mahendragarh and Kanina Khas in Haryana (as outlined in Table 4.1 and Figure 4.1). These locations were selected based on their distinct culinary traditions, expected to enhance the diversity of LAB strains identified in the samples. The samples were carefully collected and transferred to the laboratory with utmost caution, following the methodology detailed by Zhang et al. (2022).

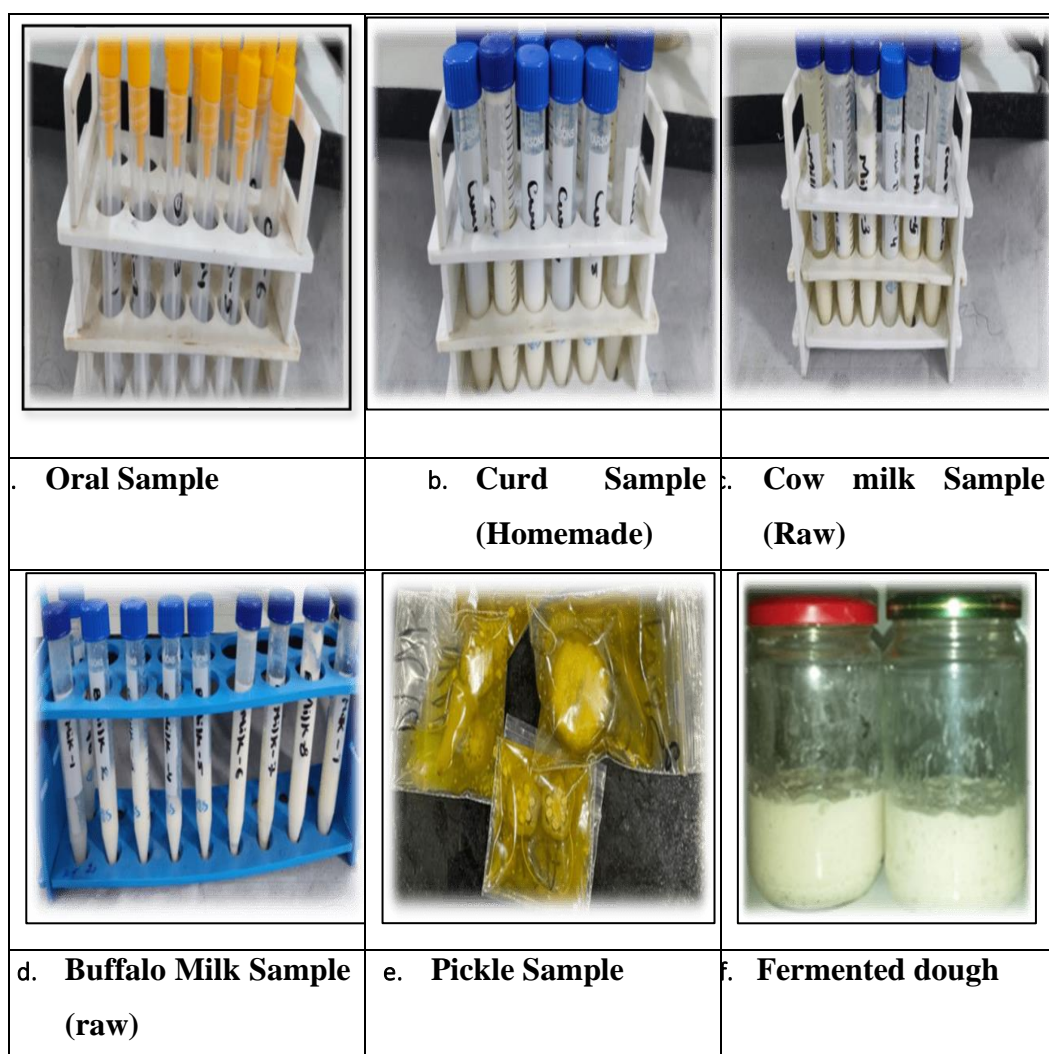


Figure 4.1: Different fermented and non-fermented samples.

Table 4.1: Details regarding the locations where food samples were collected to isolate probiotic microbes

| Place | Latitude | Longitude |
|----------------------------------|-----------|-----------|
| Amritsar district of Punjab | 31.634042 | 74.872331 |
| Jalandhar district of Punjab | 31.326015 | 75.576180 |
| Mukatsar district of Punjab | 30.480042 | 74.518204 |
| Kanina-Khas district of Haryana | 28.330292 | 76.308731 |
| Mahendragarh district of Haryana | 28.268347 | 76.150932 |

4.2 Isolation and Screening of lactic acid bacteria

Milk, curd, and pickles are typical examples of foods that contain probiotics. In one research, 50 samples were taken from diverse food sources, including buffalo's milk, cow's milk, curd, pickles, and refined wheat bread, while 10 samples were taken from human mouths. According to Pumriw et al. (2021), MRS agar media was employed to isolate LAB or probiotic bacteria (Figure 4.1). 48 hours were spent incubating the samples at 37°C. As indicated in Table 4.2, labels with the name and serial number of the sample (e.g., 1, 2, 3, etc.) were applied to the resultant colonies on the MRS agar.



a. CMI



b. OS1



c. C2



d. C3



Figure 4.2: LAB isolated from different sources on MRS Agar plates

Table 4.2: Different samples were employed to isolate LAB, and the probiotic qualities of a specific number of isolates were evaluated.

| S. No. | Sample Type | Abbreviations | Bacterial Isolates |
|--------|------------------|---------------|--------------------|
| 1 | Cow's Milk | CM | CM1 to CM10 |
| 2 | Buffalo's Milk | BM | BM1 to BM10 |
| 3 | Curd | C | C1 to C10 |
| 4 | Oral cavity | OS | OS1 to S10 |
| 5 | Fermented Batter | FB | FB1 to FB10 |
| 6 | Pickle | PK | PK1 to PK10 |

4.3 Phenotypic Characterization

4.3.1 Preliminary Identification of Isolated Bacteria

Sixty distinct colonies exhibiting diverse morphological characteristics were selected for further examination. These colonies were isolated and collected from the oral cavity and various food sources. Notable morphological features, including the colonies' size, color, shape, and elevation, were considered during the assessment. The findings were then cross-referenced with the information provided in *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) for further investigation (as detailed in Table 4.3).

Table 4.3: List of isolates along with their morphological features

| S.No. | Name of Isolates | Source | Morphology of colony | Color of Colony | Edge | Elevation |
|-------|------------------|----------|--------------------------|-----------------|--------|-----------|
| 1. | CM 1 | Cow Milk | Large, circular, regular | Creamy | Smooth | Convex |
| 2. | CM 2 | Cow Milk | Large, circular, regular | Creamy | Smooth | Convex |
| 3. | CM 3 | Cow Milk | Large, circular, regular | Creamy | Smooth | Convex |

| | | | | | | |
|-----|-------|-----------------|-------------------------------|--------|--------|--------|
| 4. | CM 4 | Cow Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 5. | CM 5 | Cow Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 6. | CM 6 | Cow Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 7. | CM 7 | Cow Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 8. | CM8 | Cow Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 9. | CM9 | Cow Milk | Large, circular, irregular | Creamy | Smooth | Convex |
| 10. | CM 10 | Cow Milk | Large, circular, regular | Creamy | Smooth | Convex |
| 11. | BM 1 | Buffalo Milk | Large, circular, irregular | Creamy | Smooth | Convex |
| 12. | BM 2 | Buffalo Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 13. | BM 3 | Buffalo Milk | Small, circular, regular | Creamy | Smooth | Convex |
| 14. | BM 4 | Buffalo Milk | Small, circular, irregular | Creamy | Smooth | Convex |
| 15. | BM 5 | Buffalo Milk | Large, circular regular | Creamy | Smooth | Convex |
| 16. | BM 6 | Buffalo Milk | Large, circular, regular | Creamy | Smooth | Convex |
| 17. | BM 7 | Buffalo Milk | Large, circular, irregular | Creamy | Smooth | Convex |
| 18. | BM 8 | Buffalo Milk | Large, circular, irregular | Creamy | Smooth | Convex |

| | | | | | | |
|-----|---------|--------------|----------------------------|--------|--------|--------|
| 19. | BM 9 | Buffalo Milk | Large, circular, irregular | Creamy | Smooth | Convex |
| 20. | BM 10 | Buffalo Milk | Large, circular, irregular | Creamy | Smooth | Convex |
| 21. | Curd 1 | Curd | Small, circular, regular | Creamy | Smooth | Convex |
| 22. | Curd 2 | Curd | Small, circular, regular | Creamy | Smooth | Convex |
| 23. | Curd 3 | Curd | Small, circular, irregular | Creamy | Smooth | Convex |
| 24. | Curd 4 | Curd | Large, circular, regular | Creamy | Smooth | Convex |
| 25. | Curd 5 | Curd | Large, circular, irregular | Creamy | Smooth | Convex |
| 26. | Curd 6 | Curd | Small, circular, irregular | Creamy | Smooth | Convex |
| 27. | Curd 7 | Curd | Small, circular, irregular | Creamy | Smooth | Convex |
| 28. | Curd 8 | Curd | Small, circular, irregular | Creamy | Smooth | Convex |
| 29. | Curd 9 | Curd | Large, circular, regular | Creamy | Smooth | Convex |
| 30. | Curd 10 | Curd | Large, circular, regular | Creamy | Smooth | Convex |
| 31. | PK 1 | Pickle | Large, circular, regular | Creamy | Smooth | Convex |
| 32. | PK 2 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |
| 33. | PK 3 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |

| | | | | | | |
|-----|-------|----------------|-------------------------------|--------|--------|--------|
| 34. | PK 4 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |
| 35. | PK 5 | Pickle | Large, circular, irregular | Creamy | Smooth | Convex |
| 36. | PK 6 | Pickle | Large, circular, irregular | Creamy | Smooth | Convex |
| 37. | PK 7 | Pickle | Large, circular, irregular | Creamy | Smooth | Convex |
| 38. | PK 8 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |
| 39. | PK 9 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |
| 40. | PK 10 | Pickle | Small, circular, regular | Creamy | Smooth | Convex |
| 41. | FB1 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |
| 42. | FB2 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |
| 43. | FB3 | Wheat Dough | Large, circular, irregular | Creamy | Smooth | Convex |
| 44. | FB4 | Wheat Dough | Large, circular, regular | Creamy | Smooth | Convex |
| 45. | FB5 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |
| 46. | FB6 | Wheat Dough | Large, circular, irregular | Creamy | Smooth | Convex |
| 47. | FB7 | Wheat Dough | Large, circular, regular | Creamy | Smooth | Convex |
| 48. | FB8 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |

| | | | | | | |
|-----|------|-------------|----------------------------|--------|--------|--------|
| 49. | FB9 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |
| 50. | FB10 | Wheat Dough | Small, circular, regular | Creamy | Smooth | Convex |
| 51. | OS1 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 52. | OS2 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 53. | OS3 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 54. | OS4 | Oral Cavity | Large, circular, irregular | Creamy | Smooth | Convex |
| 55. | OS5 | Oral Cavity | Large, circular, regular | Creamy | Smooth | Convex |
| 56. | OS6 | Oral Cavity | Large, circular, regular | Creamy | Smooth | Convex |
| 57. | OS7 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 58. | OS8 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 59. | OS9 | Oral Cavity | Small, circular, regular | Creamy | Smooth | Convex |
| 60. | OS10 | Oral Cavity | Large, circular, irregular | Creamy | Smooth | Convex |

The isolated colonies underwent a series of supplementary identification tests, as outlined in Table 4.4. 60 *Lactobacillus* spp. were initially distinguished based on their Gram staining (depicted in Figure 4.3). Both Gram staining and a catalase activity test were performed on these colonies. Among them, 21 isolates were Gram-positive rods and catalase positive. About 23 isolates were both Gram-positive cocci and catalase positive. Only 16 isolates were identified as Gram-positive rods and catalase-negative,

which included FB4, FB7, C2, C3, C4, PK5, PK6, CM9, CM1, CM6, CM2, BM3, BM2, BM1, OS6, and OS1. These isolates underwent further detailed examination. All 60 isolates were non-endospore forming and non-motile. Additionally, they demonstrated robust growth on MRS medium at 37°C while thriving in anaerobic environments. The 16 selected bacterial colonies identified as Gram-positive rods and catalase-negative were subjected to more thorough identification procedures. According to the principles outlined in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994), this included a combination of microscopic, macroscopic, and biochemical analyses.

The catalase test data indicated that the isolated bacteria could not enzymatically break down H_2O_2 into H_2O and O_2 , a defining characteristic of lactic acid bacteria (Tamang et al., 2009; Ismail et al. 2018). This absence of catalase activity was confirmed by the lack of bubble formation during the test (MacFaddin et al., 2015). The result aligns with the widely accepted understanding that *Lactobacillus* is a catalase-negative bacterium, as supported by the research of MacFaddin (2015). The isolates that tested catalase-negative were selected for further research. After purification, sixty isolates were chosen from each source (pickle, curd, and milk) for preliminary identification (as shown in Table 4.4).

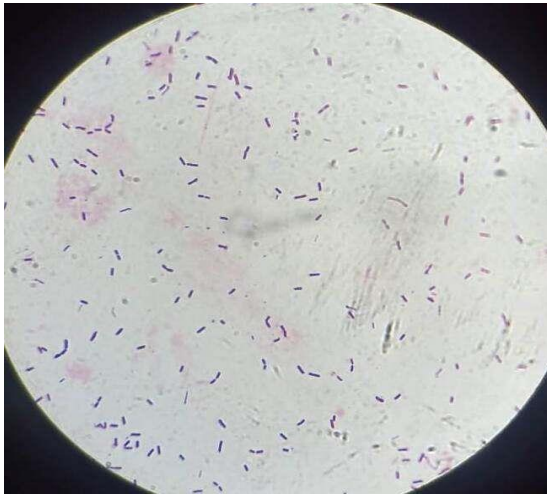
The isolates that matched the characteristics described in Bergey's Manual—such as being Gram-positive, non-motile, catalase-negative, and non-endospore forming—were selected for further analysis. Additionally, these isolates underwent further biochemical tests to assist in their identification. Previous studies by Menconi et al. (2014) and Fontana et al. (2013) also reported the identification of probiotic bacteria based on morphological and biochemical features. In these studies, the presence of Gram-positive bacteria that were catalase-negative and oxidase-negative was used as an initial selective criterion for detecting LAB with probiotic potential.

Table 4.4: Preliminary Identification of Isolates

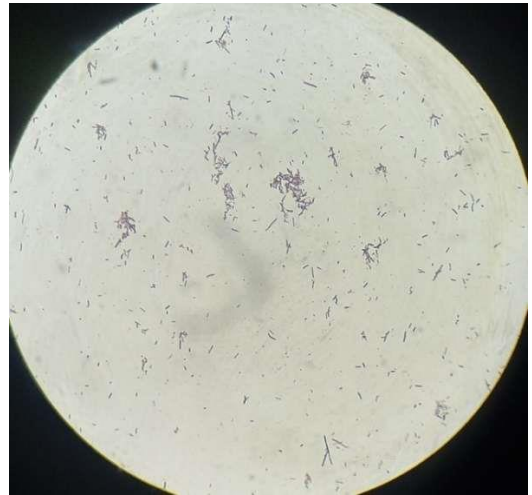
| Isolates | Morphology | Gram Staining | Motility | Endospore Test | Catalase Test |
|-----------------|-------------------|----------------------|-----------------|-----------------------|----------------------|
| CM1 | Cocci | + | - | - | - |
| CM2 | Rods | + | - | - | - |
| CM3 | Rods | + | - | - | + |
| CM4 | Cocci | + | - | - | + |
| CM5 | Cocci | + | - | - | + |
| CM6 | Rods | + | - | - | - |
| CM7 | Rods | + | - | - | + |
| CM8 | Rods | + | - | - | + |
| CM9 | Rods | + | - | - | - |
| CM10 | Cocci | + | - | - | + |
| BM1 | Rods | + | - | - | - |
| BM2 | Rods | + | - | - | - |
| BM3 | Cocci | + | - | - | - |
| BM4 | Cocci | + | - | - | + |
| BM5 | Cocci | + | - | - | + |
| BM6 | Rods | + | - | - | + |
| BM7 | Rods | + | - | - | + |
| BM8 | Rods | + | - | - | + |

| | | | | | |
|-------------|-------|---|---|---|---|
| BM9 | Cocci | + | - | - | + |
| BM10 | Rods | + | - | - | + |
| C1 | Cocci | + | - | - | + |
| C2 | Cocci | + | - | - | - |
| C3 | Rods | + | - | - | - |
| C4 | Rods | + | - | - | - |
| C5 | Rods | + | - | - | + |
| C6 | Cocci | + | - | - | + |
| C7 | Cocci | + | - | - | + |
| C8 | Rods | + | - | - | + |
| C9 | Rods | + | - | - | + |
| C10 | Cocci | + | - | - | + |
| PK1 | Rods | + | - | - | + |
| PK2 | Rods | + | - | - | + |
| PK3 | Rods | + | - | - | + |
| PK4 | Cocci | + | - | - | + |
| PK5 | Cocci | + | - | - | - |
| PK6 | Rods | + | - | - | - |
| PK7 | Cocci | + | - | - | + |
| PK8 | Cocci | + | - | - | + |
| PK9 | Cocci | + | - | - | + |

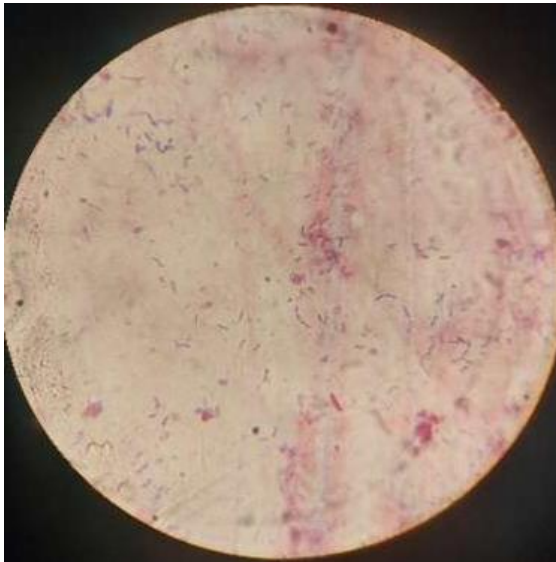
| | | | | | |
|-------------|-------|---|---|---|---|
| PK10 | Cocci | + | - | - | + |
| FB1 | Rods | + | - | - | + |
| FB2 | Cocci | + | - | - | + |
| FB3 | Cocci | + | - | - | + |
| FB4 | Rods | + | - | - | - |
| FB5 | Cocci | + | - | - | + |
| FB6 | Cocci | + | - | - | + |
| FB7 | Rods | + | - | - | - |
| FB8 | Cocci | + | - | - | + |
| FB9 | Cocci | + | - | - | + |
| FB10 | Cocci | + | - | - | + |
| OS1 | Rods | + | - | - | - |
| OS2 | Cocci | + | - | - | + |
| OS3 | Cocci | + | - | - | + |
| OS4 | Rods | + | - | - | + |
| OS5 | Cocci | + | - | - | + |
| OS6 | Rods | + | - | - | - |
| OS7 | Cocci | + | - | - | + |
| OS8 | Cocci | + | - | - | + |
| OS9 | Cocci | + | - | - | + |
| OS10 | Cocci | + | - | - | + |



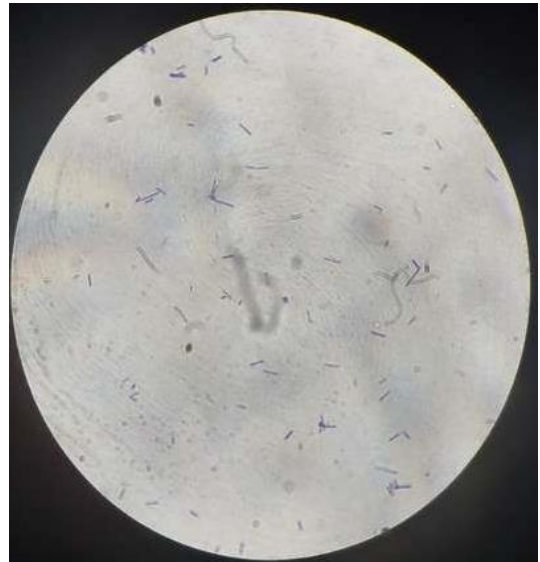
a. CM1



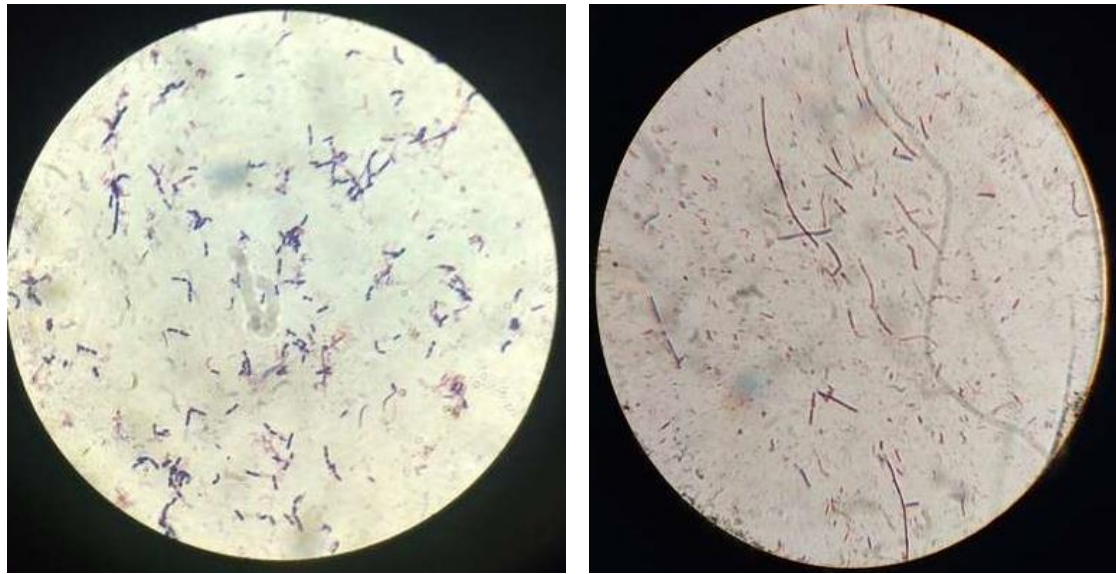
b. OS1



c. BM3



d. PK6



e. C3

f. FB7

Figure 4.3: Observation of both isolates after Gram staining under a microscope (100X)

4.4 Biochemical Tests

Based on the initial identification outcomes (as shown in Table 4.4), a subset of 16 isolates was selected to investigate the prevalence of lactic acid bacteria (LAB). This assessment involved conducting various biochemical assays, including the methyl red (MR) test, Voges-Proskauer (VP) test, hydrogen sulfide (H₂S) production, indole production, glucose-induced gas production, nitrate reduction, and citrate utilization. The results presented in Table 4.4 demonstrate that these 16 isolates showed significant LAB prevalence based on the outcomes of these biochemical evaluations. The present research employed a variety of biochemical tests on the 16 selected isolates to determine the prevalence of LAB, following the protocols outlined by Taye et al. (2021).

Among the 16 isolates, only three isolates, OS6, BM2, and CM6, yielded negative results for the MR test, indicating the absence of mixed acid fermentation by these bacteria. Furthermore, in the citrate utilization test, only one isolate, OS1, showed a

positive reaction, indicating its ability to utilize citrate as a carbon source. The remaining isolates displayed adverse outcomes for the MR, VP, H₂S, indole, citrate utilization, and nitrate reduction tests, suggesting that they may represent distinct LAB strains. According to the investigation by Khedid et al. (2009), LAB prevalence was also assessed using specific criteria, which included the capacity to produce gas from glucose, a positive malonate reaction, lack of citrate utilization, an adverse Voges-Proskauer reaction, and a harmful nitrate reduction. The findings of this study confirmed the prevalence of LAB based on these criteria.

Table 4.5: Biochemical characterization of isolates

| Isolates | MR Test | VP Test | Indole Production | Nitrate reduction | Citrate Utilization |
|-----------------|----------------|----------------|--------------------------|--------------------------|----------------------------|
| CM1 | Positive | Negative | Negative | Negative | Negative |
| CM2 | Positive | Negative | Negative | Negative | Negative |
| CM6 | Negative | Negative | Negative | Negative | Negative |
| CM9 | Positive | Negative | Negative | Negative | Negative |
| BM1 | Positive | Negative | Negative | Negative | Negative |
| BM2 | Negative | Negative | Negative | Negative | Negative |
| BM3 | Positive | Negative | Negative | Negative | Negative |
| C2 | Positive | Negative | Negative | Negative | Negative |
| C3 | Positive | Negative | Negative | Negative | Negative |

| | | | | | |
|------------|----------|----------|----------|----------|----------|
| C4 | Positive | Negative | Negative | Negative | Negative |
| OS1 | Positive | Negative | Negative | Negative | Negative |
| OS6 | Negative | Negative | Negative | Negative | Negative |
| PK5 | Positive | Negative | Negative | Negative | Negative |
| PK6 | Positive | Negative | Negative | Negative | Negative |
| FB4 | Positive | Negative | Negative | Negative | Negative |
| FB7 | Positive | Negative | Negative | Negative | Negative |

4.5 Characterization and screening of isolates for Probiotic Properties

Characterizing isolates for probiotic properties involves evaluating specific attributes and functionalities of microorganisms to determine their suitability as probiotics. As Sefcova et al. (2021) highlighted, the KIA test results illustrated notable variations in the bacterial types present among the isolates. All 16 strains displayed a positive outcome in the KIA test, indicating their ability to ferment sugars. Among them, 10 isolates (CM2, CM1, CM9, CM6, BM3, BM2, OS6, OS1, FB6, and FB4) exhibited both an alkaline slant and an alkaline butt, while one isolate (PK5) displayed an alkaline slant and an acidic butt. Five isolates (C4, C3, C2, PK6, and BM1) demonstrated an acid slant and an acidic butt during the Kligler's iron agar test, as shown in Table 4.6 and Figure 4.9. In cases where no color change occurred and no carbohydrate fermentation occurred, it indicated that the bacteria could not produce acid through sugar fermentation. Conversely, an acidic butt and an alkaline slant pointed to bacteria that could ferment glucose but were unable to break down lactose. This characteristic is consistent with bacteria incapable of metabolizing lactose, as Sefcova et al. (2021) noted.

The fermentation of glucose initially resulted in a reduction in pH; however, this was followed by a recovery to an alkaline pH due to the formation of alkaline amines near the organism's surface. This process resulted from the oxidative decarboxylation of peptides derived from medium-sized proteins in the presence of oxygen. Notably, none of the isolates exhibited hemolytic activity, confirming their safety for human consumption. In the arginine hydrolysis test, the microorganism first consumed the available glucose, indicating the color change from violet to yellow. The activation of the arginine dihydrolase enzyme occurred due to the increased acidity of the medium. The culture was maintained at 37°C for an additional 24 hours to allow the microorganisms sufficient time to utilize the arginine. The tube was observed after a total incubation time of 48 hours to obtain the final results. The arginine dihydrolase test indicates a positive result when the yellow liquid returns to its original purple color. The isolates also demonstrated protease activity, as clear zones were observed around the colonies.

Table 4.6: Characterization of isolates for Probiotic Properties

| Isolates | Casein Hydrolysis | Arginine Hydrolysis | Kliger's Iron Agar | Hemolytic Activity |
|-----------------|--------------------------|----------------------------|---------------------------|---------------------------|
| CM1 | Positive | Positive | Alk/A | γ activity |
| CM2 | Positive | Positive | Alk/Alk | γ activity |
| CM6 | Positive | Positive | Alk/Alk | γ activity |
| CM9 | Positive | Positive | Alk/Alk | γ activity |
| BM1 | Positive | Positive | A/A | γ activity |

| | | | | |
|------------|----------|----------|---------|-------------------|
| BM2 | Positive | Positive | Alk/Alk | γ activity |
| BM3 | Positive | Positive | Alk/Alk | γ activity |
| C2 | Positive | Positive | A/A | γ activity |
| C3 | Positive | Positive | A/A | γ activity |
| C4 | Positive | Positive | A/A | γ activity |
| OS1 | Positive | Positive | Alk/Alk | γ activity |
| OS6 | Positive | Positive | Alk/Alk | γ activity |
| PK5 | Positive | Positive | Alk/Alk | γ activity |
| PK6 | Positive | Positive | A/A | γ activity |
| FB4 | Positive | Positive | Alk/Alk | γ activity |
| FB7 | Positive | Positive | Alk/Alk | γ activity |

Alk – Alkaline, A- Acid, γ – gamma

4.6 Carbohydrate Fermentation

The current study examined the ability of isolated lactic acid bacteria strains to utilize sugar, looking closely at a total of 11 different sugars. The results showed that these sugars could be divided into two categories based on the proportion of bacterial strains capable of using them. The result demonstrated that various strains could ferment sugars like Glucose, Maltose, Lactose, and Fructose at about 91.63%, 83.3%, and 66.64%, respectively. On the other hand, some sugars were less utilized by the isolated strains, including 58.31% Mannose, 49.98% Galactose Mannitol, Ribose, about 41.65%

Sucrose, 16.66% Starch, and 8.33% Arabinose, showed considerably reduced consumption by the isolated microbes, showing the partial utilization of tested sugars.

Importantly, it should be emphasized that sugar use patterns varied between all the strains, highlighting the distinctive features that are unique to each other. Considering these unique sugar usage patterns and other desirable qualities, the best LAB strains may be chosen to find suitable probiotic candidates for future studies. According to Lim and Im (2009), these findings significantly improve our knowledge of the lactic acid bacteria's capacity to utilize sugar and their prospective application as probiotic agents.

Table 4.7: Carbohydrate utilization pattern of isolates

| Isolates | Glucose | Lactose | Maltose | Fructose | Sucrose | Arabinose | Mannitol | Mannose | Galactose | Starch | Ribose |
|-----------------|----------------|----------------|----------------|-----------------|----------------|------------------|-----------------|----------------|------------------|---------------|---------------|
| CM1 | + | - | + | - | + | - | - | + | + | - | - |
| CM2 | + | - | + | + | - | - | - | + | - | - | - |
| CM3 | + | + | + | + | + | - | + | + | + | - | - |
| CM4 | + | + | - | + | - | - | - | - | + | - | - |
| BM1 | + | + | - | - | + | - | - | - | - | + | + |
| BM2 | + | + | - | - | + | + | - | - | + | - | - |
| BM3 | + | + | - | + | + | - | + | - | - | - | + |
| C2 | + | + | - | + | - | - | + | - | - | - | - |
| C3 | + | + | - | - | - | - | + | - | - | - | - |
| C4 | + | + | - | - | - | - | + | + | - | - | - |
| C5 | + | + | - | - | - | - | - | + | - | - | - |
| PK5 | + | - | + | - | - | - | - | - | + | - | - |
| PK6 | - | - | + | - | - | + | - | - | - | + | - |
| PK8 | + | + | - | + | + | - | + | - | - | - | + |
| PK9 | - | - | - | + | + | - | + | - | + | - | + |

4.7 Evaluation of probiotic attributes of isolates

The probiotic characteristics of all 16 isolates were assessed, including their ability to withstand various temperature fluctuations, changes in pH levels, variations in NaCl concentrations, tolerance to bile salts, antibacterial activities, susceptibility to antibiotics, and results from the salt aggregation test, among other evaluations.

4.7.1 Temperature Tolerance Test

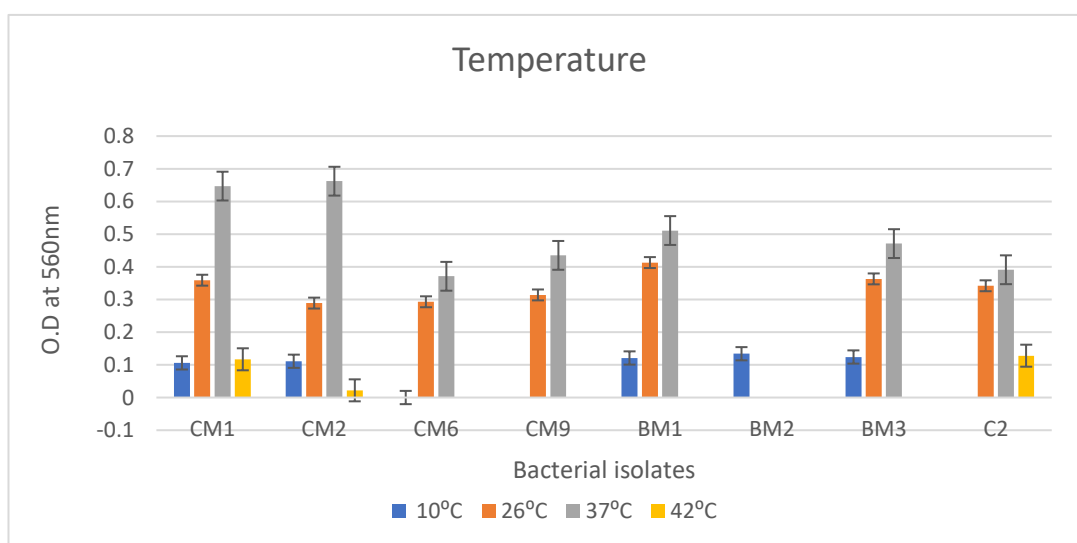
Figures 4.4 A and B display the findings of the temperature investigation, which assessed each isolate's ability to grow in MRS broth incubated at various temperatures, including 10°C, 21°C, 37°C, and 42°C. As shown in Table 4.8, approximately 37.2% of the isolates exhibited growth at 10°C, while 43.8% showed growth at 42°C, though with a notable decline compared to their growth at 37°C. At both 21°C and 37°C, most of the isolates (93.7%) demonstrated significantly improved growth. Notably, the growth rate at 37°C (the temperature corresponding to the human body) was higher than at 10°C and 42°C. Distinct letters denote statistically significant differences ($p < 0.05$), and the values shown in the figure represent the means of three replicates.

Table 4.8: Screening the isolates for their ability to sustain variable temperature (O.D at 560nm)

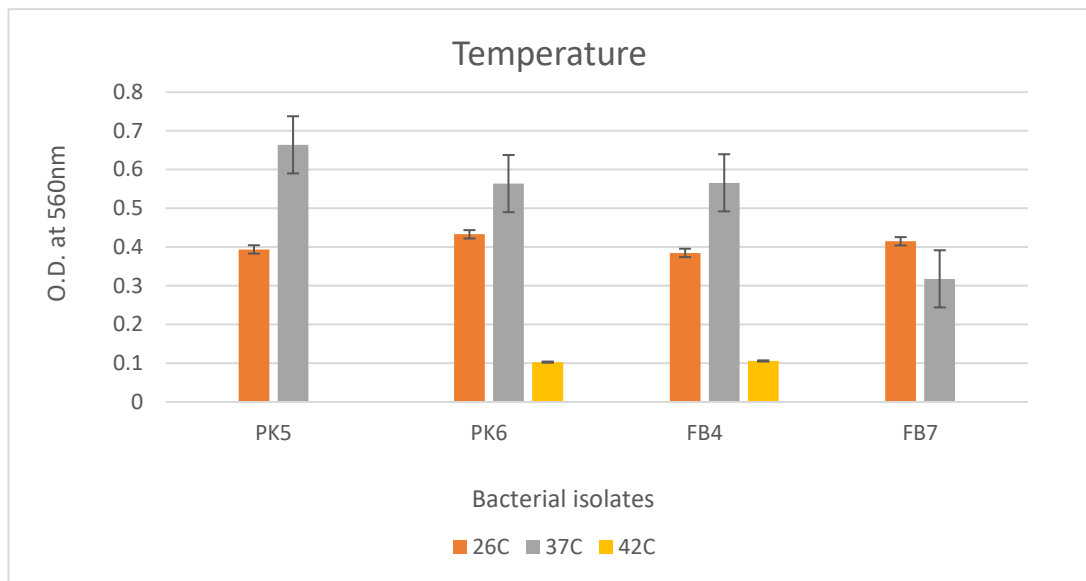
| Isolates | Temperature | | | |
|------------|-------------|-------------|-------------|-------------|
| | 10°C | 21°C | 37°C | 42°C |
| CM1 | 0.106±0.002 | 0.359±0.002 | 0.447±0.002 | 0.117±0.002 |
| CM2 | 0.111±0.002 | 0.289±0.002 | 0.625±0.002 | 0.022±0.002 |
| CM6 | - | 0.293±0.002 | 0.371±0.002 | - |
| CM9 | - | 0.343±0.003 | 0.435±0.002 | - |
| BM1 | 0.121±0.002 | 0.413±0.001 | 0.511±0.003 | - |
| BM2 | 0.134±0.001 | - | - | - |
| BM3 | 0.124±0.002 | 0.363±0.001 | 0.471±0.002 | - |

| | | | | |
|------------|-------------|--------------|-------------|-------------|
| C2 | - | 0.624±0.002 | 0.791±0.002 | 0.328±0.002 |
| C3 | - | 0.212±0.001 | 0.363±0.002 | - |
| C4 | - | 0.118±0.001 | 0.367±0.002 | 0.524±0.002 |
| OS1 | 0.217±0.002 | 0.0443±0.002 | 0.563±0.001 | 0.134±0.002 |
| OS6 | - | 0.221±0.002 | 0.511±0.002 | - |
| PK5 | - | 0.394±0.002 | 0.664±0.003 | - |
| PK6 | - | 0.433±0.001 | 0.564±0.003 | 0.103±0.002 |
| FB4 | - | 0.385±0.002 | 0.566±0.003 | 0.106±0.003 |
| FB7 | - | 0.415±0.001 | 0.318±0.002 | - |

*values in columns are mean ± Standard deviation



(A)



(B)

Figure 4.4: (A, B) The screening of isolates at different Temperatures after 24 hours of incubation.

Previous studies suggest that lactic acid bacteria isolated from humped camel milk exhibited a similar trend. In those studies, it was found that 50% of the bacteria grew at 40°C, while only 0.5% were able to grow at 10°C, indicating a preference for higher temperatures for growth and metabolism. This finding is consistent with the results of your study. Additionally, Menconi et al. (2014) reported that certain lactic acid bacteria strains, specifically strains '18' and '48d,' were able to grow at both 15°C and 45°C after 2 and 4 hours of incubation, suggesting that some strains can tolerate a wide range of temperatures and still survive and multiply.

The isolated lactic acid bacteria's ability to grow at high temperatures is considered desirable, as it correlates with increased growth and lactic acid production. Moreover, growing at high temperatures can help reduce other microorganisms' contamination, as Ibourahema et al. (2008) noted. Overall, these findings highlight lactic acid bacteria's temperature preferences and capabilities, emphasizing their ability to thrive at high temperatures and potentially outcompete other microorganisms.

4.7.2 pH Tolerance Test

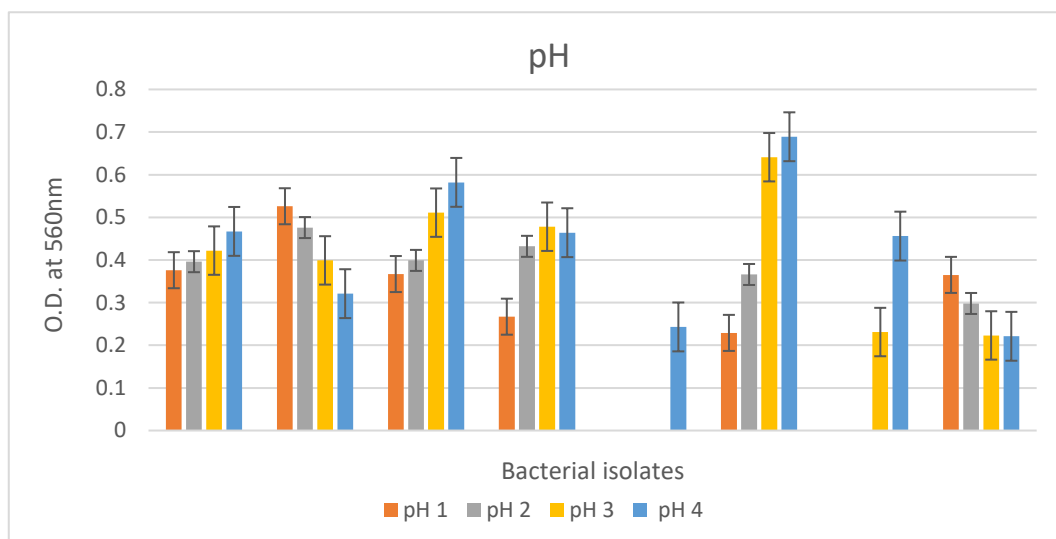
Figure 4.5 A and B outline the findings of the acid tolerance assessment conducted on various bacterial isolates. These isolates were exposed to different acidic pH levels (1, 2, 3, and 4) at 37°C for 24 hours, with a control pH 7.0. Notably, isolates such as CM9, CM1, BM3, BM2, C3, C4, OS6, OS1, FB7, and PK6 survived at pH levels ranging from 1 to 4. However, cell concentrations decreased compared to the control at pH 7 (Table 4.9). Significant differences in bacterial growth were observed between pH levels 2, 3, and 4 when compared to pH 1. Among the isolates, OS1 and CM1 demonstrated the ability to withstand acidic conditions for up to 3 hours. Most isolates showed significantly improved survival at pH 3 compared to pH 1 and 2. Although cell concentrations at pH 3 were lower than at the control pH of 7, survival was notably higher at this level. The values in the figure represent the means of three replicates, and distinct letters indicate statistically significant differences ($p < 0.05$).

Table 4.9: The screening of the isolates for their ability to sustain variable pH levels (O.D at 560nm)

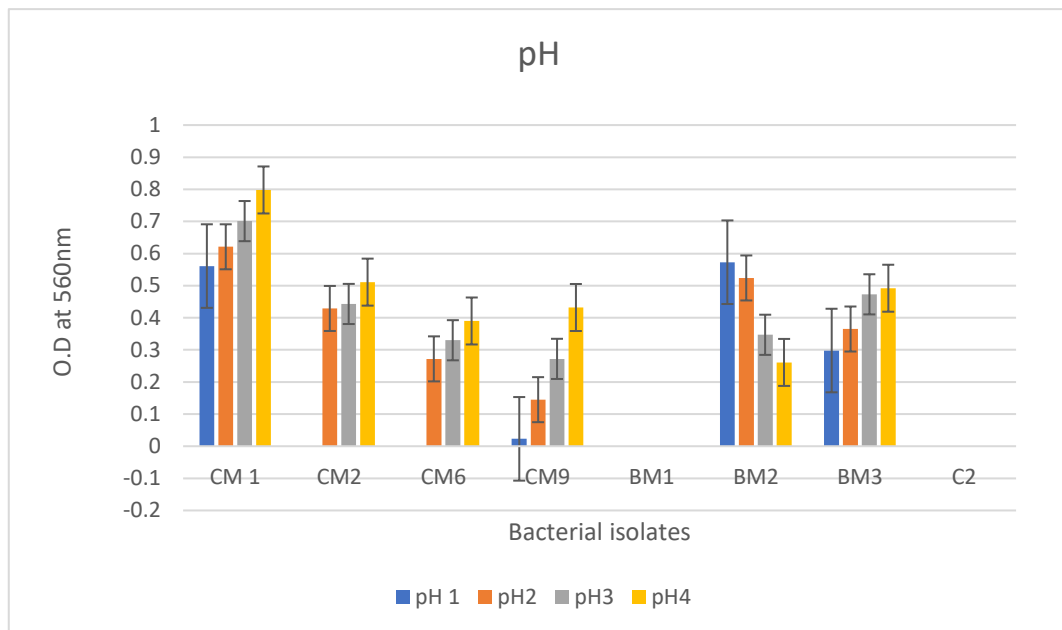
| Isolates | Ph | | | |
|----------|-------------|-------------|-------------|-------------|
| | 1 | 2 | 3 | 4 |
| CM1 | 0.561±0.003 | 0.621±0.001 | 0.701±0.002 | 0.798±0.002 |
| CM2 | - | 0.429±0.003 | 0.443±0.003 | 0.411±0.002 |
| CM6 | - | 0.272±0.002 | 0.330±0.002 | 0.390±0.001 |
| CM9 | 0.023±0.003 | 0.145±0.002 | 0.272±0.002 | 0.432±0.001 |
| BM1 | - | - | - | - |
| BM2 | 0.573±0.004 | 0.524±0.002 | 0.347±0.002 | 0.261±0.002 |
| BM3 | 0.298±0.002 | 0.365±0.003 | 0.473±0.002 | 0.492±0.002 |
| C2 | - | - | - | - |
| C3 | 0.676±0.001 | 0.796±0.002 | 0.822±0.002 | 0.867±0.002 |
| C4 | 0.526±0.003 | 0.876±0.002 | 0.799±0.002 | 0.741±0.001 |

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| OS1 | 0.376±0.002 | 0.399±0.001 | 0.511±0.001 | 0.582±0.002 |
| OS6 | 0.267±0.002 | 0.432±0.003 | 0.478±0.003 | 0.464±0.001 |
| PK5 | - | - | - | 0.243±0.002 |
| PK6 | 0.229±0.003 | 0.366±0.004 | 0.641±0.002 | 0.689±0.002 |
| FB 4 | - | - | 0.231±0.001 | 0.456±0.002 |
| FB7 | 0.365±0.003 | 0.298±0.002 | 0.223±0.002 | 0.221±0.002 |

*values in columns are mean ± Standard deviation



(A)



(B)

Figure 4.5 (A, B): The screening of isolates at different pH levels after 24 hours of incubation.

In the Menconi et al. (2014) study, the LAB18 and LAB48 strains could not survive when exposed to a pH of 2.0 for 2 or 4 hours. However, at a pH of 3.0, both strains survived after 2 and 4 hours of incubation. In comparison, the present study identified several isolates capable of withstanding a pH of 1.5 for up to 3 hours of incubation. Previous research has shown that *Lactobacillus* strains exhibit resistance to low pH, with a survival rate of 1 hour at pH 3.0. On the other hand, *Bifidobacterium* spp., another type of lactic acid bacteria, is highly sensitive to pH 2.0 and pH 3.0. This suggests that lactic acid bacteria have an acidophilic nature. However, it is essential to distinguish this from the inhibitory effect of high concentrations of free acids (H^+), which can hinder their growth. The ability to survive at pH 1.5 indicates that these strains can withstand passage through the stomach, where the pH can be as low as 1.5 to 2.0, and remain viable for at least 3 hours before reaching the intestinal tract.

4.7.3 NaCl Tolerance Test

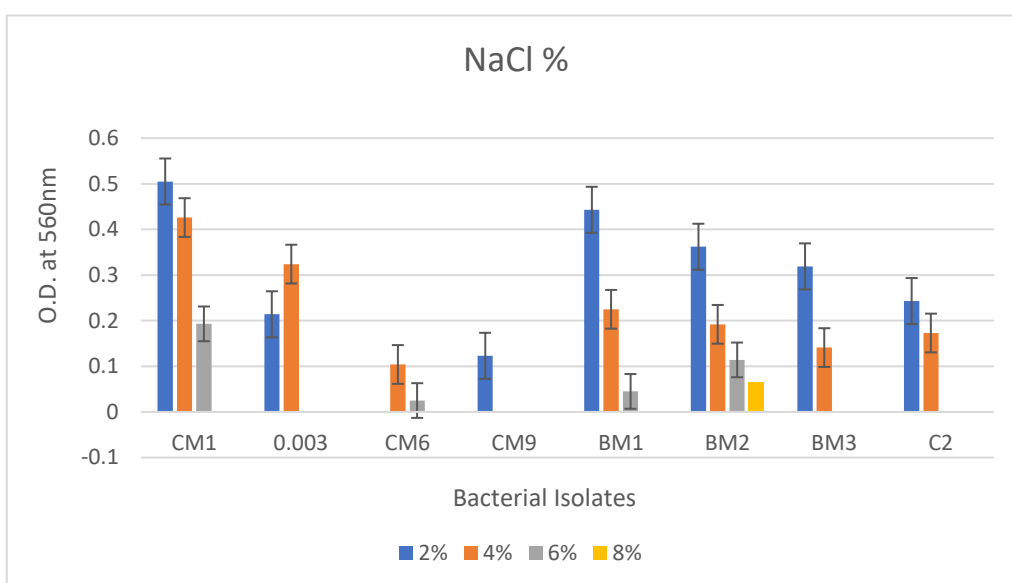
A series of tests were conducted to determine how varying amounts of NaCl affect bacterial growth. The findings of these studies are presented in Table 4.10. According to the data, 75% of the isolates showed significant growth at NaCl concentrations as high as 4%. However, when the NaCl concentration was increased to 6% and 8%, only 6 and 16 of the isolates, respectively, displayed growth, as shown in Figure 4.6 A and B. Notably, isolates OS1, PK6, and CM1 consistently showed growth at NaCl levels up to 6%. The values in the figure are based on the averages of three replicate experiments, and distinct letters indicate statistically significant differences ($p < 0.05$).

Table 4.10: Screening the isolates for their ability to sustain variable NaCl concentrations (O.D at 560nm)

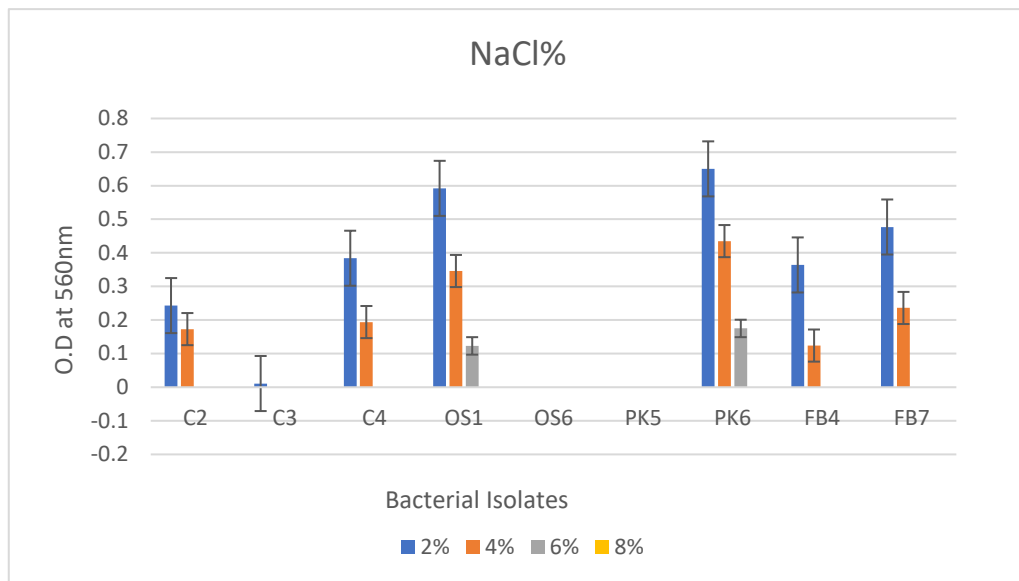
| Isolates | NaCl% | | | |
|------------|-------------|-------------|-------------|-------------|
| | 2 | 4 | 6 | 8 |
| CM1 | 0.505±0.002 | 0.426±0.002 | 0.193±0.00 | - |
| CM2 | 0.214±0.002 | 0.324±0.002 | - | - |
| CM6 | - | 0.106±0.002 | 0.025±0.002 | - |
| CM9 | 0.123±0.001 | - | - | - |
| BM1 | 0.443±0.003 | 0.225±0.003 | 0.045±0.002 | |
| BM2 | 0.362±0.004 | 0.192±0.002 | 0.114±0.001 | 0.064±0.001 |
| BM3 | 0.319±0.004 | 0.141±0.003 | - | - |
| C2 | 0.243±0.002 | 0.173±0.002 | - | - |
| C3 | 0.011±0.003 | - | - | - |
| C4 | 0.384±0.003 | 0.194±0.003 | - | - |
| OS1 | 0.592±0.003 | 0.346±0.002 | 0.123±0.002 | |

| | | | | |
|------------|-------------|-------------|-------------|---|
| OS6 | - | - | - | - |
| PK5 | - | - | - | - |
| PK6 | 0.65±0.004 | 0.435±0.002 | 0.175±0.003 | - |
| FB4 | 0.364±0.002 | 0.124±0.001 | - | - |
| FB7 | 0.477±0.002 | 0.236±0.003 | - | - |

*values in columns are mean ± Standard deviation



(A)



(B)

Figure 4.6 (A, B): The Screening of isolates at different NaCl concentrations after 24 hours of incubation.

In a previous study, lactic acid bacteria survived at different NaCl concentrations (2%, 4%, and 6.5%). The bacteria preferred growth in the presence of 2% and 4% NaCl compared to 6.5%, suggesting that increasing NaCl concentrations beyond 6.5% could inhibit the growth of lactic acid bacteria. However, some strains could still survive in 6.5% NaCl, making them potential candidates for probiotics. In a separate study by Menconi et al. (2014), it was reported that the isolated strains LAB18 and LAB48 were capable of growing in the presence of 3.5% and 6.5% NaCl for up to 4 hours, indicating their ability to tolerate high osmotic conditions.

It has been previously observed that bacterial cells cultured in high salt concentrations experience a loss of turgor pressure, affecting their enzyme activity, physiology, water activity, and metabolism. Testing for osmo-tolerance is essential because lactic acid bacteria are used as commercial strains and produce lactic acid in the broth. To counteract the excessive reduction in pH, alkali is added to the broth, converting the free acid to its salt form, which increases the osmotic pressure on the bacterial cells (Adnan and Tan, 2007).

4.7.4 Bile Tolerance Test

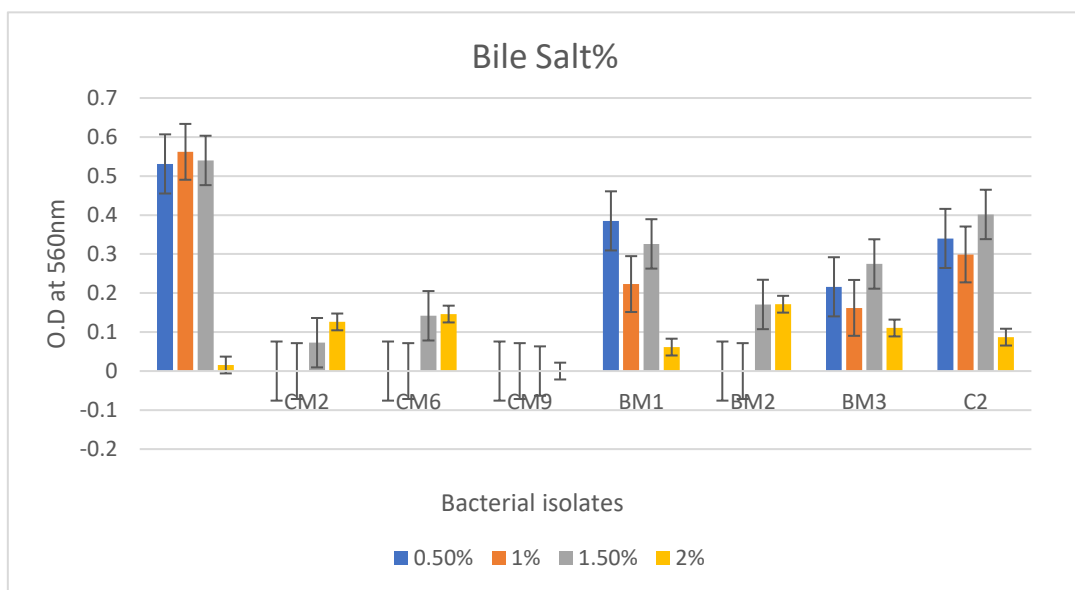
In an independent investigation, sixteen selected isolates were tested under varying concentrations of bile salts to evaluate their tolerance level. Bile salt tolerance is crucial for the survival of probiotic bacteria within the human gastrointestinal tract, as Bazireh et al. (2020) highlighted. The outcomes, shown in Table 4.11 and Figure 4.7 (A, B), revealed that out of 16 isolates, only eight isolates—BM3, BM1, CM1, OS6, OS1, C2, FB4, and PK5—exhibited distinct and significantly varied tolerance levels at bile salt concentrations up to 2%. Four isolates—BM2, CM6, CM2, and C4—experienced short-lived survival at lower bile salt concentrations ($\leq 1\%$) but could not persist for 24 hours at higher concentrations. Conversely, isolates C3, C4, FB7, CM9, and PK6 could not tolerate low bile salt concentrations (0.5%). In contrast, BM3, BM1, CM1, C2, PK5, OS6, FB4, and OS1 demonstrated flexibility across high and low bile salt concentrations, highlighting their potential as promising probiotics, as Xing et al. (2016) emphasised. The values in the figure are based on the averages of three replicate experiments, and distinct letters indicate statistically significant differences ($p < 0.05$).

Table 4.11: The Screening of the isolates for their ability to sustain variable bile concentrations (O.D at 560nm)

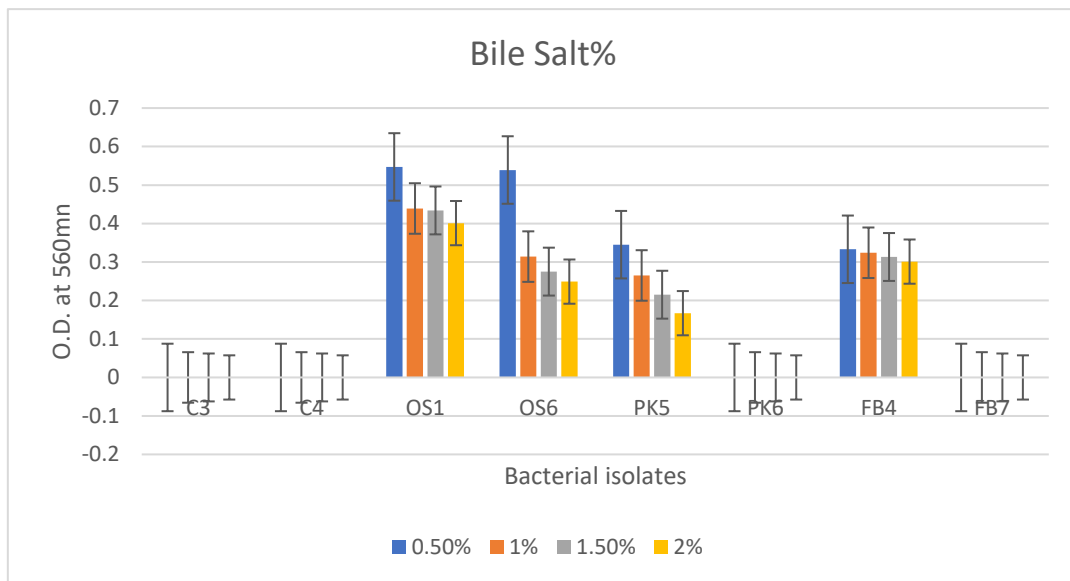
| Isolates | Bile Concentration | | | |
|------------|--------------------|-------------|-------------|-------------|
| | 0.50% | 1% | 1.50% | 2% |
| CM1 | 0.521±0.002 | 0.546±0.002 | 0.531±0.002 | 0.562±0.002 |
| CM2 | 0.291±0.002 | - | - | - |
| CM6 | 0.334±0.003 | 0.233±0.002 | - | - |
| CM9 | - | - | - | - |
| BM1 | 0.342±0.004 | 0.354±0.001 | 0.385±0.002 | 0.223±0.003 |
| BM2 | 0.321±0.001 | 0.362±0.002 | - | - |
| BM3 | 0.455±0.002 | 0.265±0.001 | 0.216±0.002 | 0.162±0.003 |
| C2 | 0.519±0.004 | 0.448±0.002 | 0.34±0.002 | 0.299±0.002 |

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| C3 | - | - | - | - |
| C4 | - | - | - | - |
| OS1 | 0.547±0.002 | 0.439±0.002 | 0.434±0.002 | 0.401±0.003 |
| OS6 | 0.539±0.001 | 0.314±0.002 | 0.275±0.002 | 0.249±0.003 |
| PK5 | 0.345±0.004 | 0.265±0.002 | 0.215±0.003 | 0.167±0.003 |
| PK6 | - | - | - | - |
| FB 4 | 0.333±0.004 | 0.324±0.002 | 0.313±0.004 | 0.301±0.003 |
| FB7 | - | - | - | - |

*values in columns are mean ± Standard deviation



(A)



(B)

Figure 4.7 (A, B): Screening of isolates at different bile concentrations after 24 hours of incubation

In previous studies, Ding and Shah (2007) found that *lactobacilli* spp. They have experienced a loss of viability when exposed to 3.0% bile, reducing viable cell count. Dhewa et al. (2009) also observed a similar pattern of cell viability loss in *Lactobacillus* strains from both human and food sources. Understanding the tolerance of lactic acid bacteria to bile salts is essential for assessing their potential as probiotics, as bile salts can act as antimicrobial molecules and influence intestinal microflora (Fontana et al., 2013). Therefore, it is necessary to evaluate the ability of lactic acid bacteria to tolerate bile salts when considering their use as probiotics (Lee and Salminen, 1995).

In their study, Menconi et al. (2014) examined the tolerance of LAB strains LAB18 and LAB48 to bile salts. The researchers grew these strains in varying concentrations of bile salts, specifically 0.4%, 0.5%, and 0.6%, for different incubation durations, namely 2, 4, and 24 hours. According to Bakari et al. (2011), the typical concentration of bile salts in the human small intestine varies between approximately 0.2% and 0.3%, with a maximum concentration of 2% (w/v), depending on factors such as the individual and the specific type and quantity of food ingested. Xanthopoulos et al. (2000) noted that the ability to tolerate bile salts varies among LAB species and even among different

strains within the same species. Similar observations were made in the current study, where each LAB species demonstrated varying abilities to withstand bile salts. The resistance to bile salts in these isolates has been attributed to bile salt hydrolase (BSH) enzymes, which hydrolyze conjugated bile and reduce its toxic effects (Toit et al., 1998). BSH activity has been predominantly reported in microorganisms derived from animal intestines or feces (Tanaka et al., 1999).

4.7.5 Antimicrobial Activity

In Figure 4.8, the results of the present study indicate that isolate CM1 displayed the most significant degree of antibacterial activity, as evidenced by zone of inhibition measurements. Specifically, the measurements were 12 mm for *S. aureus*, 11 mm for *E. coli*, 11 mm for *Bacillus cereus*, 10 mm for *S. typhimurium*, and 10 mm for *E. faecalis*. The observed diameters of the inhibition zones exhibited variability ranging from 4 to 14 mm across different isolates when tested against the indicator species, as shown in Table 4.12. In contrast, the isolates C2, C4, C3, PK5, and CM2 did not show antibacterial activity against the examined microorganisms. The inhibitory characteristics of LAB species are attributed to the generation of primary metabolites, such as lactic acid, ethanol, and carbon dioxide, as well as antimicrobial substances, including bacteriocins (Wasana et al., 2022). Based on the findings derived from evaluating tolerance to varied NaCl concentrations, bile salt concentrations, pH, and temperature, isolates OS1 and CM1 were chosen for subsequent genotypic identification due to their demonstrated ability to suppress all examined enteric pathogens.

Table 4.12: Antimicrobial activity of isolates (ZOI in mm)

| Isolates | <i>Staphylococcus Aureus</i> | <i>Escherichia Coli</i> | <i>Salmonella typhimurium</i> | <i>Enterococcus faecalis</i> | <i>Bacillus Cereus</i> |
|----------|------------------------------|-------------------------|-------------------------------|------------------------------|------------------------|
| CM1 | 12±0.5 | 11 ±1 | 10±0.1 | 10±0.2 | 11±0.2 |

| | | | | | |
|------------|-------|--------|--------|-------|--------|
| CM2 | - | - | - | - | - |
| CM6 | - | 8±0.4 | - | 9±0.3 | 14±0.4 |
| CM9 | 4±0.5 | 6±0.4 | - | - | 6±0.2 |
| BM1 | - | 5±0.5 | - | - | - |
| BM2 | 9±0.2 | 4±0.1 | - | - | - |
| BM3 | 7±0.5 | 8±0.5 | 9±0.1 | 8±0.4 | 10±0.3 |
| C2 | - | - | - | - | - |
| C3 | - | - | - | - | - |
| C4 | | | | | |
| OS1 | 9±0.5 | 10±0.5 | 10±0.1 | 8±0.4 | 9±0.3 |
| OS6 | 7±0.5 | 8±0.5 | 9±0.1 | 7±0.4 | 7±0.3 |
| PK5 | - | - | | - | - |
| PK6 | - | 9±0.4 | - | 8±0.3 | 12±0.4 |
| FB4 | - | - | - | 8±0.3 | 7±0.2 |
| FB7 | - | 7±0.4 | 13±0.4 | 9±0.3 | |

ZOI in mm, - = no ZOI. *values in columns are mean ± Standard deviation.

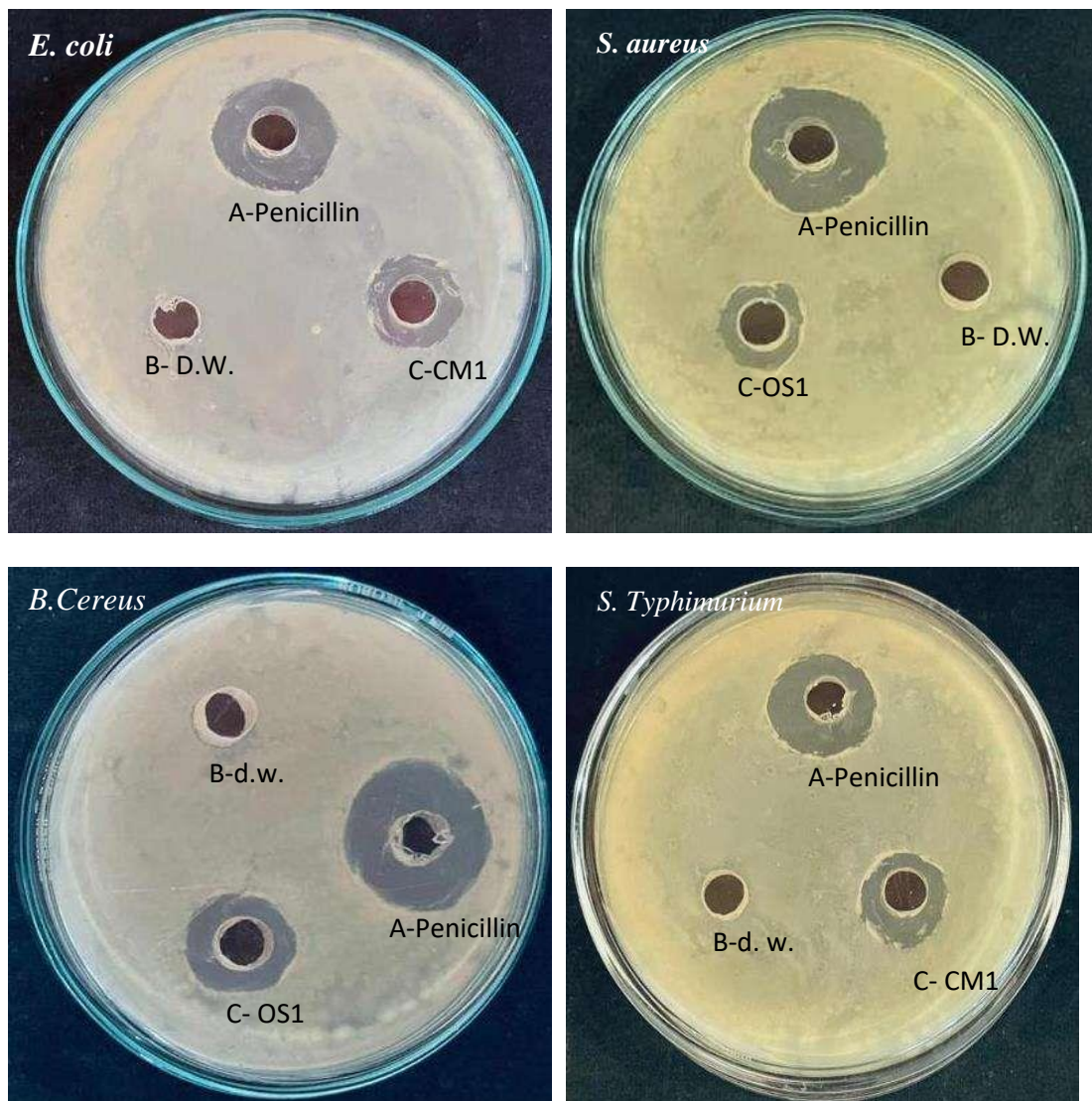


Figure 4.8: The antimicrobial activity of OS1 and CM1 against *S. typhimurium*, *B. cereus*, *S. aureus*, and *E. coli* was investigated. The experimental setup consisted of three groups: (A) the positive control group, which was treated with Penicillin; (B) the negative control group, which was treated with distilled water; and (C) the test organism group, which included CM1 or OS1.

The study conducted by Menconi et al. (2014) revealed that lactic acid bacteria can hinder the growth of *Salmonella Enteritidis*, *Escherichia coli* (O157), and *Campylobacter jejuni*. This inhibitory potential of lactic acid bacteria is attributed to synthesizing primary metabolites like lactic acid, ethanol, and carbon dioxide.

Moreover, lactic acid bacteria generate antimicrobial agents, such as bacteriocins, contributing to their suppressive effects (Rattanachaikunsopon et al., 2010).

In a distinct investigation, the antimicrobial efficacy of lactic acid was assessed against different bacterial strains, encompassing *Bacillus cereus*, *Bacillus megaterium*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The study also included three yeast strains: *Rhodotorula* sp., *Saccharomyces cerevisiae*, and *Candida albicans*. The antimicrobial potential was gauged through the disc diffusion and broth microdilution methodologies. At 32.1 mg/mL, inhibition zones spanned 24.0 mm (for *Escherichia coli*) to 38.3 mm (for *Enterococcus faecalis*) for the tested bacteria. For the yeast strains, the inhibition zones ranged from 11.3 mm (for *Saccharomyces cerevisiae*) to 14.0 mm (for *Rhodotorula* sp.). These outcomes indicate that lactic acid exhibits noteworthy antimicrobial activity across a broad spectrum of bacteria and yeasts, as evidenced by the discernible inhibition zones in the study (Stanojević-Nikolić et al., 2016).

4.7.6 Antibiotic Susceptibility test

After a 24-hour incubation period, the zone of inhibition was evaluated, and interpretations were made according to the 2015 CLSI standards. According to these rules, isolates were classified as resistant if their inhibition zone diameters were 14 mm or below, sensitive if they had diameters over 21 mm, and intermediate if they had diameters between 15 and 20 mm (Table 4.13 & 4.14). The isolates' differences in the profiles of antibiotic susceptibility were astounding. Previous studies suggested that LABs were susceptible to β -lactams and chloramphenicol. However, most isolates resisted ciprofloxacin, gentamycin, kanamycin, ampicillin, clindamycin, and Ciprofloxacin. It's vital to remember that LABs already have built-in resistance to gentamicin, vancomycin, kanamycin, and streptomycin. The lack of cytochrome-mediated electron transport in LABs, which is necessary for the uptake of these antibiotics, is the cause of this resistance. Notably, the vancomycin resistance mechanism in LABs has been the subject of the most significant research, and this is because peptidoglycan lacks a binding site. The existence of two isolates that showed

vancomycin sensitivity against the expected trend was an exciting finding. These results cast doubt on the idea that these isolates have only recently been exposed to antibiotics.

Table 4.13: Antibiotic Susceptibility of isolates

| Isolate | VM 30mcg | GM 10mcg | AC 10mcg | KM 30mcg | SM 10mcg | EM 5mcg | CM 5mcg | TC 10mcg | CP 10mcg |
|---------|-------------|-------------|-------------|-------------|-------------|------------|------------|-------------|-------------|
| CM1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6±0.2 |
| CM2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9±0.4 |
| CM6 | 6±0.3 | 0 | 0 | 0 | 7±0.2 | 0 | 0 | 12±0.2 | 6±0.2 |
| CM9 | 8±0.3 | 0 | 0 | 0 | 9±0.3 | 0 | 0 | 9±0.4 | 8±0.3 |
| BM1 | 9±0.3 | 11±0.3 | 0 | 8±0.3 | 0 | 22±0.3 | 0 | 17±0.2 | 12±0.4 |
| BM2 | 8±0.4 | 11±0.2 | 0 | 8±0.2 | 0 | 15±0.4 | 0 | 15±0.3 | 17±0.3 |
| BM3 | 7±0.4 | 9±0.2 | 0 | 9±0.3 | 13±0.2 | 16±0.2 | 0 | 23±0.2 | 21±0.4 |
| C2 | 9±0.3 | 8±0.3 | 0 | 10±0.3 | 0 | 21±0.3 | 0 | 15±0.2 | 16±0.4 |
| C3 | 7±0.2 | 12±0.3 | 0 | 13±0.2 | 0 | 23±0.3 | 0 | 18±0.3 | 23±0.2 |
| C4 | 8±0.3 | 10±0.2 | 0 | 10±0.2 | 9±0.3 | 26±0.3 | 0 | 15±0.2 | 25±0.3 |
| OS1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10±0.3 |
| OS6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8±0.4 |
| PK5 | 10±0.2 | 16±0.2 | 0 | 0 | 0 | 22±0.2 | 0 | 15±0.3 | 22±0.3 |
| PK6 | 11±0.2 | 18±0.3 | 0 | 0 | 0 | 19±0.2 | 0 | 17±0.2 | 25±0.2 |
| FB 4 | 23±0.3 | 18±0.3 | 0 | 16±0.4 | 6±0.3 | 11±0.4 | 0 | 24±0.2 | 18±0.2 |
| FB7 | 26±0.2 | 21±0.3 | 0 | 28±0.2 | 8±0.2 | 16±0.2 | 0 | 22±0.2 | 23±0.3 |

All data is expressed with mean ± standard deviation

VM- Vancomycin, GM- Gentamycin, AC- Ampicillin, KM-Kanamycin, SM- Streptomycin, EM- Erythromycin, CM- Clindamycin, TC- Tetracycline, CP- Chloramphenicol CF- Ciprofloxacin.

Table 4.14: Antibiotic Susceptibility of isolates (Resistant or sensitive)

| Isolates | VM | GM | AC | KM | SM | EM | CM | TC | CP | CF |
|----------|----|----|----|----|----|----|----|----|----|----|
| CM1 | R | R | R | R | R | R | R | S | R | R |
| CM2 | R | R | R | R | R | R | R | S | R | R |
| CM6 | R | R | R | R | R | R | R | S | R | R |
| CM9 | R | R | R | R | R | R | R | S | R | R |
| BM1 | R | R | R | R | R | S | R | I | R | R |
| BM2 | R | R | R | R | R | I | R | I | I | R |
| BM3 | R | R | R | R | R | I | R | S | S | R |
| C2 | R | R | R | R | R | S | R | I | I | R |
| C3 | R | R | R | R | R | S | R | S | S | R |
| C4 | R | R | R | R | R | S | R | I | S | R |
| OS1 | R | R | R | R | R | R | R | R | R | R |
| OS6 | R | R | R | R | R | R | R | R | R | R |
| PK5 | R | S | R | R | R | S | R | I | S | R |
| PK6 | R | S | R | R | R | S | R | I | S | R |
| FB 4 | S | S | R | S | R | S | R | S | S | R |
| FB7 | S | S | R | S | R | I | R | S | S | R |

R- Resistant, I- Intermediate, S- susceptible.

4.7.7 Cell Hydrophobicity of Isolates

In Table 4.15, the results of the experiment showed that isolate CM1 displayed the most significant degree of cell hydrophobicity, as indicated by optical density values of approximately 0.054, 0.059, 0.062, 0.122, and 0.132, corresponding to different concentrations of ammonium sulfate (0.01 M, 0.05 M, 0.125 M, 0.25 M, and 0.5 M, respectively). In addition to OS1, CM1 exhibited the maximum cell hydrophobicity, with values of about 0.046, 0.052, 0.057, 0.114, and 0.123 at the same concentrations of ammonium sulfate. The optical density of cell hydrophobicity varied from 0.009 to 0.154 for different isolates across the various concentrations of ammonium sulfate. In

contrast, isolates BM1, C2, PK5, and PK6 did not display any cell hydrophobicity in response to the different concentrations of ammonium sulfate (Wasana et al., 2022).

Table 4.15: Cell Hydrophobicity of isolates

| Isolates | 0.01 M | 0.05M | 0.125M | 0.25M | 0.5M |
|-----------------|---------------|--------------|---------------|--------------|-------------|
| CM1 | 0.054 | 0.059 | 0.062 | 0.122 | 0.132 |
| CM2 | - | 0.011 | 0.019 | 0.022 | 0.027 |
| CM6 | 0.021 | 0.026 | 0.039 | 0.044 | 0.048 |
| CM9 | 0.009 | 0.011 | 0.015 | 0.021 | 0.027 |
| BM1 | - | - | - | - | - |
| BM2 | 0.020 | 0.025 | 0.029 | 0.032 | 0.089 |
| BM3 | 0.023 | 0.028 | 0.033 | 0.039 | 0.041 |
| C2 | - | - | - | - | - |
| C3 | 0.028 | 0.032 | 0.037 | 0.042 | 0.045 |
| C4 | 0.033 | 0.039 | 0.043 | 0.048 | 0.052 |
| OS1 | 0.046 | 0.052 | 0.057 | 0.114 | 0.123 |
| OS6 | 0.011 | 0.014 | 0.019 | 0.022 | 0.029 |
| PK5 | - | - | - | - | - |
| PK6 | - | - | - | - | - |
| FB4 | 0.012 | 0.018 | 0.023 | 0.029 | 0.036 |
| FB7 | 0.015 | 0.027 | 0.034 | 0.039 | 0.043 |

4.7.8 β -galactosidase activity test

In this β -galactosidase activity test, all 16 isolates, namely CM6, CM9, CM1, CM2, BM3, BM2, BM1, C4, C3, C2, OS6, OS1, PK6, PK5, FB7, and FB4, showed β -galactosidase activity as they produced an intense yellow color after incubation (Table 4.16). The isolates FB4, FB7, and PK6 were light yellow, indicating low β -galactosidase activity. A similar study conducted by Casarotti et al. (2000) revealed that the ability to produce β -galactosidase activity was present in most LAB species. *Lactobacillus casei* SJRP146, *Lactobacillus* SJRP50, and *Lactobacillus delbrueckii* SJRP76 all showed a bright yellow color, indicating that they contained β -galactosidase activity. Although all other strains, except for *L. casei* SJRP145, showed positive results, some strains' yellow color intensity varied. The *L. rhamnosus* GG reference strain and the *L. casei* SJRP145 strain were negative for β -galactosidase activity, as evidenced by the absence of color change.

Table 4.16: Characterization of probiotic bacteria

| Isolates | β -galactosidase activity | Lactic acid Production |
|----------|---------------------------------|------------------------|
| CM1 | Positive | Positive |
| CM2 | Positive | Positive |
| CM6 | Positive | Negative |
| CM9 | Positive | Positive |
| BM1 | Positive | Positive |
| BM2 | Positive | Positive |
| BM3 | Negative | Negative |
| C2 | Positive | Positive |
| C3 | Positive | Positive |
| C4 | Positive | Positive |
| OS1 | Positive | Positive |

| | | |
|------------|----------|----------|
| OS6 | Positive | Positive |
| PK5 | Negative | Positive |
| PK6 | Positive | Positive |
| FB4 | Positive | Positive |
| FB7 | Positive | Positive |

4.7.9 Lactic acid production

In Table 4.16, it was observed that out of 16 isolates, 14 isolates CM2, CM9, CM6, CM1, BM2, BM3, BM1, C4, C3, C2, OS6, OS1, FB4, and PK5 produced lactic acid and only two isolates FB7 and PK6 were not able to produce lactic acid. These results are very similar to the study by Kylvä-Nikkilä et al., 2000. In this study, many strains from various genera, such as *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Oenococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Lactococcus*, *Tetragenococcus*, *Vagococcus*, *Weissella*, and *Pediococcus*. These factors collectively impact the outcome of LA production and the productivity of the process. The production yield and efficiency of lactic acid (LA) are influenced by several factors, including pH levels within the range of 3.5 to 9.6, temperature ranging from 5 to 45°C, and the presence of essential nutrients like amino acids, peptides, nucleotides, and vitamins.

4.8 Genotypic Characterization

To analyze the genetic features of the two chosen isolates, OS1 and CM1, which exhibit considerable potential as probiotics, total genomic DNA was isolated individually from both isolates. The amplification of the 16S rRNA gene, which acts as a distinguishing marker for bacteria, was the method utilized to extract genomic information to determine the identity of the isolates. The decision to employ 16S rRNA gene sequencing was deliberate, as it facilitates the classification of bacteria at the genus and species levels, as emphasized by Sadrani et al. (2014). The polymerase chain reaction (PCR) products of CM1 and OS1 exhibited the expected size, measuring 1,500 base pairs. These PCR products and a molecular weight marker were further analyzed using

gel electrophoresis. The research effectively showcased the precise targeting of the 16S rRNA gene. The gel's electrophoretic analysis provided additional confirmation of amplicons with a size of around 1,500 base pairs, as anticipated.

After completing the molecular identification procedure, a phylogenetic analysis was carried out on the complete genomes of both CM1 and OS1 isolates. This study identified the CM1 isolate as *Lactobacillus acidophilus* and the OS1 isolate as *Lactobacillus delbrueckii*, as illustrated in Figures 4.9 and 4.10. Subsequently, the sequence data for both isolates were submitted to the National Center for Biotechnology Information (NCBI) GenBank, generating distinct accession numbers. The primary function of these identifiers is to differentiate individual genomes, thereby aiding in future research efforts and serving as reference points. The isolation of the 16S rRNA gene and its subsequent amplification proved an effective method for determining the identities of the OS1 and CM1 isolates. Additionally, the genomes of *L. delbrueckii* OS1 and *L. acidophilus* CM1 were submitted to NCBI GenBank, each assigned a unique accession number—OP824643 for *L. delbrueckii* OS1 and OP811266 for *L. acidophilus* CM1.

1. CM1- *Lactobacillus acidophilus*

```
ATAGATCATGTCAGGACGAACGTTGGCGGCGTGCCTAATACAAGCAAGTC
GAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGA
GCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGAT
ACCACTTGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGAT
CACCTTATAAAAGGCGGCGTAATGTCGCTATGGGATGGCCCCGCGGTGCA
TTAGCTAGTTGGTAGGGTAACGGCCTACCTAGGCAATGACATAGCCGAGT
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GGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTG
GTGAGGATAGAGGTAGTAACTGGCCTTTATTTGACCGTAATCACAGAAAA
TCACGGCTAACAACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCT
GATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACAGTTTTT
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GAGTGCAGAAGAGGAGAGTGGA ACTCCATGTGTAGCGGTGGAATGCAGT
 ATGGAAGAACC ACTGGCGAAGGCGGCTCTCTGGTCTGCAACTCACGCTGA
 GGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATG
 CCGTAAACGATGAGTGCTAAGTGTGGCAGGTTTCCGCCTCTCAGTGCTGC
 AGCTTACGCATTAAGCACTCCGCCTGAGTACGACCGCAAGGTTGAAACTC
 AAAATTGACGGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTGAAGCAA
 CGCGAAGAACCTTACCAGGTCTTGACATCTAGTGCAATCCGTAGAGATAC
 GGAGTACCCTTCGGGGACACTAAGACAGGTGCTGCATGGCTGTCGTCAGC
 TCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGTC
 ATTACCAGCATTAAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACGG
 GAGGAAGGTGGGGATGACGTCAAGTCATCATCCCCCTTATGACCTGGGCT
 ACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGAAGGC
 AAGCGAATCTCTTAAAGCTCTTCTCAGTTCGGACCAGTCTGCAACTCGACT
 GCACGAAGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGT
 TCCCGGGCCTAGTACACACCGCCCGTCACACCATGGGAGTCTGCAACCAA
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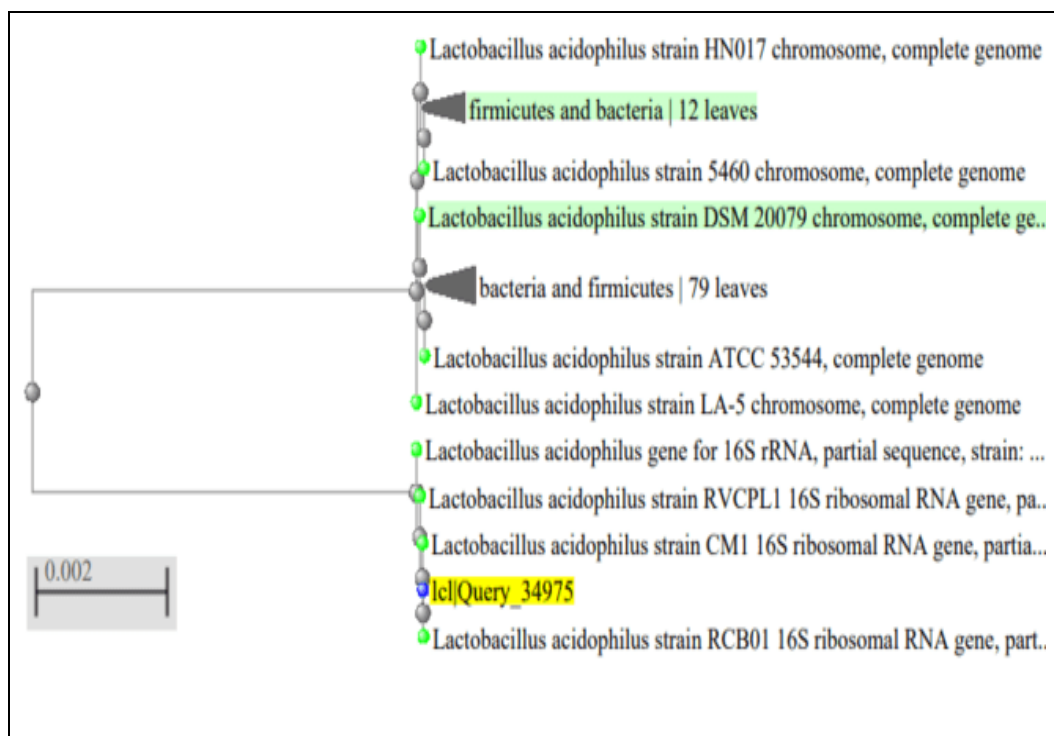
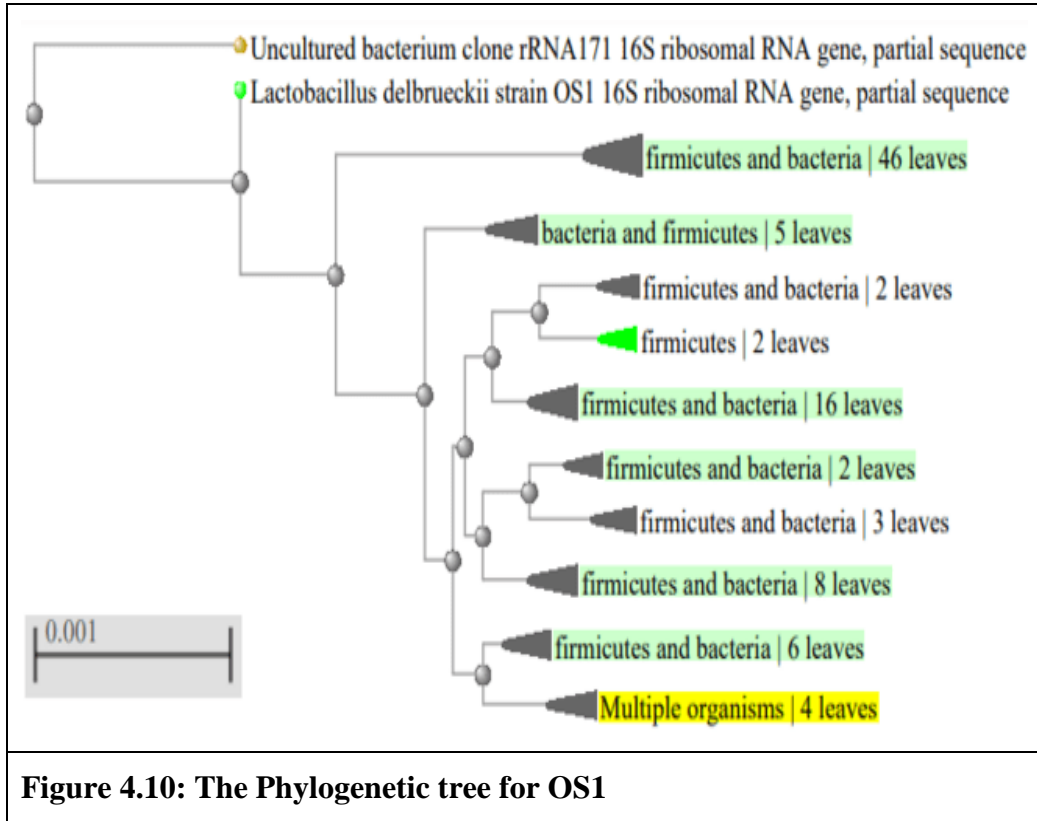


Figure 4.9: The Phylogenetic tree for CM1

2. OS1- *Lactobacillus delbrueckii*

TGGGACTGAGACACGGCCAAACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCACAATGGCAAGTCTGATGGAGCAACGCCGCGTGGAAGAAG
GTCTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGCA
GTA ACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGA ACTGCATCGGAAACTGTCATTCTT
GAGTGAAGAGGAGAGTGGAACTCCATGAGCGGTGGAATGCGTAGATA
TATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGA
CGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATAACCCTGG
TAGTCCATGCCGTA AACGATGAGCGCTAGGTGTTGGGGACTTTCCGGT
CCTCAGTGCCGCAGCAAACGCATTAAGCGCTCCGCCTGGGGAGTACG
ACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCCTGCGCTACACAGAGATAGGTGGTTCCCTTCGGGGACG
CAGAGACAGGTGGTGCATGGCTGTTCGTCAGCTCGTGTTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCATCAT
TAAGTTGGGCACTCTAAAGAGACTGCCGGTGACAAACCGGAGGAGTG
GGGATGACGTCAAGTCATCATGCCCTTATGACCTGGGCTACACACGT
GCTACAATGGGCAGTACAACGAGAAGCAAACCCGCGAGGGTAAGCG
GATCTCTTAAAGCTGCTCTCAGTTCGGACTGCACTGCAACTCGCCTGC
ACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTCTG
CAATGCCCAAAGTCGGTGAGATAACCTTTATAGGAGTCAGCCGCCTA
AGGCAGGGCAGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGG
AGAACCTGCGGCTGGATCACCTCCTTTCTAAGGAAAACGGATGGATG

GAGAGCAGAAATGCTAAAAGAAGTCCATCAGTTACGGAAGCACACTG
 CAAAAGAAACTTTGTTTCAGT



4.9 Statistical optimization of physical factors and media to achieve high yield of cells

4.9.1 Plackett Burman's design for optimizing physical factors and media to achieve a high yield of cells.

Before conducting Response Surface Methodology (RSM), Prioritization by Difference (PBD) was employed to identify the most significant factors due to its effectiveness in assessing the major effects of each variable. The selected factors for PBD analysis included pH, temperature, sodium chloride, bile salt, inoculum size, incubation period, ascorbic acid, ammonium citrate, magnesium sulfate, manganese sulfate, and calcium carbonate. Two distinct levels were set for each variable: -1 and 1. The experiments, totalling 12 runs, were carried out to account for various combinations of variables. The biomass production ranged from 0.543 to 1.14 g/100

ml (Table 4.17). The standardised impacts of the variables are represented as a single column on the Pareto diagram.

Table 4.18 displays a statistical analysis of Cell growth (g/100ml) for the P.B. design as an ANOVA table. Furthermore, Figure 4.23 shows a Pareto chart illustrating the impact of variables on cell growth.

| Runs | Ph | Temperature (Celsius) | Bile salt Chloride (%) | Sodium Chloride (%) | Inoculum Size (%) | Incubation Period (hours) | Magnesium Sulphate (%) | Manganese Sulphate (%) | Ascorbic Acid (%) | Ammonium Citrate (%) | Calcium Carbonate (%) | Cell growth Predicted Values | Cell growth Observed Values |
|------|----|-----------------------|------------------------|---------------------|-------------------|---------------------------|------------------------|------------------------|-------------------|----------------------|-----------------------|------------------------------|-----------------------------|
| 1 | 1 | 25 | 2 | 2 | 3 | 96 | 0.1 | 2 | 2 | 0.5 | 0.1 | 0.706 | 0.721 |
| 2 | 4 | 25 | 2 | 8 | 3 | 24 | 0.1 | 0.1 | 2 | 0.1 | 2 | 0.519 | 0.543 |
| 3 | 4 | 45 | 0.5 | 2 | 0.5 | 96 | 0.1 | 2 | 2 | 0.1 | 2 | 1.009 | 0.995 |
| 4 | 1 | 45 | 2 | 2 | 3 | 96 | 2 | 0.1 | 0.1 | 0.1 | 2 | 0.848 | 0.872 |
| 5 | 4 | 25 | 0.5 | 2 | 3 | 24 | 2 | 2 | 0.1 | 0.5 | 2 | 1.030 | 1.021 |
| 6 | 1 | 25 | 0.5 | 2 | 0.5 | 24 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.940 | 0.912 |
| 7 | 1 | 25 | 0.5 | 8 | 0.5 | 96 | 2 | 0.1 | 2 | 0.5 | 2 | 0.330 | 0.321 |
| 8 | 1 | 45 | 2 | 8 | 0.5 | 24 | 0.1 | 2 | 0.1 | 0.5 | 2 | 0.643 | 0.672 |
| 9 | 4 | 45 | 2 | 2 | 0.5 | 24 | 2 | 0.1 | 2 | 0.5 | 0.1 | 1.176 | 1.191 |
| 10 | 1 | 45 | 0.5 | 8 | 3 | 24 | 2 | 2 | 2 | 0.1 | 0.1 | 0.574 | 0.546 |
| 11 | 4 | 45 | 0.5 | 8 | 3 | 96 | 0.1 | 0.1 | 0.1 | 0.5 | 0.1 | 0.495 | 0.472 |
| 12 | 4 | 25 | 2 | 8 | 0.5 | 96 | 2 | 2 | 0.1 | 0.1 | 0.1 | 0.488 | 0.498 |

Table 4.18: ANOVA and Regression analysis of PB design on nine factors for microbial yield

| Source | Sum of Squares | Df | Mean Square | F-value | p-value | |
|--|----------------|----|-------------|---------|---------|-------------|
| Model | 0.7800 | 9 | 0.0867 | 257.42 | 0.0039 | Significant |
| A-PH | 0.0381 | 1 | 0.0381 | 113.11 | 0.0087 | |
| B-Temp | 0.0447 | 1 | 0.0447 | 132.63 | 0.0075 | |
| C-Bile Salt | 0.0044 | 1 | 0.0044 | 13.09 | 0.0686 | |
| D-NaCl | 0.5896 | 1 | 0.5896 | 1751.39 | 0.0006 | |
| E-Inoculum Size | 0.0143 | 1 | 0.0143 | 42.42 | 0.0228 | |
| F-Incubation Period | 0.0843 | 1 | 0.0843 | 250.50 | 0.0040 | |
| G-MgSO ₄ .7H ₂ O | 0.0015 | 1 | 0.0015 | 4.44 | 0.1695 | |
| H-MnSO ₄ .4H ₂ O | 0.0017 | 1 | 0.0017 | 4.99 | 0.1551 | |
| J-Ascorbic acid | 0.0014 | 1 | 0.0014 | 4.18 | 0.1775 | |
| Residual | 0.0007 | 2 | 0.0003 | | | |
| Cor Total | 0.7807 | 11 | | | | |

df = degree of freedom, SS = sum of squares, MSE = Mean square error

R² = 99.68%; R²_{adjusted} = 98.24%; R²_{predicted} = 88.48%

The calculated Model F-value of 257.42 suggests that the model has statistical significance. The probability of observing an F-value of this magnitude solely owing to random variation is estimated to be only 0.39%.

P-values below the threshold of 0.0500 imply that the model terms exhibit statistical significance. Model terms A, B, D, E, and F are significant in the present scenario. Values exceeding 0.1000 imply that the model terms lack statistical significance. If a

substantial number of model terms are deemed insignificant, excluding those necessary to maintain a hierarchy, the model reduction process may enhance the model.

B- temperature (p-value = 0.0087),

D- pH(p-value = 0.00369),

D- NaCl (p-value = 0.0006), and

H- Incubation Period (p-value = 0.004).

Pareto Chart

Cell Growth

- A: PH
- B: Temp
- C: Bile Salt
- D: Nacl
- E: Inoculum Size
- F: Incubation Period
- G: MgSO4.7h20
- H: MnSO4.4H2O
- J: Ascorbic acid
- K: Ammonium Citrate
- L: CaCO3

- Positive Effects
- Negative Effects

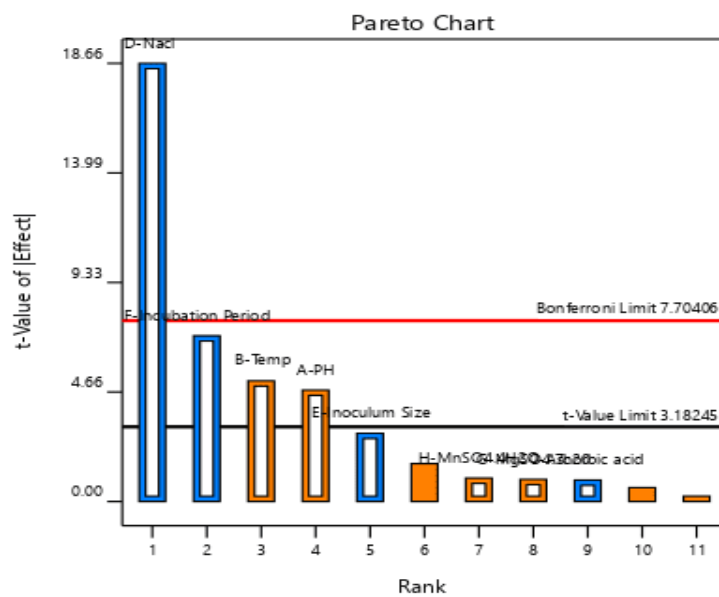


Figure 4.11: Pareto chart of standardized effect of growth parameters for the growth produced by *Lactobacillus acidophilus* CM1. Four factors (NaCl, pH, temperature, and Inoculum size) out of eleven show significant effects as they crossed above the t-value limit.

A Pareto chart is a visual tool that illustrates the relative weights of various elements in a Plackett-Burman (PB) design. Based on their impact on the response variable, the variables are ranked in a bar chart from the most to the least important. The Pareto chart in Figure 4.11, which included nine variables for determining maximum cell growth,

revealed that four—NaCl, pH, temperature, and inoculum size—were above the t-value threshold of 4.30. These variables were found to have a significant impact on cell growth. The height of each bar in the chart indicates the magnitude of each factor's influence, arranged in decreasing order of magnitude. The Pareto chart can be used to prioritize which aspects should be further studied or improved and identify the most important variables influencing the response variable. Clear and succinct communication of the PB design's findings can also be beneficial.

Predicted vs. Actual values

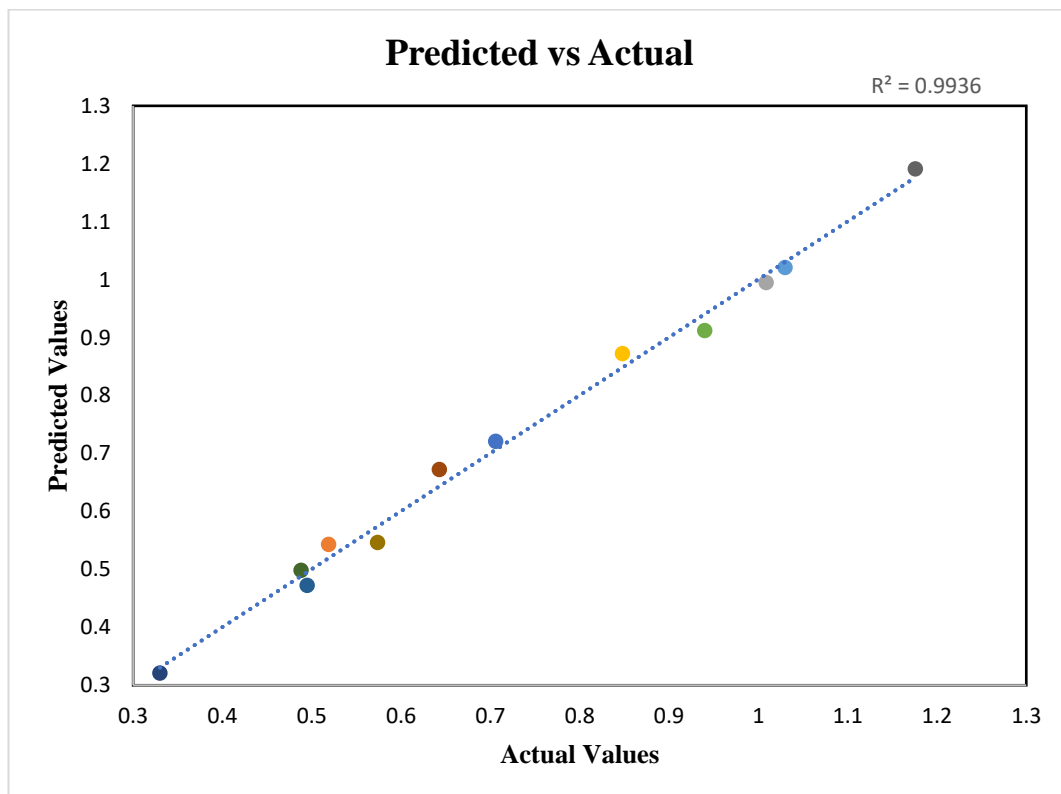


Figure 4.12: The predicted vs. actual bacterial cell yields of Plackett-Burman design for 12 runs of eleven factors

The projected vs. actual plot can be used to evaluate the validity and accuracy of the PB design model (Fig 4.12). The PB design model is used to calculate the anticipated values, while the experimental data is used to determine the actual values. If the PB design model is valid, the points on the plot should follow a line with a slope of 1.0. Figure 4.12 shows that the data from 12 runs for 11 variables fall along a straight line,

indicating that the data is reliable and outliers-free. However, suppose there is any departure from this straight line. In that case, the PB design model may not be valid, or additional factors may impact the response variable that were not considered. The anticipated vs. actual plot can also assist in locating any outliers or other data patterns that may compromise the reliability of the PB design model. Data points known as outliers considerably deviate from the overall trend of the data and may indicate errors or unique conditions during the experiment. By comparing the projected vs. actual plot, researchers can verify whether the PB design model is accurate and dependable and determine whether any changes or adjustments are needed.

BOX-COX Transformation

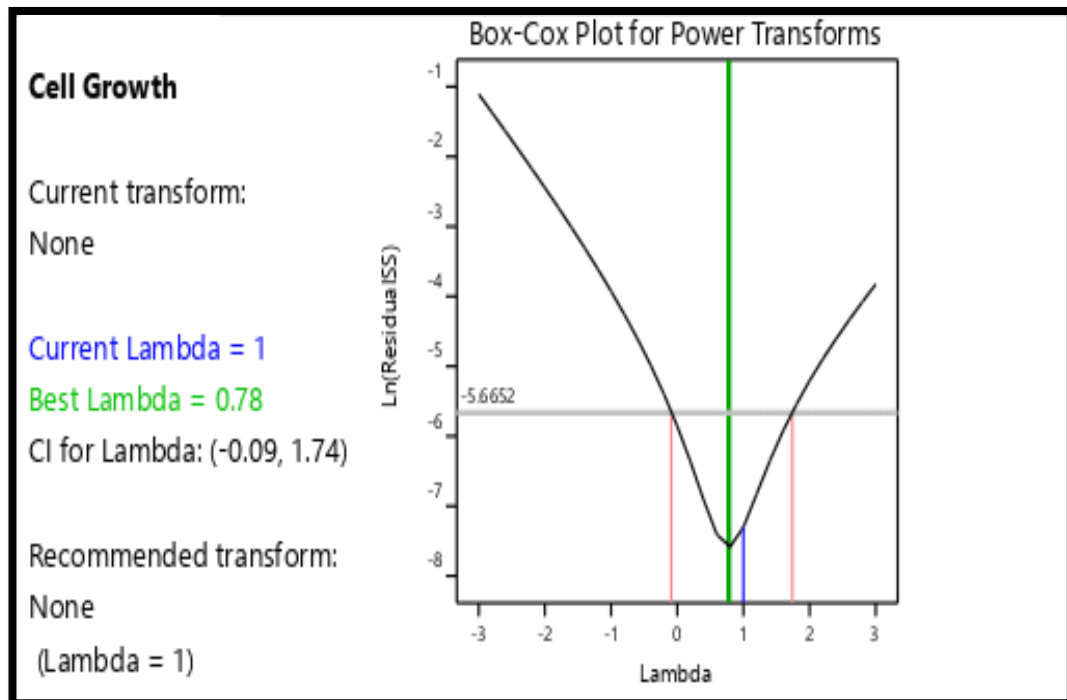


Fig 4.13: Box-Cox plot of PB design for eleven factors. The Box-cox plot here represents that the data is normalized and does not require any transformation.

The Box-Cox transformation (Fig 4.13) is a statistical technique commonly used to modify data to meet the assumptions of normality and equal variance, which are necessary for many statistical methods. It involves applying a power transformation to the data, which helps reduce the influence of outliers and other sources of variability.

When combined with perturbation in a Plackett-Burman (PB) design, both the Box-Cox transformation and perturbation can enhance the reliability and robustness of the results.

Initially, a PB design is conducted, and if the researcher finds it necessary, they can perturb the system by altering the levels of the factors, as explained earlier. However, if the response variable is not normally distributed or does not exhibit equal variance, the non-normality or heteroscedasticity may affect the results of the PB design. The researcher can employ a Box-Cox transformation on the response variable to address this issue. This transformation helps to normalize the data and mitigate the impact of outliers.

Nonetheless, in Figure 4.13, the Box-Cox plot indicates no need for transformation since the data or values obtained from the PB design involving eleven factors are already normalized (Kepli et al., 2019).

Regression Formula for the model's cell growth g/100ml

$$0.935019 + (0.037556 \times \mathbf{pH}) + (0.006100 \times \mathbf{Temperature}) + (0.025556 \times \mathbf{Bile\ Salt}) - (0.073889 \times \mathbf{NaCl}) - (0.027600 \times \mathbf{Inoculum\ size}) - (0.002329 \times \mathbf{Incubation\ period}) + (0.011754 \times \mathbf{MgSO_4 \cdot 7H_2O}) + (0.012456 \times \mathbf{MnSO_4 \cdot 4H_2O}) - (0.011404 \times \mathbf{Ascorbic\ acid}).$$

The primary goal of the experimental screening design was to investigate the variables that significantly impact the growth of microbial cells. Plackett-Burman studies were conducted to gain an initial understanding of which production factors might be influential. These studies aimed to identify important factors before conducting a more specific screening process. Experimental variables were selected based on their close association with production in previous shake flask cultures. The Pareto chart was used to visually represent the standardized effect of each variable on production, providing a clear overview.

In conclusion, the concentrations of NaCl, pH, temperature, and incubation period were found to affect cell growth. The study revealed that temperature and pH played a significant role in increasing cell growth.

The Plackett-Burman design has been effectively utilized in numerous studies to optimize the parameters for cell growth. However, this study differs from previous research as it selects factors before PBD due to variations among different strains. The influence of physical factors, such as temperature and pH, on cell growth has already been examined, and Li et al. (2002) reported the significance of medium composition on cell growth. The factors identified through the Plackett-Burman design were optimized using Response Surface Methodology (RSM) with a Box-Behnken experimental plan. The obtained results were analyzed using Design-Expert software and subjected to appropriate analysis of variance (ANOVA) based on the experimental design (Table 4.17). The highest cell growth obtained was 1.191 g/100 ml. The close agreement between the experimental and predicted data demonstrates the model's validity. The model's quality was further evaluated using various criteria. The regression equation derived from quadratic regression analysis on the experimental data describes the relationship between cell growth and the variables representing the optimization of various parameters.

The significance of the medium's composition in bacterial growth was also demonstrated in previous research. Many studies have successfully employed Plackett-Burman Design (PBD) to optimize cell mass production. However, this study differs from previous ones in selecting one factor at a time before applying PBD due to variations in factors across different strains. Moreover, recent efforts have been made to reduce the cost of the medium. In this study, specific nitrogen sources, namely peptone and yeast extract, were utilized in the MRS medium to decrease expenses. Earlier research by Li and colleagues in 2002 indicated that inoculum size and temperature are two significant factors that positively influence the growth of the cells.

The R^2 value, which represents the coefficient of determination for predicted values, is 0.9689, indicating a solid agreement. This aligns closely with the adjusted R^2 value of 0.9953, validating both the experimental and predicted levels of bacteriocin production. The fact that the R^2 value is close to 1.0 suggests that the model is reliable and makes accurate predictions about the response. The difference between the predicted R^2 of 0.9689 and the adjusted R^2 of 0.9953 is less than 0.2, indicating that the two values are comparable. The signal-to-noise ratio determines the level of precision. It is preferable

to have a ratio greater than 4. Your ratio of 52.777 suggests that the signal is strong enough. Using this paradigm, one can navigate more quickly through the design space. By conducting this statistical analysis, we could also assess the contribution of experimental factors (signals) compared to noise. The signal needs to be significantly more significant than the noise, as Kumar et al. (2010) emphasized.

The model's F-value was 257.42, with a p-value of 0.0039, indicating the significance of the model terms. Specifically, the factors A, B, AC, BC, B², and C² were significant for cell growth, as Gobikrishnan et al. (2013) stated. The ANOVA corresponding to these findings can be found in Table 4.18.

4.9.1 Response Surface methodology

A Central Composite Design (CCD) model optimized the medium components using Response Surface Methodology (RSM). X1, ranging from 9.29 g/L to 32.6 g/L; X2, ranging from 7.34 g/L to 54.7 g/L; and X3, ranging from 5.76 g/L to 62.8 g/L, were three independent variables whose optimal concentrations had to be determined. According to Table 4, these concentrations were shown as coded units and actual values.

The design matrix included 20 runs with different combinations of the independent variables, and Table 4.19 contains the associated experimental results. The studies revealed biomass production that ranged from 1.094 to 1.948 g/L.

Unexpectedly, run 19, with a temperature of 35°C, pH of 2.5, and an incubation period of 30 hours, produced the maximum amount of biomass. Under these optimal settings, the most advantageous outcome for biomass production during the experimentation process was achieved.

Final Equation in Terms of Coded Factors

$$Y = +1.36 - 0.0615 A - 0.0983 B + 0.0038 C + 0.1167 D + 0.0088 AB - 0.022 AC - 0.0181 AD - 0.0152 BC - 0.0034 BD + 0.0134 CD$$

The coded factors equation can be used to predict the response for given levels of each factor. By default, the high levels of the factors are coded as +1, and the low levels are coded as -1. Comparing the faded equation helps identify the relative impact of the factors by comparing the factor coefficients.

The relevance of the quadratic regression model, which includes linear, squared, and interaction variables, is demonstrated by the analysis of variance shown in Table 4.18 of this article. The model's vital significance is indicated by the derived F-value of 33.22, which has a meager probability value($p > F$) of 0.0001. The F-value is the ratio of the mean square regression to the mean square residual. To evaluate the model's validity, the critical value of F (8, 10, 0.05) from the F distribution table was 3.07. The null hypothesis can be rejected since the estimated F-value significantly exceeds the critical value of F, indicating that the model is highly significant in explaining the relationship between the variables. The model's goodness of fit is gauged by the coefficient of determination (R^2), which was found to be 0.9676. This indicates that the model can account for about 96.76% of the response's overall variability, leaving about 3.24% unaccounted for. R^2 values range from 0 to 1, and larger values—those closer to 1—indicate that the model is more accurate in its predictions. In this instance, the model can be deemed appropriate because it can predict the response and has an $R^2 > 0.75$.

Table 4.19: Response surface methodology for media optimization for *Lactobacillus* yield

| Std | Runs | Ph | Temperature | Nacl% | Incubation Period (Hours) | Expected Values | Observed Values |
|-----|------|-----|-------------|-------|---------------------------|-----------------|-----------------|
| 22 | 1 | 2.5 | 35 | 5 | 60 | 1.364 | 1.342 |
| 12 | 2 | 4 | 45 | 2 | 96 | 1.321 | 1.296 |
| 15 | 3 | 1 | 45 | 4 | 96 | 1.467 | 1.426 |
| 27 | 4 | 2.5 | 35 | 3 | 60 | 1.357 | 1.302 |

| | | | | | | | |
|-----|----|-----|----|---|----|-------|-------|
| 1 | 5 | 1 | 25 | 2 | 24 | 1.359 | 1.321 |
| 25 | 6 | 2.5 | 35 | 3 | 60 | 1.357 | 1.294 |
| 4 | 7 | 4 | 45 | 2 | 24 | 1.058 | 1.215 |
| 7 | 8 | 1 | 45 | 4 | 24 | 1.177 | 1.232 |
| 3 | 9 | 1 | 45 | 2 | 24 | 1.182 | 1.201 |
| 29 | 10 | 2.5 | 35 | 3 | 60 | 1.357 | 1.321 |
| 8 | 11 | 4 | 45 | 4 | 24 | 1.063 | 1.094 |
| 18 | 12 | 5.5 | 35 | 3 | 60 | 1.234 | 1.198 |
| 14 | 13 | 4 | 25 | 4 | 96 | 1.497 | 1.392 |
| 520 | 14 | 2.5 | 55 | 3 | 60 | 1.160 | 1.204 |
| 28 | 15 | 2.5 | 35 | 3 | 60 | 1.357 | 1.324 |
| 23 | 16 | 2.5 | 35 | 3 | 24 | 1.240 | 1.256 |
| 16 | 17 | 4 | 45 | 4 | 96 | 1.281 | 1.342 |
| 10 | 18 | 4 | 25 | 2 | 96 | 1.477 | 1.445 |
| 21 | 19 | 2.5 | 35 | 1 | 60 | 1.349 | 1.314 |
| 11 | 20 | 1 | 45 | 2 | 96 | 1.418 | 1.486 |
| 9 | 21 | 1 | 25 | 2 | 96 | 1.609 | 1.492 |
| 17 | 22 | 0.5 | 35 | 3 | 60 | 1.480 | 1.467 |
| 6 | 23 | 4 | 25 | 4 | 24 | 1.266 | 1.221 |
| 5 | 24 | 1 | 25 | 4 | 24 | 1.415 | 1.354 |
| 30 | 25 | 2.5 | 35 | 3 | 60 | 1.357 | 1.348 |
| 2 | 26 | 4 | 25 | 2 | 24 | 1.299 | 1.254 |
| 13 | 27 | 1 | 25 | 4 | 96 | 1.718 | 1.684 |
| 26 | 28 | 2.5 | 35 | 3 | 60 | 1.357 | 1.741 |

| | | | | | | | |
|----|----|-----|----|---|----|-------|-------|
| 19 | 29 | 2.5 | 35 | 3 | 60 | 1.553 | 1.948 |
| 24 | 30 | 2.5 | 35 | 3 | 32 | 1.266 | 0.994 |

Fit Statistics

| | | | |
|------------------|--------|--------------------------------|--------|
| Std. Dev. | 0.1533 | R² | 0.5661 |
| Mean | 1.35 | Adjusted R² | 0.3377 |
| C.V. % | 11.35 | Predicted R² | 0.1897 |
| | | Adeq Precision | 7.0546 |

The difference between the predicted R² of 0.1897 and the adjusted R² of 0.3377 is less than 0.2, indicating reasonable agreement between the two values.

Adeq Precision measures the signal-to-noise ratio. A ratio bigger than 4 is preferable. Your ratio of 7.055 demonstrates that the signal is sufficient. Using this paradigm, one may move more quickly through the design space.

ANOVA for 2FI model

Response 1: R1

| Source | Sum of Squares | Df | Mean Square | F-value | p-value | |
|---------------------|----------------|----|-------------|---------|---------|-------------|
| Model | 0.5824 | 10 | 0.0582 | 2.48 | 0.0425 | Significant |
| A-pH | 0.0907 | 1 | 0.0907 | 3.86 | 0.0643 | |
| B-Temperature | 0.2319 | 1 | 0.2319 | 9.87 | 0.0054 | |
| C-NaCl | 0.0003 | 1 | 0.0003 | 0.0147 | 0.9048 | |
| D-Incubation Period | 0.2384 | 1 | 0.2384 | 10.14 | 0.0049 | |
| AB | 0.0012 | 1 | 0.0012 | 0.0529 | 0.8206 | |
| AC | 0.0080 | 1 | 0.0080 | 0.3390 | 0.5672 | |

| | | | | | | |
|------------------|--------|----|--------|--------|--------|--------------------|
| AD | 0.0052 | 1 | 0.0052 | 0.2222 | 0.6428 | |
| BC | 0.0037 | 1 | 0.0037 | 0.1571 | 0.6963 | |
| BD | 0.0002 | 1 | 0.0002 | 0.0080 | 0.9295 | |
| CD | 0.0029 | 1 | 0.0029 | 0.1230 | 0.7297 | |
| Residual | 0.4464 | 19 | 0.0235 | | | |
| Lack of Fit | 0.2954 | 14 | 0.0211 | 0.6985 | 0.7269 | not significant |
| Pure Error | 0.1510 | 5 | 0.0302 | | | |
| Cor Total | 1.03 | 29 | | | | |

Factor coding involves utilizing coding techniques to represent categorical variables numerically. The term "sum of squares" refers to the mathematical calculation that involves summing the squares of a set of numbers. The third type of rewriting is designated as Type III - Partial. This approach transforms the user's text into an academic style without introducing additional information, aiming to enhance clarity. The calculated Model F-value of 2.48 indicates that the model possesses statistical significance. The probability of observing an F-value of this magnitude purely due to random variation is approximately 4.25%.

P-values below the threshold of 0.0500 suggest that the model terms exhibit statistical significance. In this case, model terms B and D are significantly influential. Conversely, values exceeding 0.1000 indicate that the model terms lack statistical significance. Implementing model reduction strategies could enhance the model's overall quality if a substantial portion of the associated terms are deemed superfluous, except for those necessary to maintain the hierarchy.

The **F-value** of 0.70 for the Lack of Fit indicates that it is not statistically significant compared to the pure error. The probability of observing a Lack of Fit F-value of this magnitude solely owing to random variation is estimated to be 72.69%. A non-significant lack of fit is desirable as it indicates that the model is fitting well.

4.10 Analyze the millet varieties for various physicochemical properties

4.10.1 Physical analysis of millet grains

4.10.1.1 Seed Weight

Based on the information provided, the data in Table 4.20 and Fig. 4.14 indicate no significant differences in seed weight among the three millet varieties: finger millet, foxtail millet, and barnyard millet. The seed weight for these varieties ranged between 7.6 ± 0.03 and 10.4 ± 0.03 grams per thousand seeds. Similar findings were reported for six pearl millet genotypes cultivated in the semi-arid Kitui County of Kenya. Among these genotypes, the lowest seed weight for 1000 grains was recorded for genotype Kimberee (6.1 g), while the highest seed weight (15 g) was observed for Pvs-Pm 1005 (Benard et al., 2016). The study also noted that each genotype exhibited variations in 1000-grain weight across different seasons, an important aspect to consider when developing various products.

Furthermore, the study concluded that seed weight varied with each season and that medium quality was consistent for all millet varieties in terms of seed weight. The 1000-seed weight values obtained in the present investigation were higher than the reported value of 9.40 g (Varsha, 2003). According to Sehgal and Kawatra (2002), the 1000-kernel weight of various pearl millet ranged from 6.75 to 8.76 grams, lower than the values obtained in the present study. This indicates that the seed weight of the millet varieties in the current study was higher than the findings of Sehgal and Kawatra. Similarly, Cheik et al. (2006) reported a more comprehensive range for seed weight in four pearl millet cultivars, ranging from 5.53 to 13.13 grams. This suggests that the seed weight variation observed in the present study is within the range reported by Cheik et al. for foxtail millet cultivars.

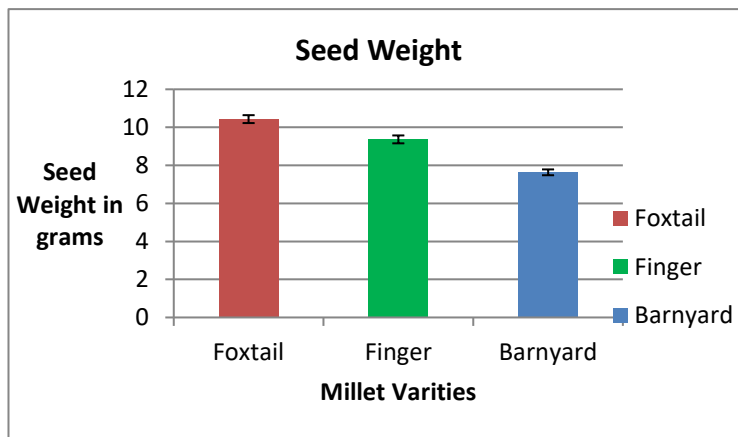


Figure 4.14: Seed weight analysis

4.10.1.2 Seed Density

Upon examining the data from Table 4.20 and Fig. 4.15, it can be observed that foxtail millet had a higher seed density (0.748 ± 0.02 g/ml), equivalent to 748 kg/m^3 , compared to finger millet (0.671 ± 0.03 g/ml), equivalent to 671 kg/m^3 , and barnyard millet (0.487 ± 0.02 g/ml), equivalent to 487 kg/m^3 . Among the three millet varieties, barnyard millet had the lowest seed density. Similar findings were reported for foxtail millet cultivated at the Lake Chad Research Institute in Maiduguri, Nigeria, where seed density varied among different varieties based on moisture content. For example, the variety Ex-Borno exhibited different seed densities at various moisture percentages (811.4, 684.8, 679.2, 646.4 kg/m^3), while SOSAT C88 had varying seed densities at different moisture percentages (817.64, 728.8, 726.0, 725.6 kg/m^3) (Ojediran et al., 2010).

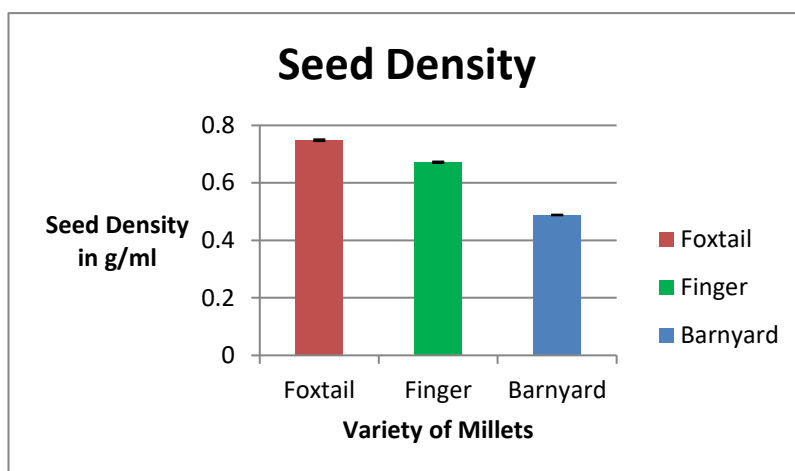


Figure 4.15: Seed Density analysis

Table 4.20: Physical analysis of millet grains

| Characteristics | Foxtail Millet | Finger Millet | Barnyard Millet |
|-----------------------------|----------------|---------------|-----------------|
| | (Mean ± SE) | (Mean ± SE) | (Mean ± SE) |
| Seed weight(g) | 10.4±0.03 | 9.3±0.02 | 7.6±0.03 |
| Seed density(g/ml) | 0.748±0.02 | 0.671±0.03 | 0.487±0.02 |
| Seed volume(g/ml) | 0.034±0.02 | 0.031±0.01 | 0.028±0.01 |
| Bulk density(g/ml) | 0.771±0.15 | 0.695±0.10 | 0.422±0.11 |
| Hydration capacity (g/seed) | 0.002±0.02 | 0.004±0.01 | 0.003±0.02 |
| Hydration index | 0.004±0.02 | 0.005±0.02 | 0.003±0.01 |
| Swelling capacity (ml/seed) | 0.005±0.04 | 0.007±0.03 | 0.004±0.02 |
| Swelling index | 5.56±0.03 | 6.32±0.01 | 4.32±0.02 |
| Germination percent (%) | 85 ±0.4 | 73.6±0.03 | 63±0.03 |

4.10.1.3 Seed Volume

The data from Table 4.20 and Fig. 4.16 revealed marginal differences in seed volume between the local and hybrid millet varieties. However, these differences were not statistically significant, indicating no notable variations in seed volume among the three varieties. Foxtail millet had a recorded weight of 0.034 ± 0.02 g/ml, finger millet 0.031 ± 0.01 g/ml, and barnyard millet 0.028 ± 0.01 g/ml. The non-significant differences in seed volume suggest that all three varieties have similar seed sizes in terms of volume.

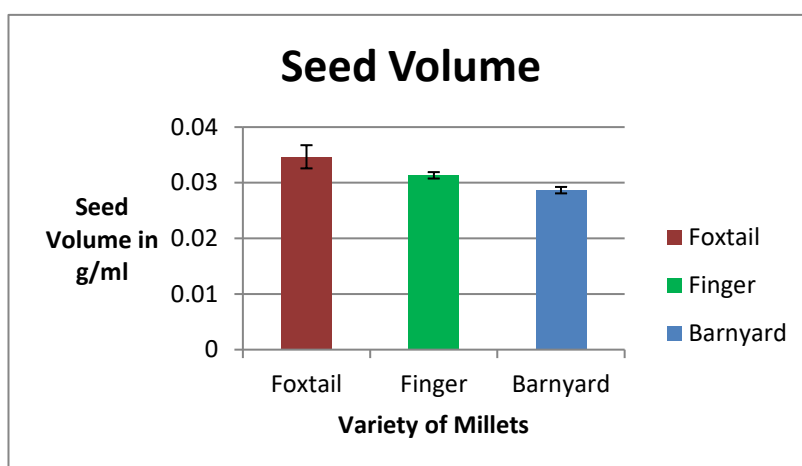


Figure 4.16: Seed Volume analysis

4.10.1.4 Bulk Density

The data presented in Table 4.20 and Fig 4.17 indicate a significant change in the bulk density of the three millet varieties under study. However, it was found that the foxtail millet had a slightly higher bulk density (0.771 ± 0.15 g/ml) compared to the finger millet (0.695 ± 0.10 g/ml) and barnyard millet (0.422 ± 0.11 g/ml). Although the difference was statistically significant, it suggests a slight variation in bulk density between the three varieties.

Previous studies have also reported similar findings, where the bulk density of millet varieties varied with different moisture contents. For instance, in the variety Ex-Borno, it was observed that as moisture content increased from 10% to 20%, there was a significant drop in bulk density from 811.4 to 646 kg/m³. A similar trend was observed in the variety SOSAT C88, with a decrease in bulk density from 817 to 725 kg/m³ as

moisture content increased from 10% to 20%. These findings highlight the direct relationship between moisture content and bulk density, emphasizing the need to consider moisture levels during product preparation (Ojediran et al., 2010). Bulk density is an important parameter that influences the packaging requirements of a product (Chandi et al., 2007). Therefore, understanding the bulk density variations of millet varieties, particularly about moisture content, is crucial for proper packaging and product development considerations.

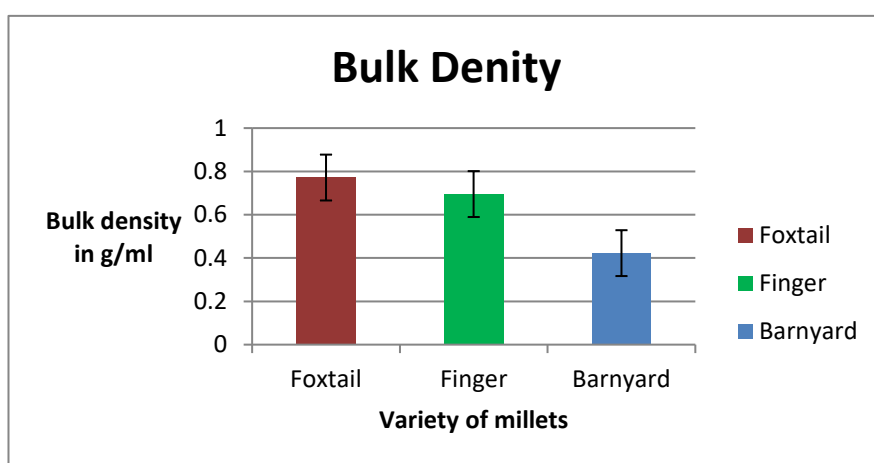


Figure 4.17: Bulk density analysis

4.10.1.5 Hydration Capacity and Index

Upon analyzing Table 4.20 and Figs. 4.18 & 4.19, it becomes evident that finger millet exhibited a higher hydration capacity of 0.004 g per seed (equivalent to 4.0 g per 1000 seeds) compared to foxtail and barnyard millet, which had hydration capacities of 0.002 g and 0.003 g per seed, respectively (equivalent to 2.0 g and 3.0 g per 1000 seeds). The hydration capacity was determined using the hydration index, with finger millet showing a higher hydration index of 0.005 ± 0.02 . In contrast, foxtail millet had a hydration index of 0.004 ± 0.02 , and barnyard millet had a hydration index of 0.003 ± 0.01 .

In a previous study by Sehgal and Kawatra (2002), the hydration index of different pearl millet varieties ranged from 0.181 to 0.500, indicating significant variations in hydration capacity. Another study conducted by Sibian et al. (2013) investigated the soaking capacity of pearl millet seeds and found that the use of additives such as NaOH (0.1%), NaHCO₃ (0.5%), and MgCl₂ (0.5%) could alter the soaking capacity. These additives were found to have a positive effect by increasing the soaking capacity. For example, adding 0.5% NaHCO₃ increased the soaking index to 10.82 ± 0.04 per 1000 seeds compared to distilled water, resulting in a 7.49 ± 0.07 per 1000 seeds.

The soaking index was lower for all three varieties in the present study, as no additives or chemicals were used in the soaking water. This accounts for the lower soaking index observed in the current study compared to studies where additives were employed.

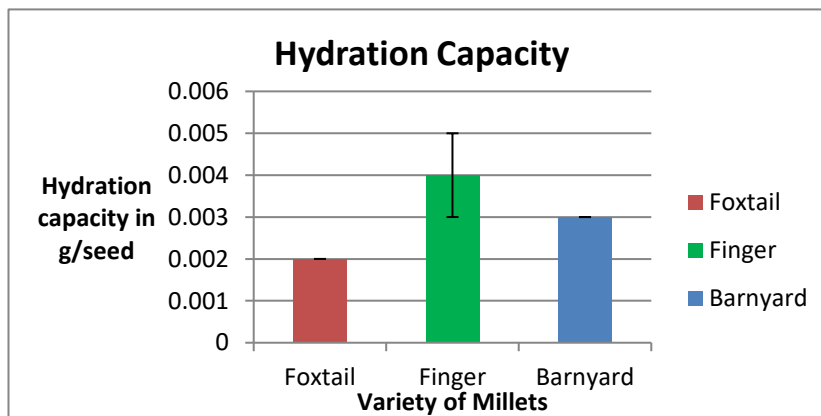


Figure 4.18: Hydration capacity analysis

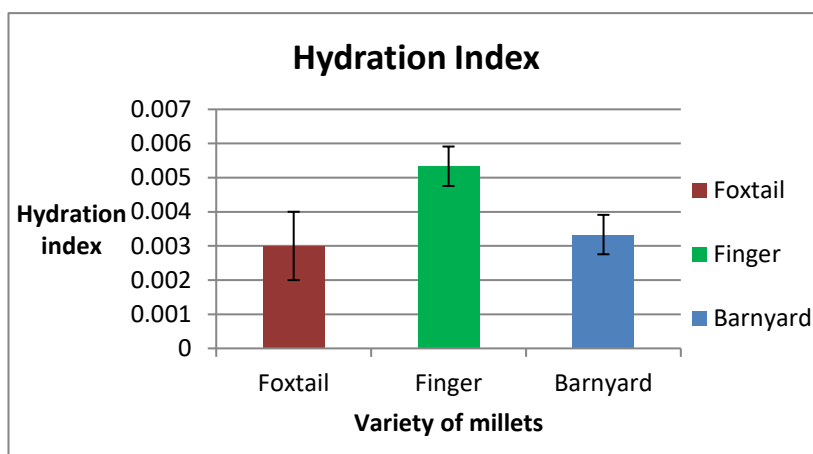


Figure 4.19: Hydration index analysis

4.10.1.6 Swelling Capacity and Index

The data in Table 4.20 and Fig 4.20 & 4.21 demonstrate that the finger millet variety exhibited a higher swelling capacity than the foxtail and barnyard millet varieties. The finger variety had a swelling capacity of 0.007 ± 0.03 ml per seed (equivalent to 9 per 1000 seeds), while the foxtail and barnyard variety had a swelling capacity of 0.005 ± 0.04 and 0.004 ± 0.002 ml per seed (equivalent to 5 and 4 per 1000 seeds) respectively. Additionally, the swelling index, which considers the volume increases relative to a standard volume (10 ml in this case), was also significantly higher for the finger variety (6.32 ± 0.01 ml/10 ml or per 1000 seeds) compared to the foxtail and barnyard (5.56 ± 0.03 and 4.32 ± 0.02 ml/10 ml or per 1000 seeds respectively).

In a study by Sehgal and Kawatra (2002), the swelling capacity of eleven pearl millet varieties ranged from 0.004 to 0.009 ml per seed, while the swelling index ranged from 0.357 to 0.538 ml per seed. Sabian et al. (2013) reported that the average swelling capacity of pearl millet seeds with distilled water was 0.006 ± 0.00 per seed. However, the swelling capacity could be enhanced by treating the seeds with additives such as 0.1% NaOH (0.007 ± 0.00), 0.5% NaHCO₃ (0.005 ± 0.00), and 0.5% MgCl₂ (0.006 ± 0.00) per seed. These findings suggest that treatment with additives can improve the swelling capacity and index of pearl millet seeds. Water absorption capacities, including swelling capacity, are influenced by factors such as starch and protein contents, as well

as the particle size distribution of the ingredient. These properties significantly determine the sample's functionality as bulking, swelling, or thickening agents in formulations or foods with relatively high water activity (Florence Suma, 2012).

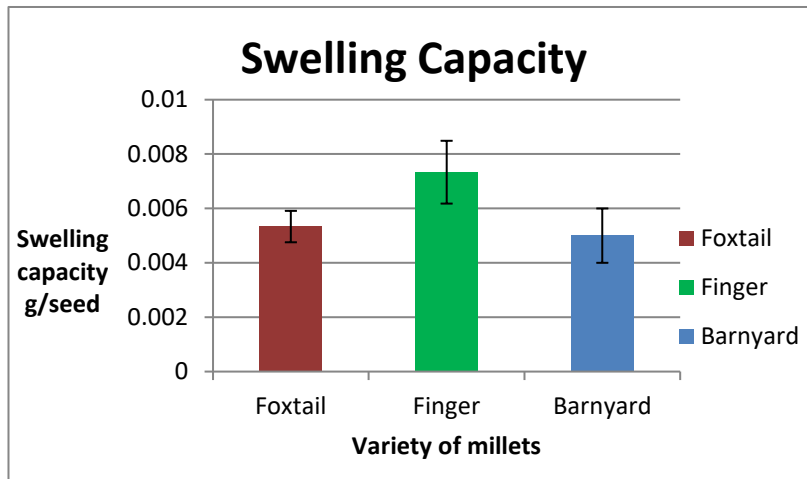


Figure 4.20: Swelling capacity analysis

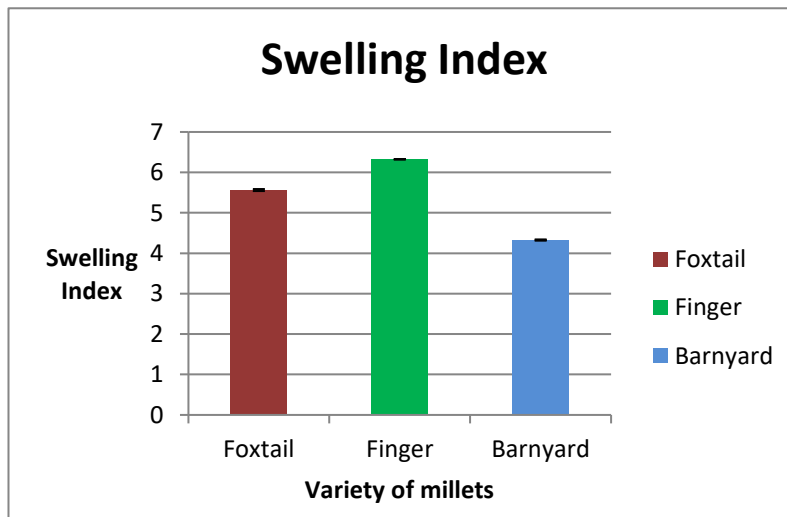


Figure 4.21: Swelling index analysis

4.10.1.7 Germination Capacity

Upon examining Table 4.20 and Fig. 4.22, it is evident that there were significant differences in germination percentage between the three millet varieties. The foxtail millet variety exhibited a higher germination percentage of 85 ± 0.04 than the finger millet and barnyard millet varieties, which had germination percentages of 73.6 ± 0.3 and 63 ± 0.03 , respectively. Previous studies have also shown that various factors can affect the germination of foxtail millet.

One such factor is salinity, where increased salinity levels have been found to impact germination negatively. For instance, research by Sam and Idris (2015) demonstrated that germination and seedling growth decreased with increasing salt concentration. Higher salt concentrations, such as 1.5% NaCl, resulted in lower germination percentages and reduced seedling growth. Similarly, exposure to higher temperatures can also affect germination. Garcia-Huidobro et al. (1985) found that germination percentages decreased with exposure to higher temperatures, such as 50°C.

Optimal germination conditions for pearl millet have also been investigated. Newman et al. (2010) reported that the best germination was achieved at temperatures ranging from 32.77 to 35 degrees Celsius. Additionally, Haryanto et al. (1997) found that germination of pearl millet at low temperatures, such as 15°C, was lower than germination at average temperatures. Based on the present study, it can be concluded that optimal germination for all three varieties—foxtail (85%), finger (73.6%), and barnyard millet (63%)—was achieved at a growth temperature of 27-32°C. The variations in germination percentage observed at ambient temperatures could be attributed to differences in genotype or cultivation practices.

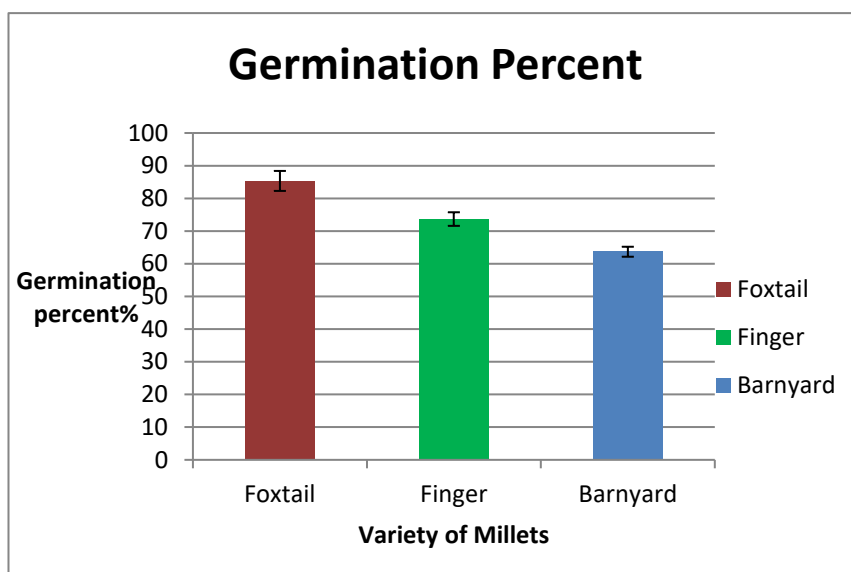


Figure 4.22: Germination capacity analysis

4.10.2 Chemical analysis of millet grains

Millet grains are small-seeded, nutrient-dense grains, and their composition and nutritional value are often ascertained using several chemical analysis procedures. Depending on the exact variety of millets (such as barnyard millet, finger millet, or foxtail millet) and their development circumstances, millets can have a variety of compositions. Here are a few typical millet-related chemical analyses:

Table 4.21: Chemical analysis of millet grains

| Characteristics | Foxtail Millet (Mean ± SE) | Finger Millet (Mean ± SE) | Barnyard Millet (Mean ± SE) |
|-----------------|-------------------------------|------------------------------|--------------------------------|
| Moisture (%) | 9.43±0.01 | 8.73±0.02 | 6.34±0.02 |
| Total Ash (%) | 1.45±0.01 | 1.66±0.01 | 1.53±0.01 |

| | | | |
|------------------------|-----------|-----------|-----------|
| Total Fat (%) | 5.86±0.02 | 5.20±0.01 | 4.65±0.01 |
| Protein (%) | 9.66±0.01 | 8.56±0.02 | 9.10±0.02 |
| Crude fiber (%) | 1.22±0.02 | 1.76±0.02 | 1.56±0.00 |

4.10.2.1 Moisture content

According to the findings presented in Table 4.21 and Fig. 4.36, the analysis of various millet varieties revealed that the moisture content in the foxtail millet variety remained slightly higher ($9.43 \pm 0.01\%$) compared to the finger millet ($8.73 \pm 0.04\%$) and barnyard millet ($6.34 \pm 0.02\%$). Previous research by Cheik et al. (2006) indicated that moisture content in different millet varieties ranged from 5.97% to 10.47%. Similarly, Sehgal et al. (2002) reported 10.24% to 13.30% moisture content in various millet varieties. Sabian et al. (2013) also found a similar moisture content of 9.93% in foxtail millet seeds. The design and development of processing methods and equipment need to have a thorough understanding of the physical and functional qualities, as well as their relationship with the amount of moisture present in the material, as highlighted by Chandi et al. (2007).

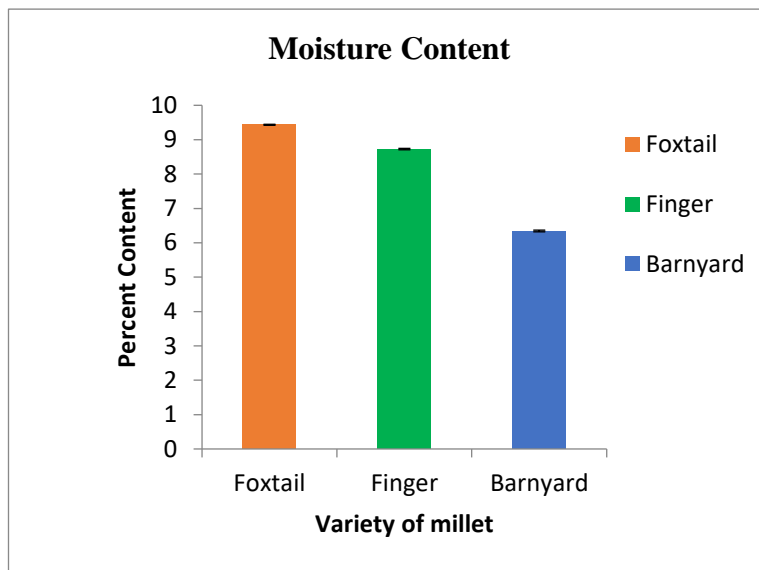


Figure 4.23: Moisture content analysis

4.10.2.2 Total Ash Content

The findings presented in Table 4.21 and Fig. 4.24 also reveal that the ash content in different millet varieties ranged from 1.40% to 1.83%. The finger millet variety exhibited the highest ash content ($1.66 \pm 0.01\%$) compared to the foxtail millet ($1.45 \pm 0.01\%$) and barnyard millet ($1.53 \pm 0.01\%$). Ash content represents the mineral content or non-combustible fraction of the sample. Previous research conducted by Cheik et al. (2006) reported ash content ranging from 2.16% to 3.44% in 14 cultivars of pearl millet. Similar results were observed by Marston and Hosney (1972) in the pearl millet cultivars "RMP 1I (S) CI" with an ash content of 1.4% and "HMP1700" and "Serere" with an ash content of 1.6 percent. Therefore, the findings of this study are consistent with earlier reports.

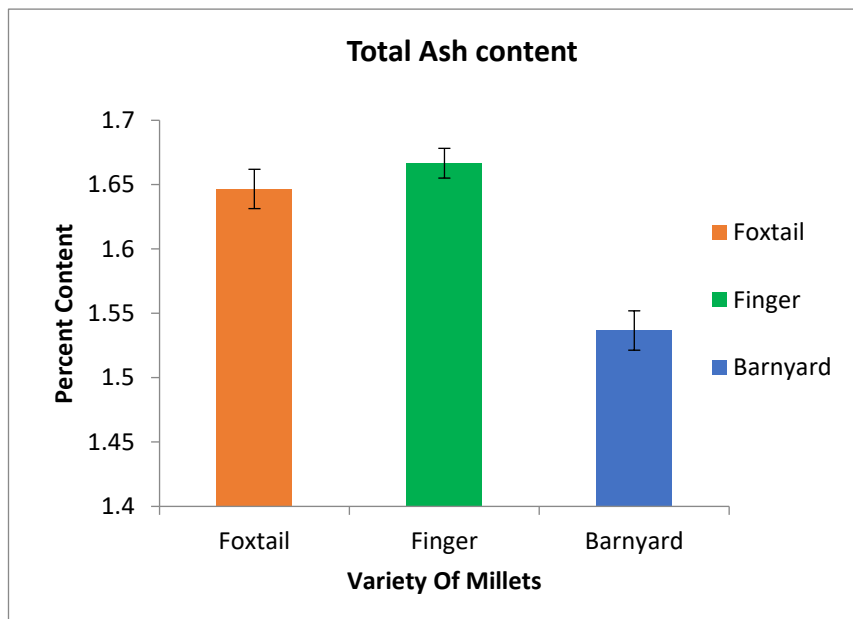


Figure 4.24: Total Ash Content Analysis

4.10.2.3 Total Fat Content

The results presented in Table 4.21 and Fig. 4.25 indicate a significant difference in the total fat content between foxtail millet ($5.86 \pm 0.02\%$), finger millet ($5.21 \pm 0.08\%$), and barnyard millet ($4.56 \pm 0.01\%$). Another study on pearl millet flour reported a lower fat content of 2.8% (Sawaya et al., 1984). The variation in fat content could be attributed to factors such as geographical location, soil conditions, and cultural practices, which can influence the composition of millet varieties. Abdalla et al. (1998) reported a wide range of fat content, ranging from 1.25% to 6.45%, in different millet varieties. Similarly, Fasasi (2009) reported a fat content of 5.70% in foxtail millet. Basahy (1996) found a fat content of approximately 5.3% in finger millet grain and highlighted its good nutritive value according to the FAO/WHO.

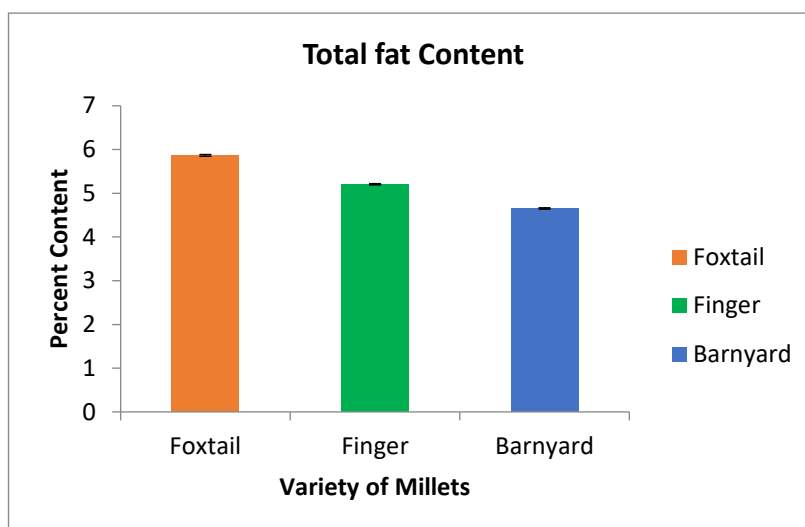


Figure 4.25: Total Fat Content Analysis

4.10.2.4 Total Protein Content

The results from Table 4.21 and Fig. 4.26 demonstrate that the protein content in different millet varieties ranged from 8.91% to 11.43%. The foxtail millet variety exhibited the highest protein content ($9.66 \pm 0.01\%$), which was statistically significant compared to the finger millet ($8.56 \pm 0.02\%$) and barnyard millet ($9.10 \pm 0.02\%$). Previous studies by Modu et al. (2005) reported protein content ranging from 10.73% to 12.97% in pearl millet cultivars, while Cheik et al. (2006) found protein content ranging from 8.66% to 17.11% in 14 cultivars of pearl millet. Hassan et al. (2014) reported a consistent protein content ranging from 8.97% to 9.55% in different pearl millet varieties. Additionally, Basahy (1996) reported a protein content of 12.3% in crude pearl millet, highlighting the presence of essential amino acids such as leucine, isoleucine, valine, threonine, lysine, and sulfur-containing amino acids. In comparison to these findings, the hybrid variety demonstrated superior protein content compared to the local variety.

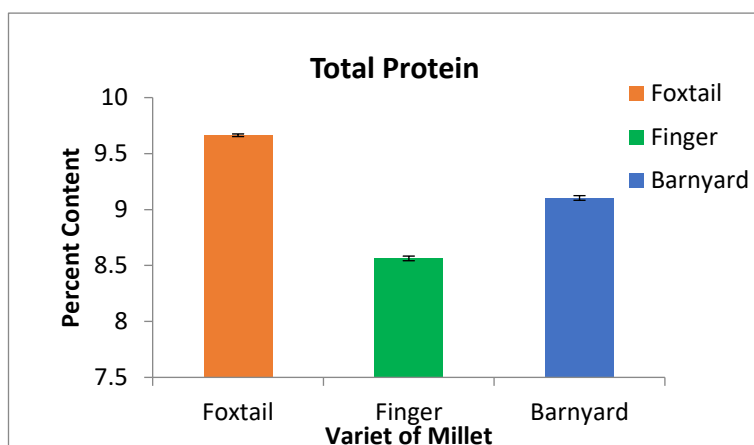


Figure 4.26: Total protein content

4.10.2.5 Crude Fibre

Upon examining Table 4.21 and Fig. 4.27, it is evident that the crude fibre content in different millet varieties ranged from 1.22% to 1.76%. Finger millet exhibited a higher crude fibre content ($1.76 \pm 0.02\%$) compared to foxtail millet ($1.22 \pm 0.02\%$) and barnyard millet ($1.56 \pm 0.02\%$). As Nambiar et al. (2011) and NIN (2003) highlighted, fibre is beneficial for patients suffering from constipation. Previous studies have reported a lower crude fiber content of 1% compared to the present local variety ($1.25 \pm 0.05\%$), as Malik (2015) noted. Conversely, another study on pearl millet flour from Saudi Arabia reported a very high fiber content of approximately 2.8%, suggesting the superior characteristics of that particular variety (Sawaya et al., 1984). Catelan et al. (2012) reported a crude fiber content of 2.80% in pearl millet, emphasising its richness in fiber. Compared to these findings, the current varieties showed significantly lower fiber content when analyzed.

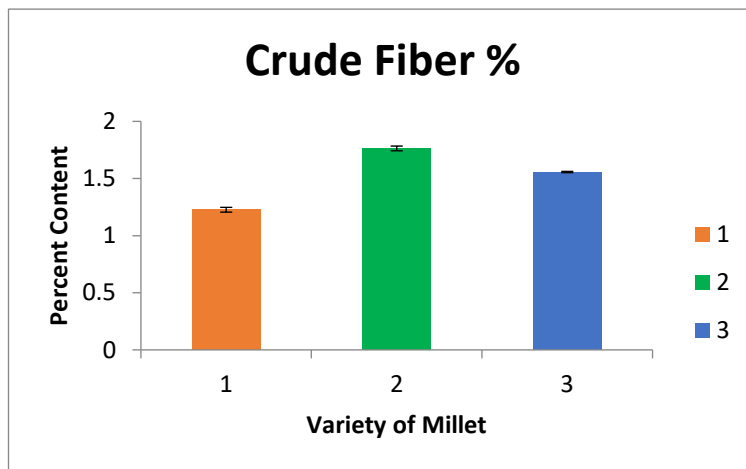


Figure 4.27: Crude fiber content analysis

4.11 Development of probiotic beverage using millet and fruit juice

All three millet varieties from the above experiment were further utilized to develop millet milk-based probiotic beverages. The probiotic beverage was developed by blending millet milk with different proportions of apple and pineapple juice (Fig.4.28). Further, the developed beverages were analyzed during fermentation for various chemical changes.

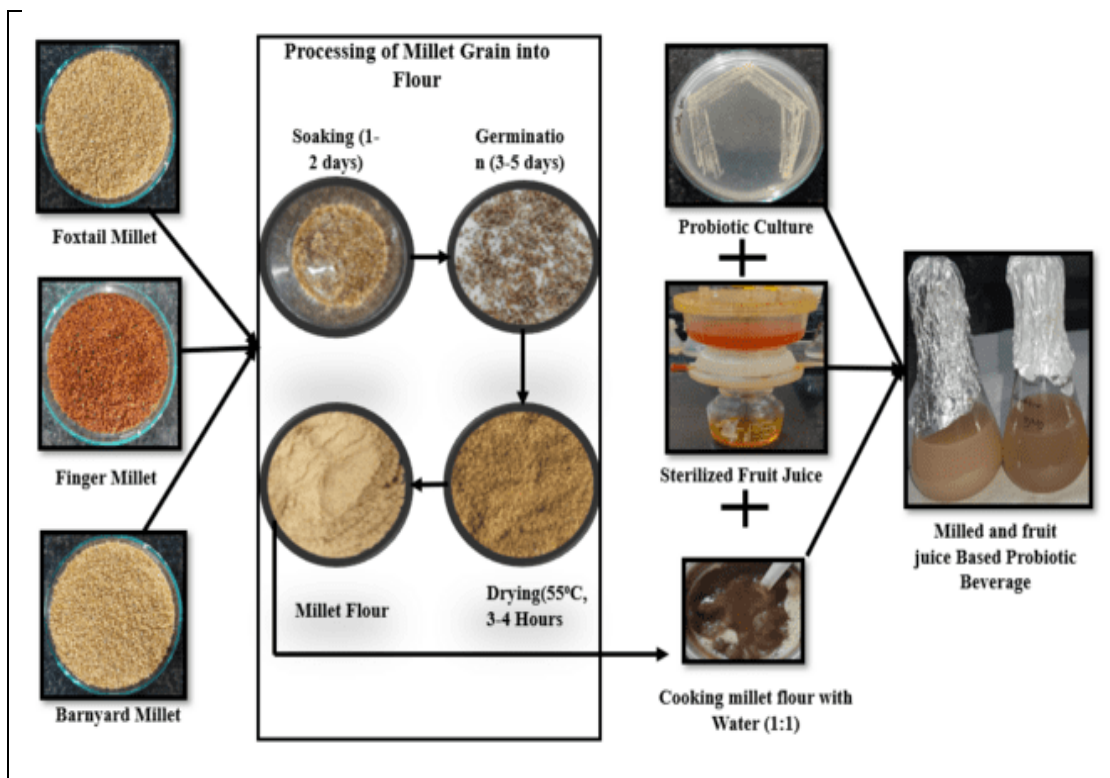


Figure 4.28: Development of probiotic beverage using millet and fruit juice

Table 4.22: Finger millet and apple juice-based beverage containing CM1 as a probiotic organism

| Test Parameters | Unit | Results |
|------------------------------|-------------|----------------|
| Total Fat | gm/100ml | 0.21 |
| Saturated Fat | gm/100ml | 0 |
| Monounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.24 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 38.07 |
| Carbohydrate ⁸⁸⁸⁸ | gm/100ml | 9.03 |
| Sodium | mg/100ml | 2 |
| Cholesterol | Mn/100ml | 0 |
| Total sugar | gm/100ml | 9.0 |
| Dietary Fiber | gm/100ml | 0 |
| Potassium | mg/100gm | 42 |

Table 4.23: Foxtail millet and fruit juice-based beverage containing CM1 as a probiotic organism

| Test Parameters | Unit | Results |
|------------------------|-------------|----------------|
| Total Fat | gm/100ml | 0.13 |
| Saturated Fat | gm/100ml | 0 |
| Monounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.68 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 38.37 |

| | | |
|---------------|----------|-------|
| Carbohydrate | gm/100ml | 8.62 |
| Sodium | mg/100ml | 4 |
| Cholesterol | Mn/100ml | 0 |
| Total sugar | gm/100ml | 10.41 |
| Dietary Fiber | gm/100ml | 0 |
| Potassium | mg/100gm | 10.1 |

Table 4.24: Barnyard millet and fruit juice-based beverage containing CM1 as a probiotic organism

| Test Parameters | Unit | Results |
|------------------------|-------------|----------------|
| Total Fat | gm/100ml | 0.16 |
| Saturated Fat | gm/100ml | 0 |
| Monounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.37 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 42.36 |
| Carbohydrate | gm/100ml | 9.86 |
| Sodium | mg/100ml | 5 |
| Cholesterol | Mn/100ml | 0 |
| Total sugar | gm/100ml | 9.6 |
| Dietary Fiber | gm/100ml | 0 |
| Potassium | mg/100gm | 48 |

Table 4.25: Finger millet and fruit juice-based beverage containing OS1 as a probiotic organism

| Test Parameters | Unit | Results |
|------------------------|-------------|----------------|
| Total Fat | gm/100ml | 0.17 |
| Saturated Fat | gm/100ml | 0 |

| | | |
|---------------------|------------|-------|
| Monounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.60 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 53.93 |
| Carbohydrate | gm/100ml | 12.50 |
| Sodium | mg/100ml | 2 |
| Cholesterol | Mn/100ml | 0 |
| Total sugar | gm/100ml | 16.03 |
| Dietary Fiber | gm/100ml | 0 |
| Potassium | mg/100gm | 68 |

Table 4.26: Foxtail millet and fruit juice-based beverage containing OS1 as a probiotic organism

| Test Parameters | Unit | Results |
|------------------------|-------------|----------------|
| Total Fat | gm/100ml | 0.10 |
| Saturated Fat | gm/100ml | 0 |
| Moounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.45 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 74.78 |
| Carbohydrate | gm/100ml | 18.02 |
| Sodium | mg/100ml | 3 |
| Cholesterol | Mn/100ml | 0 |
| Total sugar | gm/100ml | 17.14 |
| Dietary Fiber | gm/100ml | 0 |
| Potassium | mg/100gm | 57 |

Table 4.27: Barnyard millet and fruit juice-based beverage containing OS1 as a probiotic organism

| Test Parameters | Unit | Results |
|---------------------|------------|---------|
| Total Fat | gm/100ml | 0.19 |
| Saturated Fat | gm/100ml | 0 |
| Monounsaturated Fat | gm/100ml | 0 |
| Polyunsaturated Fat | gm/100ml | 0 |
| Protein | gm/100ml | 0.52 |
| Trans Fat | gm/100ml | 0 |
| Energy | Kcal/100gm | 58.71 |
| Carbohydrate | gm/100ml | 13.73 |
| Sodium | mg/100ml | 1 |
| Cholesterol | mg/100ml | 0 |
| Total sugar | gm/100ml | 14.70 |
| Dietary Fiber | gm/100ml | 0.13 |
| Potassium | mg/100gm | 64 |

4.11.1 Chemical composition of Millet and fruit juice - based beverage

Upon examining the data in Tables 4.22 to 4.27, it is evident that there were significant variations in the protein content of the probiotic beverages derived from millet milk blended with apple. The protein content ranged from 0.24% to 0.68% among the treatments. The highest protein content of 0.68% was observed in the treatment where foxtail millet was blended with apple juice in a 50:50 ratio. Conversely, the lowest protein content of 0.24% was recorded in the treatment involving finger millet and apple juice in a 50:50 ratio. Similarly, there were notable differences in the fat content among the drinks prepared by blending millet milk with pineapple and apple juice. The fat content ranged from 0.10% to 0.19% across the various treatments. The treatment with the highest fat content was 0.19%, which consisted of barnyard millet and

pineapple juice in a 50:50 ratio. On the other hand, the treatment with the lowest fat content was 0.10%, comprising millet milk and apple juice in a 50:50 ratio.

Regarding fiber content, the results indicated significant variances among the treatments involving the blending of millet milk with pineapple and apple juice. The fiber content ranged from 0.13% to 0.32%. The drink with the highest fiber content (0.32%) was prepared by blending foxtail millet with pineapple juice in a 50:50 ratio. Conversely, the lowest fiber content (0.13%) was observed in the drink blending barnyard millet milk with pineapple juice in a 50:50 ratio.

4.11.2 Detection of food-borne pathogens

The information provided in Table 4.28 indicates that no foodborne pathogens were detected in any of the treatments. The data clearly demonstrate that all treatments tested negative for the presence of *Salmonella* spp., *Listeria monocytogenes*, *E. coli*, and *Staphylococcus aureus*. This result confirms the product's safety prior to its use in sensory evaluation.

Table 4.28: Detection of food-borne pathogens

| Treatments | <i>Salmonella</i> <i>spp.</i> | <i>Listeria</i> <i>monocytogenes</i> | <i>E. coli</i> | <i>Staphylococcus</i> <i>aureus</i> |
|--|----------------------------------|---|----------------|--|
| Foxtail millet and apple juice + CM1 | Ab | Ab | Ab | Ab |
| Finger millet and apple juice + CM1 | Ab | Ab | Ab | Ab |
| Barnyard millet and apple juice + CM1 | Ab | Ab | Ab | Ab |

| | | | | |
|---|----|----|----|----|
| Foxtail millet and Pineapple juice + OS1 | Ab | Ab | Ab | Ab |
| Finger millet and Apple juice + OS1 | Ab | Ab | Ab | Ab |
| Barnyard millet and apple juice + OS1 | Ab | Ab | Ab | Ab |

4.11.3 Sensory Evaluation of Beverage

The findings from the sensory analysis, as shown in Table 4.29, indicate notable variances in the quality attributes among the various treatments. Upon closer examination of the data, it becomes evident that treatment T5, which involved blending millet milk with apple juice in a 50:50 ratio, outperformed the other treatments regarding color, flavor, consistency, taste, overall acceptability, and other quality attributes. Conversely, the treatment consisting solely of millet milk received the lowest scores in these aspects.

Table 4.29: Overall acceptability of different millet milk-based probiotic beverages

| Sensory Score | Control | Millet Milk plus Pineapple juice | | | Millet Milk plus Pineapple juice | | |
|--------------------|-------------------|--------------------------------------|-------------------------------------|---------------------------------------|--|---|---|
| | | Foxtail millet and apple juice + CM1 | Finger millet and apple juice + CM1 | Barnyard millet and apple juice + CM1 | Foxtail millet and Pineapple juice + OS1 | Finger millet and pineapple juice + OS1 | Barnyard millet and pineapple juice + OS1 |
| Treatments | 100 % Millet Milk | 50: 50 | 50: 50 | 50: 50 | 50: 50 | 50: 50 | 50: 50 |
| Color | 10.0 | 9.7 | 8.9 | 8.5 | 9.2 | 8.2 | 7.5 |
| Flavor | 9.8 | 9.2 | 8.7 | 7.4 | 8.7 | 8.4 | 7.3 |
| Consistency | 9.6 | 9.2 | 8.6 | 7.7 | 8.9 | 7.8 | 7.7 |
| Taste | 10.0 | 9.8 | 8.9 | 7.5 | 8.6 | 7.5 | 7.1 |
| Overall acceptance | 9.6 | 9.7 | 8.7 | 7.6 | 8.7 | 7.2 | 7.5 |

4.12 Storage stability studies in different packages and storage conditions

4.12.1 Changes in pH during storage under refrigerated conditions in glass and plastic packaging

Examining Table 4.30 provides insights into the changes in pH during the storage of millet-based probiotic beverages in glass and plastic bottles under refrigerated conditions. Initially, the pH of the control millet and fruit juice beverage was 3.71 in glass packaging and 3.70 in plastic packaging. Throughout the first 20 days of storage, the pH remained relatively stable. However, after the 20-day mark, the pH significantly decreased, with plastic packaging experiencing a more significant decline (reaching 3.49) than glass packaging (reaching 3.61). The table indicates that while the pH remained stable for the first 20 days in all treatments, significant changes were observed in plastic packaging beyond that timeframe.

A study conducted by Sasi et al. (2015) noted that the pH of a fresh beverage made from a blend of whey and Aloe vera juice, fermented for 5 hours, was initially 4.40. During refrigerated storage over 30 days, the pH gradually declined to 4.00. However, when the samples were stored at ambient temperature, a significant decrease in pH was observed after 6 days.

Likewise, significant changes in acidity were also observed (as shown in Table 4.42) during the first 20 days of refrigerated storage in both glass and plastic packaging. After 20 days, the acidity increased more in plastic than in glass packaging. A study by Rodas et al. (2002) demonstrated that titratable acidity values were significantly influenced by the duration of storage for whey beverages, both under refrigerated and room temperature conditions. In the study by Sasi et al. (2015), the initial acidity of a fermented beverage made from whey and Aloe vera juice was 0.553% (as lactic acid) and increased to 1.120% after 6 days of storage. Under refrigerated conditions, the acidity increased from 0.546% to 0.870% as lactic acid after 30 days. These findings highlight the significant impact of storage duration on titratable acidity values for whey beverages, regardless of whether stored under refrigeration or at room temperature.

Table 4.30: Changes in pH of blended probiotic millet and fruit juice-based beverages packed in glass and plastic bottles during storage under refrigerated conditions

| Groups | 0 day | | 10 day | | 20 days | | 30 days | | P<0.05 |
|--|----------------|------------|----------------|----------------|------------|------------|------------|---------|--------|
| | | | Glass | Plastic | Glass | Plastic | Glass | Plastic | |
| Control | 3.618±0.0 3 | 3.515±0.03 | 3.526±0.0 2 | 3.414±0.0 3 | 3.486±0.02 | 3.384±0.03 | 3.334±0.02 | ** | |
| Foxtail millet and apple juice + CM1 | 3.636±0.0 2 | 3.445±0.03 | 3.426±0.0 2 | 3.414±0.0 3 | 3.386±0.02 | 3.384±0.03 | 3.114±0.02 | ** | |
| Finger millet and apple juice + CM1 | 3.566±0.0 2 | 3.555±0.03 | 3.560±0.0 3 | 3.485±0.0 3 | 3.476±0.03 | 3.376±0.02 | 3.315±0.02 | ** | |
| Barnyard millet and apple juice + CM1 | 3.367±0.0 1 | 3.311±0.03 | 3.304±0.0 2 | 3.286±0.0 2 | 3.274±0.03 | 3.264±0.03 | 3.104±0.02 | ** | |
| Foxtail millet and Pineapple juice + OSI | 3.670±0.0 2 | 3.662±0.02 | 3.664±0.0 4 | 3.415±0.0 4 | 3.555±0.04 | 3.394±0.03 | 3.314±0.02 | ** | |
| Finger millet and pineapple juice + OSI | 3.577±0.0 2 | 3.568±0.04 | 3.566±0.0 3 | 3.414±0.0 3 | 3.064±0.02 | 3.382±0.03 | 3.265±0.05 | ** | |
| Barnyard millet and pineapple juice + OSI | 3.428±0.0 2 | 3.414±0.03 | 3.416±0.0 2 | 3.326±0.0 3 | 3.365±0.03 | 2.964±0.04 | 3.107±0.03 | ** | |

4.12.2 Changes in viable count during storage under refrigerated conditions in glass and plastic packaging

The data provided in Table 4.31 shows that the probiotic millet and juice beverage (control) had a 4.2×10^8 initial viable count. Both plastic packaging (4.0×10^8) and glass packaging (2.2×10^8) continued to be counted until the 20th day. After the 20th day, there was a noticeable decrease in the viable count, especially in the plastic packaging (1.2×10^7) instead of the glass packaging (2.1×10^7). Until the twentieth day, this pattern persisted across all treatments. According to the international standard established by FIL/IDF, probiotic products must have at least 10^6 live probiotic bacteria per milliliter to operate as intended for human consumption (Samona & Robinson, 1991; Roy, 2005). It is important to note that all of the beverages in our experiment maintained viability levels between 10^{-7} and 10^{-8} cfu mL⁻¹. The overall viable count of *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Bifidobacterium bifidum* decreased in a whey-based probiotic beverage held at 4°C 1°C, according to findings from previous studies (Heller et al., 2001). Prior research has also noticed the probiotic *Lactobacillus rhamnosus*'s strong stability in a mixed vegetable juice with a somewhat high pH (4.35) during a two-week storage period at 4°C.

Table 4.31 Changes in viable count during storage under refrigerated conditions in glass and plastic packaging

| | 0 day | | 10 days | | 20 days | | 30 days | |
|---|----------------------|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| | Glass | Plastic | Glass | Plastic | Glass | Plastic | Glass | Plastic |
| Control | 4.2 x10 ⁸ | 4.2 x10 ⁸ | 4.0x10 ⁸ | 2.0x10 ⁸ | 3.4x10 ⁸ | 1.2x10 ⁸ | 2.1x10 ⁷ | 1.2x10 ⁷ |
| Foxtail millet and apple juice + CM1 | 5.7x 10 ⁸ | 2.6x 10 ⁸ | 6.7x10 ⁸ | 2.3x10 ⁸ | 5.8x10 ⁸ | 3.4x10 ⁸ | 5.4x10 ⁷ | 2.2x10 ⁷ |
| Finger millet and apple juice + CM1 | 4.5x 10 ⁸ | 2.4x 10 ⁸ | 7.3x10 ⁸ | 1.5x10 ⁸ | 6.4x10 ⁸ | 4.1x10 ⁸ | 4.5x10 ⁷ | 1.5x10 ⁷ |
| Barnyard millet and apple juice + CM1 | 5.4 x10 ⁸ | 4.4 x10 ⁸ | 5.9x10 ⁸ | 3.9x10 ⁸ | 6.5x10 ⁸ | 2.9x10 ⁸ | 3.5x10 ⁷ | 1.4x10 ⁷ |
| Foxtail millet and Pineapple juice + OS1 | 5.5 x10 ⁸ | 3.6 x10 ⁸ | 5.9x10 ⁸ | 2.8x10 ⁸ | 6.4x10 ⁸ | 2.5x10 ⁸ | 4.3x10 ⁷ | 1.7x10 ⁶ |
| Finger millet and Pineapple juice + OS1 | 5.4x10 ⁸ | 2.8x10 ⁸ | 5.8x10 ⁸ | 3.5x10 ⁸ | 6.5x10 ⁷ | 2.6x10 ⁷ | 4.6x10 ⁷ | 1.5x10 ⁶ |
| Barnyard millet and pineapple juice + OS1 | 5.2 x10 ⁸ | 2.6 x10 ⁸ | 5.9x10 ⁸ | 2.1x10 ⁸ | 5.2x10 ⁸ | 1.2x10 ⁸ | 3.4x10 ⁶ | 2.0x10 ⁶ |

It has been recommended to seal beverages with either a natural cork stopper or a plastic stopper and then store them in glass containers to preserve their sensory qualities and reduce oxygen exposure. Glass is transparent, has excellent barrier characteristics against gases and vapors, is durably stable, and is readily recyclable. It protects against chemical and environmental factors, including gases and vapours. According to Ramos et al. (2015), glass offers transparency, remarkable stability, and ease of recyclability. As a result, the current study also indicates that alterations are more pronounced in plastic bottles than in glass bottles.

4.13 DISCUSSION

Probiotics are present in diverse food items, with dairy products as the predominant source. The study conducted by Asadi et al. (2022) provides evidence that consuming probiotic supplements might have many advantageous effects on human health. The advantages encompass alleviating ailments such as lactose intolerance, control of hypercholesterolemia, and control and treatment of cardiovascular disorders. The presence of various probiotic products in the market supports these findings. The integration of probiotics into a well-balanced diet has the potential to augment overall health. Microorganisms are regarded as viable contenders for probiotic utilization owing to their capacity to enhance the host's immunity against diseases, their aptitude to establish themselves within the host's body, and their capability to oppose detrimental infections. The authors Al-Dhabi et al. (2020) have also observed that probiotics contribute to enhancing general well-being. Furthermore, a study conducted by the authors indicates that probiotics have the potential to elicit immune system functionality and outcompete pathogens for vital nutrients and attachment sites. Nevertheless, understanding the specific mechanisms through which probiotics exert their effects remains limited (Al-Dhabi et al., 2020). Notwithstanding this, probiotics present a range of benefits, as evidenced by studies conducted by the group of authors Abdel-Gawad et al. (2021) and Zhang et al. (2022). These benefits include inhibiting bacterial development and a positive impact on the host, such as increased immune response and growth, the competitive exclusion of pathogenic microorganisms, the activation of helpful enzymes, the suppression of toxic hormones, and the enhancement of immune system responses. Because of their pre-existing familiarity with the gastrointestinal tract (GIT), probiotic strains that are derived from their indigenous host or edible sources are preferred, as this enables them to survive and exhibit the desired beneficial effects more efficiently than strains obtained from substitute origins (Asadi et al., 2022). As a result, it is necessary to design probiotics adapted to the particular host to maximize the potential health benefits they may offer. In addition, directly testing the prospective probiotics in *in vivo* experiments can be time-consuming and expensive, necessitating a significant investment of resources. Because of this, the evaluation of probiotics conducted in a laboratory under controlled conditions, referred to as an *in vitro* evaluation, is the primary factor considered when making a choice. According to Hatami et al. (2022), finding the

strain that demonstrates the maximum level of efficacy, appropriateness, and ideal qualities in proportion to its positive attributes is the goal.

The present research acquired lactic acid bacteria (LAB) strains from the oral cavity and food samples. These samples were grown on an MRS medium with a pH range of 6.4 ± 0.2 to 6.5 ± 0.2 , which was used to isolate the strains, and the pH range of the medium was measured. Following this, the strains underwent a series of tests to determine their ability to act as probiotics. According to earlier studies (Anjum et al., 2023), an appropriate pH range for the culture and evaluation of LAB has been recommended to be between 6.2 and 8.5, which has been suggested to be optimal. Oral cavity samples (10 strains) and food samples (50 strains) were combined to obtain 60 different LAB strains.

Before the strains were subjected to any molecular analysis, further research was conducted to investigate their macroscopic, microscopic, biochemical, and existing in vitro probiotic properties. During this research, sixty unique strains were discovered, with 38 exhibiting rod-shaped morphology and 22 showing cocci morphology. Eighteen different isolates gave a negative response when subjected to the catalase test. These isolates have been determined to be Gram-positive bacteria and do not produce spores when grown in culture. Within the arid region of Algeria, Adjoudj and colleagues (2020) conducted research in which they extracted LAB from ruminant animal milk and other fermented samples. The researchers found that all isolates exhibited Gram-positive characteristics, could not produce spores, and tested negative for catalase. The research suggested that LAB, being obligate anaerobes, flourish when oxygen is absent from their environment. It has been found that these microbes are incapable of producing either catalase enzymes or cytochromes (Bazireh et al., 2022; Zourari et al., 1992). The production of hydrogen peroxide by lactic acid bacteria as a natural consequence of their metabolic processes makes these bacteria susceptible to the damaging effects of oxidative stress.

According to research by Zhang et al. (2020), these bacteria cannot combat the harmful effects of hydrogen peroxide because they do not possess the catalase enzyme necessary for its breakdown. The enzyme catalase, found in aerobic organisms, plays a vital role in the decomposition of hydrogen peroxide, as demonstrated by the following reaction:

the conversion of hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen gas (O₂) is represented by the chemical equation provided. The absence of catalase activity was identified as a crucial property of the probiotic bacteria isolated during this investigation. Because these probiotic bacteria do not produce catalase, they are unable to catalyze the breakdown of hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂) due to this lack of activity. According to the results of the experiments conducted by Bazireh et al. (2020), the absence of visible bubbles during the experiment indicated no catalase activity in the environment. Catalase activity is a characteristic commonly associated with *Lactobacillus* bacteria.

Lactic acid bacteria are distinguished because they are catalase-negative, as the enzyme is necessary to break H₂O₂ into water and oxygen. This is a trait that sets them apart from other types of bacteria. For their research, Forouhandeh et al. (2010) worked on isolating bacteria capable of producing lactic acid from standard cheese and yogurt and locally produced yogurt and cheese. Afterward, they investigated the biochemical properties of these bacteria by exposing them to various carbon sources and observing the results. During this inquiry, it was found that the fermentation of glucose in Durham's tube resulted in acid production without gas, which was a significant finding. In addition, 91.63% of the bacterial strains could utilize sugars like glucose, 83.33% could ferment lactose, and 66.64% could ferment both maltose and fructose. A wide range of fermentation activity was observed in the various sugars tested, including mannose, galactose, sucrose, starch, and arabinose, with utilization rates of 58.31%, 49.98%, 41.63%, 16.66%, and 8.33%, respectively. These researchers isolated 25 strains of lactic acid bacteria from humpbacked camel milk and investigated their capacity to ferment glucose, lactose, and maltose. The results of the present study coincided with the findings reported by Khedid et al. (2009). The approach for carbohydrate fermentation may differ from one investigation to another. For example, some research has employed standard rapid biochemical test kits (Chammas et al., 2006), while other research may use a panel of eight different carbohydrate substrates. In their respective study, Chandok et al. (2014) incorporated eight distinct carbohydrate variants into their analyses. Khedid et al. (2009) conducted their study to determine the prevalence of LAB by using a particular set of factors to arrive at their conclusions.

These criteria included evaluating several aspects, such as the capacity to generate gas from glucose, a positive result for methyl red, negative results for Voges-Proskauer and citrate consumption, and positive results for nitrate reduction. Many isolates presented a positive methyl red response due to their capacity to ferment glucose into pyruvic acid. The fact that the isolates were examined this way allows us to attribute this ability to them. Subsequently, these isolated organisms undergo pyruvic acid metabolism via the mixed acid pathway, producing various acids, such as formic acid, acetic acid, and lactic acid. Because of this metabolic process, the pH falls below 4.4, ultimately leading to a positive methyl red response. Despite this, the outcomes of the VP test were adverse, indicating that the microorganisms could not produce the compound known as acetyl-methyl-carbinol during the glucose metabolization process (Khusro et al., 2021). During this experiment, it was observed that the H₂S generation test did not yield any noticeable blackening of the medium or change in the color of the media. This was one of the findings. Based on these data, it would appear that the isolates examined did not possess the ability to transform sulfur molecules into sulfide enzymatically. According to the study, the combination of sulfide and iron compounds may have impeded the organisms' ability to convert sulfur compounds into sulfide, creating the black precipitate known as FeS (Chen et al., 2022). Additionally, the indole production test results showed that the isolates could not utilize the tryptophanase enzyme, which is necessary to produce indole from tryptophan. Adding Kovac's reagent did not result in a noticeable change to the surface layer, as the layer continued to exhibit its yellow hue rather than undergoing a color change to a more coppery shade. Only one of the 16 isolates tested could utilize citrate, and that isolate was designated OS1 based on the results of the citrate utilization tests. Conversely, none of the other 15 isolated strains could utilize citrate. The findings of the current studies coincide with those of Ayhan et al. (2005), who likewise observed that nine strains derived from fermented goat milk had negative results when tested for citrate. These previous researchers arrived at the same conclusion as those conducting the current work. The citrate test demonstrated that the isolated bacteria could not utilize citrate as their only energy and carbon source, as determined by their failure in the test. This was evident because none of the test tubes turned blue in the presence of citrate. Consequently, the bacteria could not produce

either ammonia or sodium bicarbonate independently. This was a direct consequence of the issue.

The concept of "probiotics" refers to strains of bacteria, and studies have shown that taking these strains can improve one's overall health and well-being. According to Hou et al. (2023), the Food and Agriculture Organization (FAO) recommends using probiotics because of their well-established safety profile for the organism they are being administered. For safety purposes, it is strongly suggested that you utilize strains that do not demonstrate any signs of hemolytic activity. This proves the strains in question do not exhibit the virulence features typically associated with infectious diseases. In the course of the research currently being conducted on LAB strains and the varying degrees of hemolytic activity that each strain possesses, it was found that 16 of the strains had a phenotype that was not hemolytic. According to this research, these bacteria contain positive qualities that make them ideal for potential probiotic utilization. The results revealed in this study align with the findings reported in a previous investigation conducted by Asadi et al. (2022). In previous investigations, the scientists identified multiple non-hemolytic *Lactobacillus* species in diverse alcoholic beverages and food products derived from millet. According to a study conducted by Wang et al. (2018), it was observed that LAB derived from food products that undergo natural fermentation and are free of dairy ingredients—in China had no probiotic hemolytic activity. One of the findings of the investigation was that. Several experiments conducted by Chino et al. (2023) have consistently shown that probiotics cannot break down hemoglobin during hemolytic activity. Consequently, this raises inquiries concerning the safety of their utilization as probiotics. The KIA test is employed to quantify the bacterial metabolic utilization of dextrose and can be conducted in either a fermentative or oxidative manner. The ability of the lactic acid bacteria found in this mixture to engage in lactose or dextrose fermentation, leading to noticeable alterations in the pH indicator's color (phenol), proves valuable in distinguishing between various strains of lactic acid bacteria. The observed variations in hue can be attributed to the generation of acids during sugar fermentation. Following the insertion of a needle into the agar of the KIA medium slants and subsequent distribution of the culture across the slants' surfaces, contamination was observed in the KIA medium slants. The technique was executed continuously for one

day. Altarugio et al. (2018) reported that modifications in the hue or inclination of the posterior region, the generation of hydrogen sulfide (H₂S), and the formation of gas exhibited analogous results when subjected to incubation at 37°C temperature for a duration of 18 to 24 hours.

After being incubated, our investigation unveiled that, except for BM2, all the strains of LAB that were examined exhibited indications of proliferation. In a study conducted by Amin et al. in 2023, it was observed that the growth of LAB (lactic acid bacteria) experienced inhibition within the temperature range of 10 to 42 degrees Celsius. These findings are substantiated by prior studies that yielded comparable results (Wei et al., 2022). Furthermore, it is noteworthy that all the laboratory isolates exhibited outstanding resistance to different salt concentrations, even when subjected to a concentration of 2% sodium chloride (NaCl) (Khusro et al., 2021). This discovery was documented in the scholarly publication authored by Khusro et al. in 2021. Among the several strains that were subjected to testing, it was observed that only one strain, namely BM2, exhibited notable resistance when exposed to a 10% concentration of sodium chloride (NaCl). This finding is visually represented in Figure 4.16 (A, B). This observation was made after conducting experiments on various strains. In contrast, when subjected to a sodium chloride (NaCl) solution with a concentration of 6.0%, six distinct strains exhibited a notable inhibition in their growth. A study conducted by Isono et al. (1994) demonstrated that four strains of lactic acid bacteria derived from fermented milk displayed the ability to endure in an environment with a 4% sodium chloride (NaCl) concentration. The findings of this inquiry were disseminated in the scholarly journal *Microbiology*. Furthermore, it has been shown that one of the isolated organisms exhibits the ability to survive in a medium with a higher sodium chloride content (6.5%). Khedid et al. (2009) conducted a study that yielded comparable findings, determining that only 0.5% of the strains exhibited signs of growth under the influence of sodium chloride (NaCl) concentrations of 8%. Conversely, it was observed that 50% of the LAB isolated from camel milk exhibited the ability to survive at a temperature of 40°C. Based on the research conducted by Di Martino et al. (2023) and Hatami et al. (2022), it has been observed that the *Bacillus licheniformis* (BAL) bacterial group exhibits a notable preference for environments with elevated salinity

levels. These studies indicate that the optimal growth of BAL bacteria requires a salt concentration ranging from 5 to 30 percent. This observation is derived from recognizing that this particular group has been scrutinized. Various types of lactic acid bacteria exhibit variable degrees of survival potential on agar plates containing different concentrations of sodium chloride (NaCl). As an illustration, Bazireh et al. (2020) found that throughout the fermentation process, three genera, namely *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, could flourish and multiply under high salinity conditions. The findings point to the potential of LAB probiotics, as it has been demonstrated that these probiotics boost the synthesis of beneficial metabolites and encourage the proliferation of beneficial bacteria. The qualities of the LAB strains discussed thus far are of the utmost significance when applied to the context of manufacturing processes, the promotion of innovation, and the guarantee of long-term preservation.

According to the findings of the current study, it was observed that the LAB strains tested exhibited significant growth and maximum efficiency at a temperature of 37°C, regardless of the type of LAB strain. Additionally, these strains demonstrated remarkable resistance to a NaCl concentration of 2% ($p < 0.05$) despite their growth being inhibited at temperatures of 10°C and 42°C. The findings of this study align with those of other studies and indicate that LAB strains have the potential to function as probiotics. These strains offer desirable qualities for both industrial and preservation applications. The ability of probiotics to tolerate the harsh conditions of the digestive system—including exposure to bile and acid, as well as resistance to antibiotics—has been linked to various health advantages (Yan et al., 2022; Xing et al., 2016). Thus, it is crucial to characterize potential probiotics to determine their growth potential and viability within the gastrointestinal tract (GIT). Therefore, conducting in vitro experiments using traditional methods is necessary. The ability of prospective probiotic bacteria to survive intestinal bile salts significantly influences the selection of bacteria for probiotic use. This factor is important (Le et al., 2023; Amin et al., 2023) in facilitating the growth and sustenance of organisms within the GIT. To successfully colonize and thrive within the GIT of their host, probiotics must resist both bile salts and acidic environments. Upon entering the GIT, most exogenous bacteria experience a high mortality rate due to exposure to acidic

gastric juice, characterized by a pH of 2.0, leading to the extinction of microorganisms. Previous studies have emphasized the resistance of probiotic bacteria to acidic environments with pH levels from 1.0 to 4.0 and high concentrations of bile salts (approximately 2.5% w/v) for a minimum duration of 90 minutes (Lim & Im, 2009; Xing et al., 2016). This capability enables probiotic bacteria to flourish in conditions that may harm other bacterial species. This study examined 16 distinct strains to assess their resilience under low pH (1) and high bile salt concentrations (2.5% w/v) over 180 minutes. A total of ten isolates exhibited significant growth and high survival rates exceeding 68%. Notably, the strains C4, C3, CM1, and BM2 demonstrated exceptional growth and resistance in acidic environments, particularly at a pH of 1. The strains CM2, OS1, and OS6 could also withstand pH values as low as 2. The membrane structure of LAB strains likely contributes to their extraordinary tolerance capabilities, enabling these strains to improve their stress tolerance rapidly. Earlier research has revealed that *Lactobacillus* strains can withstand harsh conditions more than bacteria from other genera within the LAB group (Zhang et al., 2020). The results obtained lend support to these conclusions. In an acidic environment, various observations were made regarding the effect of bile salts on probiotic bacterial cells. Additionally, it was revealed that the probiotics' resistance to bile salts was unexpectedly and noticeably higher than their levels of acid tolerance. Prior studies conducted by Wang et al. (2023) found that chicken-specific LAB strains maintain good viability when exposed to an acid-rich environment with a pH of 2.0, simulating gastric juice. The findings of the research, as mentioned above, support this observation. Research by Cvrtila Fleck et al. (2012) showed that fermented sausages contain similar strains of LAB, such as *L. plantarum* and *L. pentosus*, which thrive in acidic environments. Due to their greater tolerance to high bile salt conditions and low pH (>1% w/v), we selected the CM1 and OS1 LAB strains for molecular identification in our study. This choice was made to compare them with other LAB strains and evaluate their performance. These findings highlight the importance of selecting potential probiotic strains capable of surviving and thriving efficiently in the gastrointestinal tract, providing their hosts with the necessary health benefits. Our work focused on determining the extent to which the isolated *Lactobacillus delbrueckii* OS1 and *Lactobacillus acidophilus* CM1 possessed antibacterial properties and how those properties compared to one another. According to our research, these

strains demonstrated higher antibacterial activity levels than other types of bacteria, suggesting their potential use as probiotics. The findings of Lashani et al. (2020) further corroborate our research, demonstrating that these strains release numerous compounds contributing to their antimicrobial activity, including bacteriocins, biosurfactants, hydrogen peroxide, and organic acids. Additionally, our research indicated that Gram-positive bacteria such as *B. cereus*, *S. aureus*, and *E. faecalis* were more susceptible to the effects of *L. acidophilus* CM1 than Gram-negative bacteria such as *S. typhimurium* and *E. coli*. *L. delbrueckii* OS1 exhibited significantly higher antagonistic activity against Gram-negative bacteria. Our findings are consistent with those reported in earlier research conducted by Shin et al. (2008), Taheri et al. (2009), GarcaHernández et al. (2016), Oyewole et al. (2018), and Ayodeji et al. (2017). In light of the findings described above, the addition of the probiotic strains *L. acidophilus* strain CM1 and *L. delbrueckii* strain OS1 to chicken feed holds the potential to preserve and improve the health of the gastrointestinal tract and to offer some measure of defense against infectious diseases caused by pathogens. In a study conducted by (Kizerwetter-Swida et al., 2005), it was found that LAB strains exhibited more pronounced inhibitory effects against Gram-positive pathogens, including *Clostridium perfringens* and *Staphylococcus aureus*, compared to their effects on Gram-negative pathogens, including *E. coli* and *Salmonella*. The conclusions drawn in this study were derived from a comparative analysis of the inhibitory effects demonstrated by the two distinct categories of pathogens. Based on the research conducted by De Almeida Jnr. et al. (2015), no significant link could be determined between the Gram type of pathogens examined and the extent of antagonistic activity exhibited by LAB. Based on the research conducted by Spanggaard et al. (2001), it has been determined that probiotics can eliminate pathogens and thus inhibit the proliferation of heterochthonous bacteria within the gastrointestinal tract (GIT). This finding lends weight to the concept that the administration of indigenous microbial flora in the form of probiotics can substantially impact the elimination of infectious diseases. In the research carried out by Jose et al. (2015), it was found that Lactic acid bacterial strains acquired from the rumen were significantly more effective at preventing the growth of pathogens than LAB strains obtained from dairy sources. This observation was made based on the finding that LAB strains originating from the rumen exhibited a higher degree of effectiveness in suppressing the development of pathogens. During our

investigation, we encountered two distinct probiotic species. Subsequently, both species were characterized, and genotypic evidence proved that they belong to the *Lactobacillus* genus. Each indigenous probiotic strain exhibits distinct characteristics that could make it valuable in several domains, including cosmetics, food, and pharmaceuticals. The outcomes of this research highlight the significance of oral and dietary sources as potential repositories of novel probiotic bacteria with advantageous functional traits and properties.

An examination of the data in Tables 4.22 to 4.27 indicates significant variations in protein content among the treatments involving blending all three types of millet milk with apple and pineapple juice separately. The protein content varied between 0.24% and 0.68% across the treatments. The highest protein content, at 0.68%, was observed in the treatment where foxtail millet milk was blended with apple juice in a 50:50 ratio. Conversely, the lowest protein content of 0.24% was recorded in the treatment involving finger millet milk blended with apple juice at the same ratio.

Furthermore, there were noteworthy differences in fat content among the treatments that combined all three types of millet milk with pineapple and apple juice. The fat content across these treatments ranged from 0.10% to 0.19%. The treatment containing a 50:50 blend of foxtail millet milk and pineapple juice exhibited the highest fat content at 0.19%. In contrast, the treatment consisting of a 50:50 blend of barnyard millet milk and pineapple juice displayed the lowest fat content at 0.10%.

The data presented in Tables 4.22 to 4.27 indicate significant variations in fiber content among the treatments that involved blending millet milk with pineapple and apple juice. The fiber content ranged from 0.13% to 0.41% across these treatments. Notably, the treatment combining foxtail millet milk with apple juice in a 50:50 ratio exhibited the highest fiber content at 0.41%. The lowest fiber content of 0.13% was observed in the treatment involving barnyard millet milk with apple juice at the same ratio. Additionally, the results in Table 4.30 highlight significant differences in pH among the various treatments that combined millet milk with apple and pineapple juice. The pH values ranged from 3.11 to 3.67, with the highest pH recorded in the treatment consisting of foxtail millet milk blended with pineapple juice. Conversely, the lowest pH was observed

in the treatment involving barnyard millet milk blended with pineapple juice in a 50:50 ratio.

To maintain sensory characteristics and minimize exposure to oxygen, beverages have traditionally been sealed with either natural or plastic corks and packaged in glass containers. Glass containers offer several advantages, including excellent resistance to gases and vapors, long-term stability, transparency, and recyclability. They provide an almost perfect barrier against environmental factors such as gases and vapors, ensuring high stability and efficiency in recycling (Ramos et al., 2015). In our current research, we also observed that plastic bottle changes were more noticeable than glass bottles. Furthermore, it is essential to note that refrigerated storage is preferable to ambient conditions for prolonging the shelf life of probiotic beverages.

Chapter 5

Summary and conclusion

Summary and conclusion: The investigation titled "Production and Nutrient Analysis of Novel Millet and Fruit Juice-based Probiotic Beverage Using Probiotic Lactic Acid Bacterial Strains" yielded several key findings, summary, and conclusions:

- Isolation of Lactic Acid Bacterial (LAB) Strains: The investigation commenced by employing a random selection process to choose 60 bacterial isolates sourced from a diverse range of food origins, encompassing milk, curd, pickle, fermented batter, and oral cavity samples. Following an initial screening process and subsequent biochemical analysis, 16 bacterial isolates were selected for further examination. These isolates were subsequently identified as lactic acid bacteria (LAB).
- Characterization of Probiotic Properties: Among the 16 LAB isolates, two CM1 and OS1 strains exhibited probiotic characteristics. These strains displayed high resistance to bile salts at various concentrations (0.5, 1, 1.5, and 2), tolerance to acidic pH levels (1, 2, 3, and 4), and demonstrated broad-spectrum inhibition against microbial pathogens, including *Staphylococcus aureus*, *E. coli*, *Enterococcus faecalis*, *Salmonella typhimurium*, and *Bacillus cereus*.
- Identification via 16S rRNA Characterization: The two probiotic strains, CM1 and OS1, were further identified through 16S rRNA characterization. CM1 was identified as *Lactobacillus acidophilus*, while OS1 was identified as *Lactobacillus delbrueckii*. These sequences were submitted to the NCBI database and assigned accession numbers OP811266 for CM1 and OP824643 for OS1.
- Selection of Starter Cultures: CM1 and OS1 were the starter cultures used to develop the millet-based probiotic beverage. This selection was likely based on their probiotic attributes and suitability for fermentation.
- Statistical optimization of physical factors and media to achieve high yield of cells: Plackett-Burman (P-B) design for analyzing significant variables for the growth of particular micro-organisms. Optimizing the medium using the Response Surface Method (RSM) involved central composite design (CCD) within Design Expert version 12 statistical software. This allowed for the determination of optimal experimental conditions, the creation of response surface visualizations, and the statistical assessment of collected data.
- Selection of Millet Variety: The study also indicated that selecting one millet variety over another is essential for the development of the probiotic beverage. The

three millet varieties, Finger millet, foxtail millet, and barnyard millet, were found to have higher levels of fat, proteins, carbohydrates, and various minerals.

- Beverage Development and Evaluation: The probiotic beverage was developed by blending the selected millet variety with fixed apple and pineapple juice concentrations. The final products were evaluated for various physicochemical characteristics, viability of the probiotic strain, and sensory acceptability.
- Millet blended with apple and pineapple juices was prepared and subjected to analysis for its physicochemical properties. During fermentation using the *L. acidophilus* strain CM1 and *L. delbrueckii* strain OS1 starter culture at 37°C for 48 hours, a notable decrease in pH and a concurrent increase in acidity were detected across all treatments. Additionally, after fermentation, all treatments exhibited a total viable cell count of 10^8 CFU/ml.
- Among the different treatment combinations, foxtail millet milk and apple juice, in a 50:50 ratio, emerged as the most favorable choice based on a comprehensive evaluation of various physicochemical characteristics, strain viability, and sensory attributes.
- Throughout the storage period, a slight decline was observed in the physicochemical and sensory attributes of the beverages. Notably, the choice of packaging material played a significant role, with glass bottles outperforming plastic containers in maintaining the quality of the probiotic beverages. Moreover, the storage conditions had a pronounced impact, as the beverages stored under refrigerated conditions exhibited superior stability to those kept under ambient conditions. The samples stored at ambient temperature had to be discarded after 15 days due to increased acidity and an inferior taste, whereas the refrigerated samples demonstrated a longer shelf life.
- Crucially, the probiotic bacteria could retain the recommended viable cell concentration of 10^6 CFU/ml until the end of the 21-day storage period when stored under refrigerated conditions. This underscores the suitability of millet milk and fruit juices as ideal matrices for delivering and preserving probiotics.
- The study revealed that blending millet milk with apple and pineapple juices and fermenting them with CM1 and OS1 yielded a probiotic beverage with favorable characteristics. Proper packaging in glass bottles and storage under refrigerated

conditions were critical factors in maintaining beverage quality and shelf life. These findings emphasize the potential of millet-based beverages as effective carriers for probiotics, offering a nutritious and probiotic-rich product.

- **Storage Stability Assessment:** The developed beverages were then stored in glass and plastic bottles under refrigerated and ambient conditions to assess their storage stability over time.

In summary, this study has yielded several valuable conclusions that all three millet varieties exhibited superior physicochemical and nutritional characteristics compared to the hybrid millet. This finding highlights the potential of local millet as a preferred choice for producing nutritious and probiotic-rich beverages. The isolated *Lactobacillus acidophilus* CM1 and *Lactobacillus delbrueckii* OS1 demonstrated all the necessary characteristics to serve as an influential starter culture for developing probiotic beverages. Its probiotic attributes made it an ideal choice for fermentation. Among the various formulations tested, the beverage prepared by blending Foxtail millet with apple juice in a 50:50 ratio exhibited the highest quality characteristics. This formulation excelled in physicochemical properties, strain viability, and sensory scores compared to other treatments. Glass bottles, when stored under refrigerated conditions, were found to be the most effective in maintaining beverage quality. These conditions resulted in minimal acidity, pH, and viable cell count changes over time. Overall, this investigation has successfully developed a process for producing a probiotic millet milk-based beverage with improved sensory qualities. Using underutilized crops like foxtail millet to create value-added products with healthful properties is a promising approach. This probiotic foxtail millet milk-based fermented beverage can potentially enhance nutritional status and energy density, addressing the issue of malnutrition effectively.

In conclusion, these findings suggest that the study successfully identified and characterized probiotic LAB strains from diverse food sources, selected a suitable strain for beverage development, and demonstrated the nutritional advantages of using a local millet variety. The development of millet-based probiotic beverages could offer consumers a nutritious and probiotic-rich option. This research has also paved the way for the production of probiotic-rich millet-based beverages that offer health benefits

and efficiently use locally available resources. Such products have the potential to contribute positively to nutrition and combat malnutrition issues.

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APPENDIX - I

Media used

1. Lactobacillus MRS Agar

| Ingredients | Gms/liter |
|--------------------------------|-----------|
| Proteose peptone Final | 10.000 |
| HM Peptone B # | 10.000 |
| Dextrose (Glucose) | 20.000 |
| Tween 80 (Polysorbate 80) | 1.000 |
| Ammonium citrate | 2.000 |
| Yeast extract | 5.000 |
| Sodium acetate | 5.000 |
| Magnesium sulphate | 0.100 |
| Manganese sulphate | 0.050 |
| Dipotassium hydrogen phosphate | 2.000 |
| Agar | 12.000 |
| Final pH (at 25°C) | 6.5±0.2 |

2. Lactobacillus MRS broth

| Ingredients | Gms / Litre |
|-------------|-------------|
| HM peptone | 10.000 |

| | |
|----------------------------------|---------|
| HM peptone B | 10.000 |
| Diammonium citrate | 2.000 |
| Yeast extract | 5.000 |
| Dextrose (Glucose) | 20.000 |
| Dipotassium hydrogen phosphate | 2.000 |
| Manganese sulphate, tetrahydrate | 0.050 |
| Magnesium sulphate, heptahydrate | 0.200 |
| Sodium acetate, trihydrate | 5.000 |
| Final pH (at 25°C) | 5.4±0.2 |

3. Motility Test Medium

| Ingredients | Gms / Litre |
|--------------------|--------------------|
| Tryptose | 10.000 |
| Agar | 5.000 |
| Sodium chloride | 5.000 |
| Final pH (at 25°C) | 7.2±0.2 |

4. MR-VP broth

| Ingredients | Gms / Litre |
|--------------------|--------------------|
| Buffered peptone | 7.000 |

| | |
|-----------------------|----------------------|
| Dextrose | 5.000 |
| Dipotassium phosphate | 5.000 |
| Final | pH (at 25°C) 6.9±0.2 |

5. Tryptone Broth

| Ingredients | Gms / Litre |
|--------------------|--------------------|
| HM peptone | 10.000 |
| Sodium chloride | 5.000 |
| DL-Tryptophan | 1.000 |
| Final pH (at 25°C) | 7.2±0.2 |

6. Simmons citrate Agar

| Ingredients | Gms / Litre |
|-------------------------------|--------------------|
| Sodium chloride | 5.000 |
| Magnesium sulphate | 0.200 |
| Ammonium dihydrogen phosphate | 1.000 |
| Dipotassium phosphate | 1.000 |
| Sodium citrate | 2.000 |
| Bromothymol blue | 0.080 |
| Agar | 15.000 |

| | |
|--------------------|---------|
| Final pH (at 25°C) | 6.8±0.2 |
|--------------------|---------|

7. Nitrate broth

| Ingredients | Gms / Litre |
|--------------------------------|-------------|
| Peptic digest of animal tissue | 5.000 |
| Beef extract | 3.000 |
| Potassium nitrate | 1.000 |
| Agar | 12.000 |
| Final pH (at 25°C) | 6.8±0.2 |

8. Casein Agar

| Ingredients | Gms/liter |
|--------------------------------------|-----------|
| Soluble starch | 10.0 |
| Casein (Vitamin free) | 0.30 |
| KNO ₃ | 2.0 |
| MgSO ₄ .7H ₂ O | 0.05 |
| K ₂ HPO ₄ | 2.0 |
| NaCl | 2.00 |
| CaCO ₃ | 0.02 |
| FeSO ₄ .7H ₂ O | 0.01 |

| | |
|------------------|----------|
| Agar | 18.0 |
| Final pH at 25°C | 7.3 ±0.2 |

9. Kligler's Iron Agar (KIA)

| Ingredients | Gram/Liter |
|--------------------|------------|
| Beef extract | 3 gm |
| Yeast extract | 3 gm |
| Peptone | 15 gm |
| Proteose peptone | 5 gm |
| Lactose | 10 gm |
| Glucose | 1 gm |
| Ferrous sulfate | 0.2 gm |
| Sodium chloride | 5 gm |
| Sodium thiosulfate | 0.3 gm |
| Agar | 12 gm |
| Phenol red | 0.024 gm |
| Final pH | 7.4 |

10. Blood Agar

| Ingredients | Gms / Litre |
|-----------------|-------------|
| Tryptone | 14.000 |
| Peptone | 4.500 |
| Yeast extract | 4.500 |
| Sodium chloride | 5.000 |
| Agar | 12.500 |

| | |
|--------------------|---------|
| Final pH (at 25°C) | 7.3±0.2 |
|--------------------|---------|

11. Arginine dihydrolase broth

| Ingredients | Gms / Litre |
|--------------------------------|-------------|
| Peptic digest of animal tissue | 1.000 |
| Sodium chloride | 5.000 |
| Dipotassium hydrogen phosphate | 0.300 |
| L-Arginine | 10.000 |
| Bromo cresol purple | 0.016 |
| Agar | 3.000 |
| Final pH (at 25°C) | 6.0±0.2 |

12. Mueller Hinton Agar

| Ingredients | Gms / Liter |
|-------------------------|-------------|
| Beef, infusion from | 300.0 |
| Casein acid hydrolysate | 17.50 |
| Starch | 1.50 |
| Agar | 17.00 |
| Final pH (at 25°C) | 7.3±0.1 |

13. SIM Media

| Ingredients | Grams / litre |
|---------------------|----------------------|
| Peptone | 30.0 |
| Meat extract B# | 3.0 |
| Peptonised iron | 0.20 |
| Sodium thiosulphate | 0.025 |
| Distilled water | 1000ml |

14. EMB Agar

| Ingredients | Gms / Litre |
|--------------------------------|--------------------|
| Peptic digest of animal tissue | 10.000 |
| Dipotassium phosphate | 2.000 |
| Lactose | 5.000 |
| Sucrose | 5.000 |
| Eosin – Y | 0.400 |
| Methylene blue | 0.065 |
| Agar | 13.500 |
| Final pH (at 25°C) | 7.2±0.2 |

15. Salmonella Shigella Agar

| Ingredients | Gms / Litre |
|--------------------|--------------------|
|--------------------|--------------------|

| | |
|---------------------|---------|
| Proteose peptone | 5.000 |
| Lactose | 10.000 |
| Bile salts mixture | 8.500 |
| Sodium citrate | 8.500 |
| Sodium thiosulphate | 8.500 |
| Ferric citrate | 1.000 |
| Brilliant green | 0.00033 |
| Neutral red | 0.025 |
| Agar | 13.500 |
| Final pH (at 25°C) | 7.0±0.2 |


Appendix – II

Publications and conferences

- **Publications**

1. Role of Plant-Derived Prebiotic in Modulation of Human Gut Microflora: A Review
DOI: [10.30699/ijmm.16.5.368](https://doi.org/10.30699/ijmm.16.5.368)
Rajput, K., Dohroo, A., Devgon, I., & Karnwal, A. (2022). Role of Plant-Derived Prebiotic in Modulation of Human Gut Microflora: A Review. *Iranian Journal of Medical Microbiology*, 16(5), 368-375.
2. Characterization and selection of probiotic lactic acid bacteria from different dietary sources for development of functional foods DOI: [10.3389/fmicb.2023.1170725](https://doi.org/10.3389/fmicb.2023.1170725)
Khushboo, Karnwal, A., & Malik, T. (2023). Characterization and selection of probiotic lactic acid bacteria from different dietary sources for development of functional foods. *Frontiers in Microbiology*, 14, 1170725.

Characterization and selection of probiotic lactic acid bacteria from different dietary sources for development of functional foods

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Introduction: Dietary sources have an abundance of bacteria, mainly lactic acid bacteria (LABs), which have long been regarded as probiotics in humans and animals. Lactic acid bacteria (LAB) have been used as probiotic agents due to their ability to produce a variety of beneficial compounds for cultivars and their status as safe microorganisms.

Methods: In this current study, the lactic acid bacteria (LAB) were isolated from several dietary sources such as curd, pickle, milk, and wheat dough. The principal focus of this study was to determine the survivability of these microorganisms in the gastrointestinal tract and to use promising strains to create probiotic drinks with numerous health benefits. The isolates were identified using a combination of morphological, biochemical, molecular and sugar fermentation patterns, like phenotypic characteristics, sugar fermentation, MR-VP reaction, catalase test, urease test, oxidase test, H₂S production, NH₃ production synthesis from arginine, citrate utilization, indole test, and 16s rRNA sequencing.

Results: Two (CM1 and OS1) of the 60 isolates obtained showed the best probiotic results and were identified as *Lactobacillus acidophilus* CM1 and *Lactobacillus delbrueckii* OS1. These organism sequences were submitted to Gen bank with accession numbers OP811266.1 and OP824643.1, respectively. The acid tolerance test results indicated that most strains could survive significantly in an acidic environment with pH levels of 2 and 3. Similarly, the salt tolerance test results showed that both *Lactobacillus acidophilus* CM1 and *Lactobacillus delbrueckii* OS1 could survive at 4 and 6% NaCl levels significantly. The isolates also showed their ability to ferment sugars such as lactose xylose, glucose, sucrose, and fructose.

Discussion: In conclusion, the study showed that the bacteria isolated from different food sources were indeed probiotic lactic acid bacteria and had probiotic properties. These isolates hold potential for future

Role of Plant-Derived Prebiotic in Modulation of Human Gut Microflora: A Review

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Abstract: (1104 Views)

At the time of the coevolution of humans and microorganisms, the human digestive tract was colonized by thousands of species of bacteria. Mostly, intestine-borne microbes amount to the overall number of cells in the body tissue. The latest metagenomics study of the human intestinal microbiota confirmed the existence of some 3.3 million genes relative to only 23,000 genes found in tissue cells in the human body. There is increasing evidence for multiple beneficial functions of the gut microbiota in human health and illness. The best-described plant prebiotics is fructans and inulin. The best-known prebiotic carbohydrates comprise many plants, roots and tubers, and fruit crops, whereas prebiotic-rich grain crops contain maize, chickpea, lentil, lupin, and wheat. Some prebiotic enriched crop germplasm were documented in maize, chickpea, lentil, wheat, and yacon. Intestinal microbiota perturbation may contribute to persistent diseases such as autoimmune diseases, bowel cancers, stomach ulcers, colon disorders, and malnutrition. This can be impossible to recover the intestinal microbiome, but the usage of probiotics has contributed to a positive effect in a significant number of very well-designed (clinical) trials. Microbiomics has prompted a significant growth of interest in probiotics and prebiotics as potential mediators for the administration and regulation of gut microbiota in medicine, industry, and the general public. Developing prebiotic-rich healthy plants can mitigate the prevalent malnutrition challenge and facilitate worldwide global health. Bioinformatics and genomics tools may help to create mechanistic associations between gut microflora, a person's health status, and the outcomes of plant prebiotic drug treatments.

Keywords: Microbiota, Intestinal microbes, Microbiomics, Plant-derived carbohydrates, Probiotics

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