# PHYSICOCHEMICAL AND MICROBIAL PROFILING OF KEFIR FROM COW AND BUFFALO MILK: IMPLICATIONS FOR PROBIOTIC USE

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# RECOMMENDATION

This is to certify that the research work entitled **"Physicochemical and microbial profiling of kefir from cow and buffalo milk: Implications for probiotic use"** has been carried out by **Ms. Sushmita Soni** under my supervision. This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 653. The result presented here is her original findings. We hereby recommend this thesis for final evaluation.


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# CERTIFICATION OF EVALUATION

This is to certify that the thesis entitled "Physicochemical and microbial profiling of kefir from cow and buffalo milk: Implications for probiotic use" presented to evaluation committee by **Ms. Sushmita Soni** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Sushmita Soni

# LIST OF ABBREVIATIONS

| | |
|---|---|
| LAB | Lactic Acid Bacteria |
| GERD | Gastroesophageal Reflux Disease |
| QS | Quorum Sensing |
| TSS | Total Soluble Solids |
| FFA | Free Fatty Acids |
| HDL | High-Density Lipoprotein |
| LPS | Lipopolysaccharide |
| ACE | Angiotensin-Converting Enzyme |
| CC14 | Carbon Tetrachloride |
| IBD | Inflammatory Bowel Disease |
| GRAS | Generally Recognized As Safe |
| VSC | Volatile Sulfur Compounds |
| BSH | Bile Salt Hydrolase |
| GIT | Gastrointestinal Tract |
| TA | Teichoic Acid |
| MRS AGAR | De Man, Rogosa, and Sharpe Agar |
| YEGA | Yeast Extract Glucose Agar |
| TE | Tris-EDTA |
| 16SrRNA | 16S Ribosomal RNA |
| 18SrRNA | 18S Ribosomal RNA |
| PCR | Polymerase Chain Reaction |
| OD | Optical Density |
| PBS | Phosphate-Buffered Saline |
| MHA | Mueller-Hinton Agar |
| RID | Refractive Index Detector |
| NaCl | Sodium Chloride |
| ATCC | American Type Culture Collection |
| mL | Milliliter |
| µL | Microliter |
| mg/ml | Milligram per milliliter |

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# ABSTRACT

Kefir, a traditional fermented dairy product, is valued for its unique taste, thickness, and health benefits attributed to its rich microbial diversity. This study aimed to investigate the processing, physiochemical properties, and probiotic profile of kefir, particularly focusing on its therapeutic potential. The introduction highlights kefir's historical and contemporary significance, including its increasing popularity as a functional food. The problem statement addresses the rising prevalence of digestive issues and the need for effective dietary interventions like kefir. Kefir, with its high probiotic content, offers potential health benefits, including improved gastrointestinal health and immune function. The specific objectives include producing kefir using traditional fermentation methods, identifying the microbial strains present, and characterizing their probiotic properties. The study utilized traditional kefir fermentation methods with kefir grains. Physiochemical analyses of kefir and metagenomic profiling of lactic acid bacteria and yeasts from kefir were conducted. This study investigated the isolation, identification, and probiotic characterization of lactic acid bacteria (LAB) and yeast from kefir, identifying strains such as *Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus fermentum, Kazachstania martinaie,* and *Pichia chibdodasensis*. The isolates exhibited distinct morphological, biochemical, and genetic characteristics, confirmed through PCR amplification of the 16S rRNA and 18S rRNA genes. Among the LAB, *Lactobacillus helveticus* showed the highest acid tolerance at lower pH levels (2.5 and 3), while *L. rhamnosus* performed best at mildly acidic to neutral conditions (pH 5.6). *L. helveticus* also demonstrated superior bile salt tolerance, autoaggregation (65%), coaggregation (30%), and hydrophobicity (40%). All strains tolerated lower salt concentrations (2% and 5%) well, with *L. rhamnosus* showing the highest overall salt tolerance. *L. fermentum* exhibited the highest phenol tolerance, while *L. rhamnosus and L. fermentum* formed biofilms, unlike *L. helveticus*. Lastly, *L. helveticus* displayed the highest bile salt hydrolase activity (+++), followed by moderate activity in *L. rhamnosus and L. fermentum* (++). *L. helveticus* showed superior antibiotic susceptibility, and notable antimicrobial activity, particularly against *E. coli* and *Staphylococcus aureus*. In contrast, *L. rhamnosus* exhibited effectiveness against *Pseudomonas aeruginosa*. These findings indicate *L. helveticus* as a promising probiotic candidate, suggesting its application in enhancing gastrointestinal health. Buffalo milk kefir differs notably from cow dairy milk kefir in pH, acidity, Degree Brix, and HPLC-measured concentrations of organic acids and sugars. Buffalo milk kefir shows higher pH (4.7 ± 0.082), lower acidity (1.37 ± 0.024%), and higher lactose content (32.92 ± 0.088 mg/ml) compared to cow dairy milk kefir, which has lower pH (4.5 ± 0.082), higher acidity (1.96 ± 0.033%), and lower lactose content (18.02 ± 0.061 mg/ml). Significant yeast growth, essential for ethanol production, was observed in buffalo milk kefir, contributing to its distinctive flavor and aroma. The findings confirmed kefir's rich probiotic content and beneficial physiochemical properties. These findings support the broader use of kefir in dietary interventions aimed at improving overall health. Further research should focus on standardizing kefir production methods and conducting clinical trials to substantiate its health benefits. Additionally, promoting kefir consumption could help address common digestive health issues, especially in regions with limited access to healthcare.

Keywords : Kefir, fermented dairy product, probiotics, therapeutic potential, digestive health, lactic acid bacteria (LAB), yeasts, antimicrobial activity, functional food, dietary interventions, HPLC, lactic acid.

# संक्षिप्त विवरण

केफिर, परम्परागत रूपमा किण्वित दुग्धजन्य उत्पादन, यसको अनौठो स्वाद, बाक्लोपन, र स्वास्थ्य लाभहरूको लागि महत्त्वपूर्ण मानिन्छ, जसको कारण यसको धनी सूक्ष्मजीव विविधता हो। यस अध्ययनको उद्देश्य केफिरको उत्पादन, भौतिक-रासायनिक गुणहरू, र प्रोबायोटिक प्रोफाइल अनुसन्धान गर्नु थियो, विशेषगरी यसको औषधीय सम्भावनामा केन्द्रित थियो। परिचयले केफिरको ऐतिहासिक र वर्तमान महत्त्वलाई उजागर गर्छ, जसमा यो कार्यात्मक खाद्यको रूपमा बढ्दो लोकप्रियता समावेश छ। समस्या विवरणले पाचन समस्याहरूको बढ्दो प्रसार र केफिरजस्ता प्रभावकारी आहार हस्तक्षेपहरूको आवश्यकता समाधान गर्छ। केफिर, यसको उच्च प्रोबायोटिक सामग्रीका साथ, सम्भावित स्वास्थ्य लाभहरू जस्तै पाचन स्वास्थ्यमा सुधार र रोग प्रतिरोधात्मक कार्यप्रणालीको सुधार प्रस्ताव गर्दछ। विशिष्ट उद्देश्यहरूमा परम्परागत किण्वन विधिहरू प्रयोग गरेर केफिर उत्पादन गर्ने, सूक्ष्मजीवहरूको उपस्थिती पहिचान गर्ने, र तिनीहरूको प्रोबायोटिक गुणहरूको वर्णन गर्ने समावेश छ। अध्ययनले परम्परागत केफिर किण्वन विधिहरू र केफिरका केफिर अन्नहरू प्रयोग गऱ्यो। केफिरको भौतिक-रासायनिक विश्लेषण र केफिरबाट लैक्टिक एसिड ब्याक्टेरिया र यीस्टहरूको मेटाजेनोमिक प्रोफाइलिङ गरियो। यस अध्ययनले केफिरबाट लैक्टिक एसिड ब्याक्टेरिया (एलएबी) र यीस्टको पृथकीकरण, पहिचान, र प्रोबायोटिक वर्णन अनुसन्धान गरेको छ, जस्तै लैक्टोबेसिलस हेल्वेटिकस, लैक्टोबेसिलस रह्मनोसुस, लैक्टोबेसिलस फर्मेन्टम, कजाचस्टानिया मार्टिनाई, र पिचिया चिब्डोडासेंसिस। पृथकहरूमा विभिन्न आकारिक, जैव-रासायनिक, र आनुवंशिक विशेषताहरू देखिएका थिए, जुन १६एस आरएनए र १८एस आरएनए जीनहरूको पीसीआर प्रवर्धन मार्फत पुष्टि गरिएको थियो। एलएबीहरूमा, लैक्टोबेसिलस हेल्वेटिकसले निम्न पीएच स्तरहरू (२.५ र ३) मा उच्चतम एसिड सहनशीलता देखायो, जबकि एल. रह्मनोसुसले हल्का अम्लीयदेखि तटस्थ अवस्थाहरूमा (पीएच ५.६) राम्रो प्रदर्शन गऱ्यो। लैक्टोबेसिलस हेल्वेटिकसले उच्चतम पित्त नुन सहनशीलता, आत्मसमूहण (६५%), सहसमूहण (३०%), र जलप्रतिकर्षण (४०%) देखायो। सबै प्रजातिहरूले निम्न नुन एकाग्रताहरू (२% र ५%) राम्रोसँग सहन गरे, जहाँ एल. रह्मनोसुसले उच्चतम समग्र नुन सहनशीलता देखायो। लैक्टोबेसिलस फर्मेन्टमले उच्चतम फिनोल सहनशीलता देखायो, जबकि एल. रह्मनोसुस र एल. फर्मेन्टमले जैवफिल्महरू बनाए, लैक्टोबेसिलस हेल्वेटिकस विपरीत। अन्ततः, लैक्टोबेसिलस हेल्वेटिकसले उच्चतम पित्त नुन हाइड्रोलस गतिविधि (+++) देखायो, लैक्टोबेसिलस रह्मनोसुस र लैक्टोबेसिलस फर्मेन्टममा मध्यम गतिविधि (++),। लैक्टोबेसिलस हेल्वेटिकसले उच्चतम एन्टिबायोटिक संवेदनशीलता र ई. कोलाई र स्टेफाइलोककस एरियस विरुद्ध उल्लेखनीय रोगाणुरोधी गतिविधि देखायो। विपरीत, एल. रह्मनोसुसले सुडोमोनास एरुजिनोसा विरुद्ध प्रभावकारिता देखायो। यी परिणामहरूले लैक्टोबेसिलस हेल्वेटिकसलाई एक सम्भावित प्रोबायोटिक उम्मेदवारको रूपमा संकेत गर्छ, जसले पाचन स्वास्थ्यलाई सुधार गर्न यसको अनुप्रयोगको सुझाव दिन्छ। भैंसीको दूध केफिरको pH, अम्लता, डिग्री ब्रिक्स, र एचपीएलसी- मापन गरिएको जैविक अम्लहरू र चिनीहरूको सघनतामा गाईको दूध केफिर भन्दा उल्लेखनीय भिन्नता देखिएको थियो। भैंसीको दूध केफिरमा उच्च pH (४.७ ± ०.०८२), निम्न अम्लता (१.३७ ± ०.०२४%), र उच्च लैक्टोज सामग्री (३२.९२ ± ०.०८८ मि.ग्रा./मि.ली.) देखिएको थियो, जबकि गाईको दूध केफिरमा निम्न pH (४.५ ± ०.०८२), उच्च अम्लता (१.९६ ± ०.०३३%), र निम्न लैक्टोज सामग्री (१८.०२ ± ०.०६१ मि.ग्रा./मि.ली.) देखिएको थियो। भैंसीको दूध केफिरमा उल्लेखनीय यीस्ट वृद्धि, जुन इथेनोल उत्पादनको लागि महत्त्वपूर्ण थियो, देखिएको थियो, जसले यसको विशेष स्वाद र सुगन्धमा योगदान पुऱ्यायो। निष्कर्षहरूले केफिरको धनी प्रोबायोटिक सामग्री र लाभदायक भौतिक-रासायनिक गुणहरू पुष्टि गऱ्यो। यी निष्कर्षहरूले समग्र स्वास्थ्य सुधारको उद्देश्यले आहार हस्तक्षेपहरूमा केफिरको व्यापक प्रयोगलाई समर्थन गर्छ। थप अनुसन्धानले केफिर उत्पादन विधिहरूलाई मानकीकरण गर्न र यसको स्वास्थ्य लाभहरू प्रमाणित गर्न क्लिनिकल परीक्षणहरू सञ्चालन गर्न केन्द्रित हुनु पर्छ। साथै, केफिरको उपभोग प्रवर्धन गर्नुले सीमित स्वास्थ्य सेवा पहुँच भएका क्षेत्रहरूमा सामान्य पाचन स्वास्थ्य समस्याहरू समाधान गर्न मद्दत गर्न सक्छ।

**प्रमुख शब्दहरू:** केफिर, किण्वित दुग्धजन्य उत्पादन, प्रोबायोटिक्स, औषधीय सम्भावना, पाचन स्वास्थ्य, लैक्टिक एसिड ब्याक्टेरिया (एलएबी), यीस्ट, रोगाणुरोधी गतिविधि, कार्यात्मक खाद्य, आहार हस्तक्षेपहरू, एचपीएलसी, लैक्टिक एसिड।

# CHAPTER 1: INTRODUCTION

## 1.1 Background

Kefir is a distinctive beverage among fermented dairy products, distinguished by its thickness, acidity, and slight alcoholic content. It is made from milk of cows, goats, camels, sheep, or buffalo fermented by a complex mixture of lactic acid bacteria (LAB) (*Leuconostocs, Lactobacilli, Streptococci, lactococci, Enterobacter, Acinetobacter, Enterococcus, and Pseudomonas spp.*), acetic acid bacteria and yeasts (*Kluyveromyces, Candida, Torulopsis, Saccharomyces, Rhodotorula* and *Zygosaccharomyces* etc.) that inhabit kefir grains or commercial starter cultures (Mitra & Ghosh, 2020). Kefir grains are the principal inoculum used in the fermentation process of milk (Farag et al., 2020). These grains, made of an inert polysaccharide and protein matrix, are home to a high abundance of lactic acid bacteria, acetic acid bacteria, and yeasts. The unique microbial composition provides the unique qualities and nutritional profile to kefir (Leite et al., 2012, 2013). All of these microbes work together to ferment lactose and break down milk components enzymatically, producing a smooth mixture of lactic acid and acetic acid, ethanol, carbon dioxide, and a wide range of aromatic chemicals (Côco et al., 2023).

Initially, the shepherds who lived in the Caucasus Mountains had discovered a fermented milk product. They noticed the milk in the leather pouch- bolsos- made of animal skins. The milk eventually fermented due to a reaction to the skin, and a tart beverage and fizzy was produced. The name “kefir” is believed to be derived from the Turkish word “keyif” meaning “feeling good” or “pleasure” which portrays the drink as pleasurable and beneficial in terms of the benefits it offers. It has been consumed since ancient times in the Caucasus region. When Russia invaded the region in the eighteen and nineteen centuries, the kefir “grains”- which are symbiotic colonies of bacteria and yeast were transported to Russia. Kefir became well-known in the 20$^{th}$ century (Wszolek et al., 2007) due to the perception that it was linked with the longevity of human (Ahmed et al., 2019). Now it is renowned for its therapeutic benefits in improving gastrointestinal health, enhancing immune function, and regulating lipid metabolism due to its probiotic bacteria content and diverse microbial composition (Liu & Li, 2016). These health-promoting properties are largely related to the presence of live probiotic bacteria in kefir, which have been

demonstrated to exert favorable effects on the gut microbiota and host physiology (Leite et al., 2015).

Industrial kefir is mostly manufactured in Russia and other former Soviet Union nations, followed by Poland, Sweden, Hungary, Norway, Finland, Germany, the Czech Republic, Denmark, and Switzerland. Kefir is also made in Greece, Austria, and Brazil (Pogačić et al., 2013). Kefir has recently gained appeal in the United States and Japan due to its status as an ethnic product. According to available statistics in Croatia, kefir is manufactured in relatively modest amounts by only a few dairy farms, with the addition of a commercial culture (Pogačić et al., 2013). Despite an extensive body of research elucidating its advantageous effects, the therapeutic potential of kefir has historically been underestimated within the scientific community, compounded by the absence of standardized clinical trial methodologies and challenges associated with maintaining consistent characteristics in industrially produced variants. Nevertheless, a variety of commercial kefir products are readily accessible, highlighting the imperative for further meticulous inquiry into its therapeutic efficacy (Reuben et al., 2020). It was thought that kefir had therapeutic properties as early as the eighteenth century. It has been passed onto generations that the beneficial attributes of kefir seem to have been underestimated by the scientific community. Nonetheless, several kefir products are commercially marketed (Reuben et al., 2020).

Kefir's chemical composition is influenced by milk type, grains, a mixture of culture, additives, and production technology (Gul et al., 2015). Buffalo milk differs from other forms of milk not only in flavor but also in chemical composition. Compared to cow's milk, it has more calcium and protein and has lower cholesterol levels. Furthermore, buffalo milk contains twice as much tocopherol as cow milk and four times as much peroxidase activity (David, 2012). Tocopherol products can provide significant antioxidant protection, and anti-inflammatory benefits, and support overall health and well-being (Vargas-Ramella et al., 2021). However, kefir produced from cow and buffalo milk using kefir grains and starter cultures showed similar chemical properties, except ethanol levels. The type of milk influenced the microbiological characteristics, amino acid, and organic acid profiles of the kefir. Additionally, these properties varied with the type of culture used during storage. Traditionally, private households prepare dairy-based kefir by incubating milk with kefir

grains. Kefir grains are added to sterilized milk and fermented at 25°C until the pH reaches 4.4. After fermentation, the grain and milk are separated using a sterilized plastic filter (Kim et al., 2018). Backslooping is a fermentation process used for sourdough, idli, sauerkraut, dry sausage, beer, cheese, and kefir. Milk is pasteurized at 90°C for 15 minutes before cooling to 25°C to enhance microbiological quality. To make kefir, mix cooled milk with 5% grains and incubate at 18-24 ◦C for 18 hours. The grains are then sorted through a sieve under aseptic conditions and kefir is stored at 4℃ (Shrivastava & Ananthanarayan, 2015).

According to the same standard, a typical kefir must contain at least 2.8 % proteins, less than 10 % fat, at least 0.6 % lactic acid, while the alcohol percentage is not determined. The total number of specified microorganisms from culture must be at least $10^7$ cfu/mL, and the number of yeasts not under $10^4$ cfu/mL. At the end of fermentation, which includes three days of cold ripening, the pH value of a typical kefir is between 4.2-4.7, it contains between 0.8-1.2 % of lactic acid, 0.5-0.7 % of ethanol and approximately 0.20 % of $CO_2$. Apart from these compounds, kefir also contains various aromatic compounds like acetaldehyde, diacetyl and acetone, and other organic acids like formic, acetic and/or propionic and isoamyl alcohol in traces (Wszolek et al., 2007). Also, many scientific studies confirm that apart from nutritive value kefir also has a strong probiotic effect (Farnworth, 2005; Lopitz-Otsoa et al., 2006; Rattray & Connell, 2011).

## 1.2 Current studies:

The search for novel bioactive chemicals is an ongoing endeavor. Kefir has a variety of functional traits, including antibacterial, anti-inflammatory, hypocholesterolemic, anticancer, wound healing, antioxidant, and digestive aid effects. Kefir's scent, flavor, and mouthfeel are altered by ingredients such as kefir grain, inulin, and sucrose. Milk type also affects kefir's texture and the rheological properties to fit consumer preferences (Farag et al., 2020).

The researchers focus on a novel method for kefir production using encapsulated starter cultures derived from yeast and bacterial strains originally isolated from kefir grains. These starter cultures were immobilized within alginate microspheres, with the encapsulation ratio reflecting their natural distribution in kefir grains. The research evaluated both the microbiological properties of these microspheres, and the microbial and chemical

properties of the kefir produced. This new kefir was then compared to traditional kefir made directly from kefir grains (Chen et al., 2009). Due to the limited shelf life of kefir and the high storage and packaging expenses, the trend toward dry kefir in powder form appears justified. Powdered kefir is made using both spray drying at 50-120°C for 24-36 hours and freeze drying at -45°C to -50°C for 36 hours or more in a vacuum of 0.06 mbar or lower can produce a stable powder. (Teijeiro et al., 2018).

There is a lack of comprehensive data comparing the specific differences between these two types of kefirs and how these differences may impact their probiotic potential. Current studies often focus on either cow milk or buffalo milk kefir separately but rarely analyze them side by side. My research aims to address this gap by providing a detailed comparison of their physicochemical properties and microbial compositions, with the goal of understanding how these factors influence their effectiveness as probiotics. This will contribute to a more nuanced understanding of kefir's therapeutic use and potentially guide more targeted applications in probiotic therapy.

## 1.3 Objectives

### 1.3.1 General objectives

To investigate the production of kefir and analyze its physiochemical properties and probiotic profile through isolation, identification, and functional characterization of its microbial strains.

### 1.3.2 Specific objectives

- To produce milk kefir through fermentation using kefir grains.
- To determine the phylogenetic relationship of lactic acid bacteria (LAB) and yeast present in kefir.
- To characterize the probiotic properties of identified lactic acid bacteria.
- To analyze the physiochemical properties of kefir.

## 1.4 Statement of the problem

Today, the issue of gut health has gained popularity due to the prevalence of digestive issues such irritable bowel syndrome, Gastroesophageal Reflux Disease (GERD), constipation, and others. With roughly 3 million people affected each year, digestive illnesses are among the most serious and expensive health problems in the US.

Additionally, in developed nations, they rank among the main causes of employee absences. But in emerging countries, digestive issues are becoming more common. Numerous lifestyle difficulties that affect a person's overall wellbeing are frequently faced by those who have digestive problems. To overcome these problems Kefir, with its high probiotic contents, has all the essential vitamins, minerals, probiotics, and nutrients that make kefir a super-rich food and a must in everybody's diet plan.

Worldwide, there has been a noticeable shift among consumers towards functional foods, driven by increased health awareness and rising healthcare costs. The recognition that probiotics can support a healthier gut microbiome has positioned probiotic foods as essential functional foods and emerged as a crucial dietary approach to enhancing human health.

Studies have indicated that lactic acid bacteria (LAB) are predominant microflora constituents in fermented foods (Hassan & Ibrahim, 2017). LAB encompass a diverse array of gram-positive, non-spore-forming coccus or rod-shaped microorganisms crucial for various food fermentation processes (Abushelaibi et al., 2017). Notably, LAB are renowned for their probiotic attributes, with species from genera including *Lactobacillus, Enterococcus*, and *Pediococcus* frequently encountered as probiotic LAB strains in food matrices (Reuben et al., 2020). Kefir not only helps in weight loss but also nourishes the complete body. It also has healing properties, which repair the damaged cells in the body and make our bodies internally stronger which is why it helps in losing weight. It is well-positioned to take advantage of this rapidly growing market for probiotics. Kefir's market potential is further increased by the need for healthy, natural alternatives to conventional dairy products, especially among vegans and people with lactose intolerance. Despite having sensory qualities like those of yogurt and curd, there is a striking lack of knowledge of kefir in the Nepalese context.

## 1.5 Research hypothesis

Null hypothesis ($H_0$): Kefir contains probiotics with several health benefits.

Alternative hypothesis ($H_1$): Kefir doesn't contain probiotics with several health benefits.

## 1.6 Rationale of the study

Although kefir is known to include a wide variety of bacteria and yeast, many probiotic strains within kefir grains are still unidentified or have not been well-defined. Understanding the potential health advantages of these probiotic strains and how they can be used in different businesses depends on their isolation and characterization. There is a need to identify specific probiotic strains in kefir that show promising probiotic characteristics considering the increased interest in probiotics and their health-promoting effects. The creation of specialized probiotic products for various medical uses can be guided by this information. Understanding the whole range of microorganisms present in kefir will help clarify any potential health advantages of its usage. Dietary advice and health-related interventions may be affected by this information.

Introducing kefir into Nepalese cuisine could offer a novel way to enhance the dietary diversity while promoting health and wellness among the population. This research aims to bridge this gap by providing insights into the local production of kefir and exploring its potential as a source of beneficial probiotic strains.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Kefir grain composition

Kefir grains resemble cauliflower in appearance, possessing an elastic, irregular, gelatinous texture with a color ranging from ivory to white and varying in size from 0.3 to 3.5 cm in diameter (Gaware et al., 2011). These grains constitute a distinctive natural ecosystem, formed through a symbiotic alliance between bacteria and yeasts. They harbor a rich microbial community, comprising more than 50 species of bacteria and yeast, alongside filamentous molds depending on their origin (Wang et al., 2008). Kefir grains host a diverse microbial community including *Leuconostoc mesenteroides* and *Kluyveromyces marxianus* (Lin & Chien, 2007). Notable species like *Lb. kefiri* and *Lb. kefiranofaciens* are named after kefir.

The microbial composition of kefir grains made up 65 to 80% *Lactobacillus* and *Lactococcus species.* such as *Lactococcus lactis subsp. lactis* with the remaining amount completed by yeasts. In kefir grains, the peripheral portion is almost entirely made up of bacteria, primarily *lactobacillus,* whereas the inner portion contains yeasts, and the interface between the inner and outer portions is made up of bacteria with long polysaccharide filaments, yeasts, and fungi (Lopitz-Otsoa et al., 2006). Yeast species such as *Saccharomyces cerevisiae, Saccharomyces unisporus, Candida kefyr, Kluyveromyces marxianus subsp. marxianus, Torulaspora delbrueckii, Pichia fermentans, Kazachastania aerobia, Lachanceae meyersii, Yarrowia lipolytica*, and *Kazachstania unispora* are present in greater numbers in kefir and kefir grains (Wang et al., 2008).

A kefir grain, in addition to multiple microbial species, is composed of a spongy fibrillated structure with reticular laminar matrix and fibrous cluster that branches and interconnects with long chains, especially in the grain center. Proteins, polysaccharides, diverse cellular constituents, and an unknown number of additional components make up this complicated structure. These grains can be preserved through conventional drying methods at 33°C or vacuum drying and maintain viability for years under stable conditions (Wszolek et al., 2007).

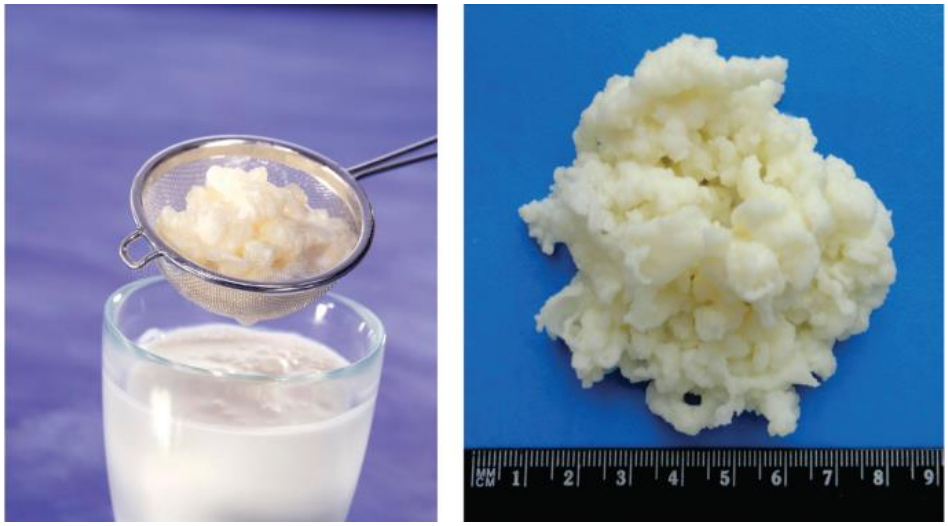


Figure 2.1: Kefir and Kefir grains (Leite et al., 2013)

## 2.2 Symbiotic interactions among microorganisms in kefir

### 2.2.1 Interactions between yeast and bacteria

Yeast and bacteria interactions are essential for the fermentation process in kefir and other fermented foods. These interactions include:

- Lactic Acid Utilization: Certain yeasts assimilate lactic acid, which helps lactobacilli thrive by preventing excessive acid buildup. This enhances kefiran production (Katakura et al., 2010).
- $CO_2$ Production and Oxygen Reduction: Yeasts like *S. cerevisiae* create a conducive environment for *Lactobacillus* species by producing carbon dioxide and reducing oxygen levels (Suharja et al., 2014).
- Nutrient Provision: The collaboration between yeast and bacteria involves nutrient exchange, with yeasts providing essential nutrients like vitamins and amino acids to bacteria, supporting their growth under nutrient-limited conditions (Ponomarova et al., 2017).

### 2.2.2 Interactions among bacteria

Bacteria-bacteria interactions in fermented foods are less studied compared to yeast-bacteria interactions. According to research conducted by Ponomarova et al., (2017) on yogurt bacteria, such as *Lb. delbrueckii subsp. bulgaricus* and *S. thermophilus*, shows protocooperative relationships. In kefir, interactions among bacterial species like *Lb. kefiranofaciens, Lb. kefir, Lc. lactis, A. fabarum,* and *L. mesenteroides* reveal competitive dynamics. For instance, *Lb. kefiranofaciens* inhibits *Lb. kefir,* promotes *L. mesenteroides*, and has no significant effect on *Lc. lactis* and *A. fabarum* (Ponomarova et al., 2017).

### 2.2.3 Interactions among yeasts

Quorum Sensing (QS) in yeasts is less understood. Studies in wine and sourdough ecosystems show that environmental factors like nitrogen levels, cell density, and ethanol concentration significantly influence QS molecule production by *S. cerevisiae*. Some *S. cerevisiae* strains release peptides that inhibit non-Saccharomyces strains, a strain-specific trait. Understanding these yeast interactions is crucial for optimizing kefir quality and functionality (Avbelj et al., 2016).

## 2.3 Production of kefir

Concerns have been raised by the growing world population about the availability of a healthy and sustainable natural food supply (Knorr & Augustin, 2023).The preferred method for creating future food components and products turns out to be fermentation, surpassing other approaches such as cellular or acellular products, edible biomass, and edible insects. Fermented products require less land, emit fewer greenhouse gases, and utilize less water than traditional farming methods.

The conventional method for producing kefir involves adding kefir grains directly as a starter to pasteurized, cooled milk. In home manufacturing, fermentation temperature and duration are not strictly controlled. The resulting product cannot be used to inoculate new milk to make kefir since the grains original balance of microorganisms has been disrupted. Kefir grains are essential to the manufacturing procedure (Farnworth, 2007). Kefir was traditionally made by artisans by inoculating milk with a quantity of grain (2–10%) and allowing the fermentation process to operate for around 24 hours to reach a predetermined pH or until the appropriate texture or taste was achieved. Fermentation is carried out between 20℃ and 25℃. A maturation stage of 8 to 10 $^{o}$C for 15 to 29 hours is frequently included. The grains are then sieved and can be utilized to begin a new fermentation or stored in fresh milk for 1-7 days. Leaving the grains in the finished product causes increased acid production and a poor flavor (Hallé et al., 2001). To make Russian style kefir, a grain-free inoculum or mother culture is made by fermenting regular kefir, filtrating the product, and inoculating the milk with the percolate. When using lyophilized starter cultures, the mother culture is made by adding 1 g of lyophilized kefir grain starter to 3 liters of milk. Approximately 1 to 3% of the mother culture is added to pasteurized milk during the process (Libudzisz & Piątkiewicz, 1990).

To produce industrial kefir, a third step involves taking a portion of Russian-style kefir and adding it (2–3%) to a fresh milk source. Then, it is necessary to repeat the fermentation (8–20 hours at 20–22 degrees Celsius) and maturation (12 hours to 7 days at 8–16 degrees Celsius) steps. These conditions promote the growth of beneficial microbes, enhance flavor development, and increase the bioavailability of nutrients and bioactive compounds (Hallé et al., 2001). Large-scale kefir manufacturing has been delayed by the difficulties associated with replicating kefir grains and generating a consistent product. However, industrial kefir production is popular in several European nations, and patents outlining the method have been filed in various countries (E. R. Farnworth & Farnworth, 2007). There are several variations to the methodology used in commercial kefir production. Initially, the set technique was employed for production. In this procedure, inoculated milk was put into bottles, fermented at a controlled temperature until a strong coagulum formed, and then cooled. However, the kefir produced was of below-average quality when compared to smaller-scale production utilizing traditional methods.

Current methods for making kefir use stirring, in which the fermentation, agitation, ripening, and cooling processes take place within a single jar. The type of milk used, the source of grains, the preparation of the mother culture (often accomplished by coarsely sieving the grains and using the percolate), the length of fermentation, the inclusion of a cooling step, and the inclusion of a maturation step all influence the final product's composition (chemical, organoleptic, and microbiological characteristics (Farnworth & Farnworth, 2007). The benefits of agitating the fermentation mix included minimizing the growth of mold on the starter surface and even encouraging distribution of metabolites and microorganisms. After agitation, the number of homofermentative lactic acid streptococci and yeast increased tenfold. However, the numbers of heterofermentative lactic acid streptococci, thermophilic lactobacilli, and acetic acid bacteria remained unchanged, nor did the amounts of volatile fatty acids in the final starter change. Kefir grains are cleaned with water once a week in certain facilities.

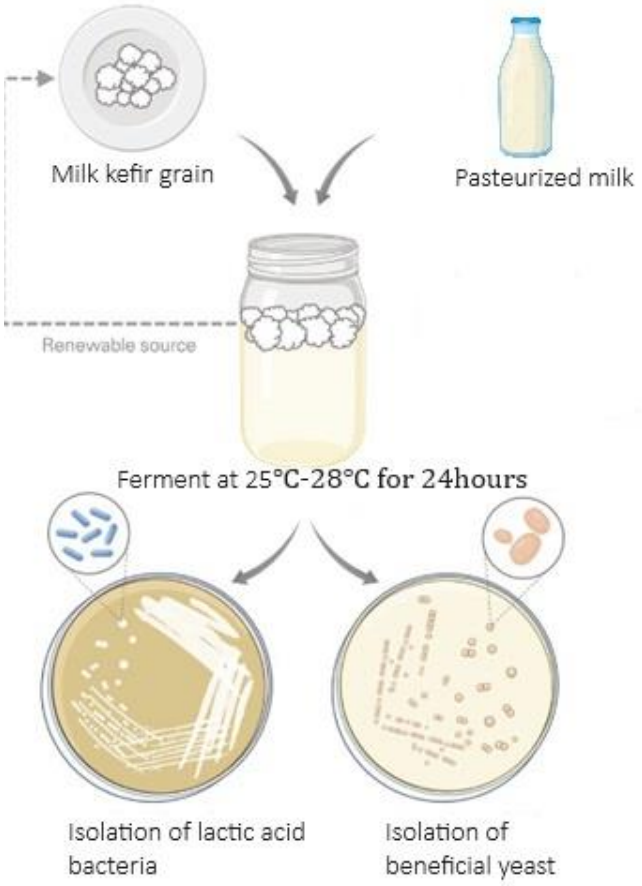



Figure 2.2: Milk kefir production. Kefir grains are added to milk and fermented at 25 °C ± 2 for a day. After the mixture is filtered, a beverage that is slightly acidic and alcoholic is obtained. Strains of lactic acid bacteria and yeasts can then be isolated from the milk kefir.

## 2.4 Physiochemical properties of Kefir

### 2.4.1 pH

The pH value is one of the most important metrics used to track the progression of the fermentation process. It detects the presence of specific chemical components that influence growth, metabolism, and the finished product. The regulation of pH is critical in milk fermentation since it regulates not only LAB growth but also metabolic activity. Milk's pH is 6.6, making it perfect for the growth of a variety of microbes. The fermentation process of LAB lowers the pH of the milk medium by creating lactic acid. The pH of finished milk kefir after 24 hours of fermentation should range between 4.0 and 4.5. If it is higher, allow it to ferment for an additional 12 hours. To eliminate the chance of pathogens existing, it must fall below 4.6 (Alves et al., 2021).

When using 1% kefir grain at a pH of 4.5 led to a decrease in alcohol level to 0.3% in goat

milk kefir, compared to 1% alcohol when using 5% kefir grain at the same pH value Goat milk kefir made with 1% kefir grain at pH 4.5 had an alcohol content of 0.3% as opposed to 1% when kefir grain with 5% pH value was used (Setyawardani et al., 2014). In certain regions of the world, such a reduction in alcohol content in kefir may be preferred, such as in Islamic countries where alcoholic beverages are prohibited.

When lactose is fermented by the kefir grains, lactic acid is produced. This process occurs mostly during the fermentation phase, but post-acidification during storage also contributes to some of the acidity of kefir. On the other hand, it is not good for there to be too much acidity after fermentation because this can lead to whey separation, textural flaws, and an extremely sour flavor that can overpower the consumer-appreciated fragrance constituents. Kefir's shelf life can also be measured by the emergence of bitter and sour flavors. Depending on the type and strain of kefir grains used, storage will cause a rise in titratable acidity and bitterness as well as a drop in pH. pH measurement is the primary method for monitoring acid development in kefir, however volumetric titration is also often used to evaluate acidity.

### 2.4.2 Total soluble solid

The measurement of all dissolved solids in a beverage, such as sugars, acids, and alcohols, is called total soluble solids, or TSS (Leite et al., 2013). Regardless of temperature, the TSS level of kefir does not drastically drop throughout fermentation as bacteria break down carbohydrates to produce $CO_2$, lactic acid, and alcohol. Time and inoculum size influence pH, titratable acidity, and antioxidant activity, but not total soluble solids. TSS is commonly tested with a hand refractometer and remains stable at approximately 2-4% throughout the 24–72-hour fermentation period (Azizi et al., 2021).

### 2.4.3 Lactic acid content

Lactic acid is the primary metabolic end product of carbohydrate fermentation in lactic acid bacteria, and this feature has long been associated with food fermentations because acidification slows the growth of spoiling organisms. Either homofermentative or heterofermentative bacteria may exist in these systems. Compared to heterofermentative lactic acid bacteria, which only make 50% lactic acid and significant amounts of ethanol, acetic acid, and carbon dioxide from glucose, homofermentative lactic acid bacteria

produce more than 85% of lactic acid from glucose. After 24 hours of incubation, the isolates were tested for lactic acid generation and residual reducing sugar (glucose) content. The pH is lowered by the growth of LAB because lactic acid is produced. The capacity of bacteria to withstand low pH, which favors probiotic properties, is necessary for their survival in gastric juice (Aswathy et al., 2008).

According to Amorim et al., (2019) lactic acid concentration is an outcome of the breakdown of lactose and sucrose during the carbohydrate metabolism process. The LAB can produce lactic acid from 95% of glucose under ideal circumstances when it is kept at room temperature (Ismaiel et al., 2011). According to Nikolaou et al., (2019), kefir's acidity ranges from 0.85% to 1%. According to Temiz & Dağyıldız, (2017), the acidity of kefir ranges between 0.57 and 0.75 percent. Mutually beneficial activity between LAB and yeast resulted in an increase in total kefir acid. Iskandar et al., (2019) indicated that throughout the fermentation process, LAB turns lactose into lactic acid, which is then converted into ethanol from the carbohydrate breakdown by *Saccharomyces cerevisiae.*

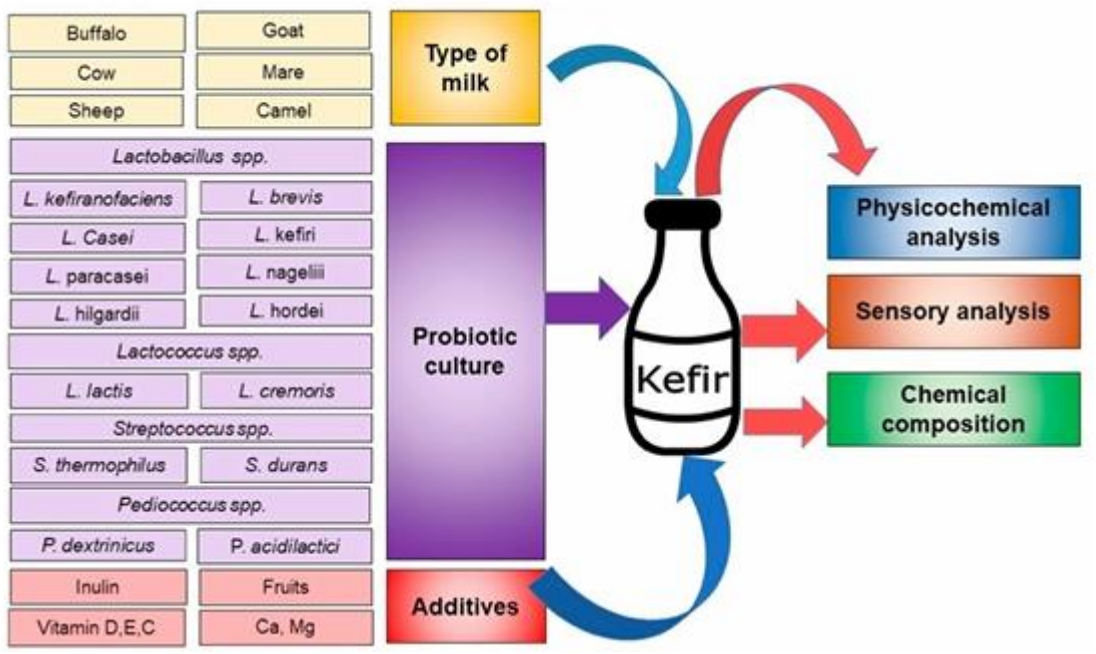



Figure 2.3: Key factors in kefir production that influence its quality and chemical composition include milk, culture, and the additives used (Farag et al., 2020).

## 2.5 Nutritional properties of kefir

### 2.5.1 Protein content

According to Shen et al., (2018), the protein content of kefir varies depending on the

milk's origin, the ingredients of the grains or cultures, and the kefir fermentation method.

### 2.5.2 Amino acid content

kefir is a rich source of amino acids, providing both essential and nonessential varieties such as threonine, serine, alanine, and lysine compared to unfermented milk, which are enhanced through the fermentation process. Additionally, partially digested proteins like caseins included in kefir help the body to absorb and digest it (Simova et al., 2006). Kefir's rich supply of vital amino acids also helps to maintain immunological response, balance energy levels, control protein, glucose, and lipid metabolism, and regulate body weight. According to studies by Bifari & Nisoli, (2017) and Grohmann & Bronte, (2010), amino acids can prevent impairment and increase the healthy life expectancy of older participants.

### 2.5.3 Fat content

According to Izquierdo-González et al.(2019), the longer the ripening process, the more rapidly the LAB proliferates and produces more lipase enzymes, which hydrolyzes more fat and lowers its levels. Kefir's unique flavor and aroma come from free fatty acids (FFAs) produced during lipolysis, which can be 5 to 10 times higher than in the original milk. These FFAs create distinct sensory notes, such as buttery, cheesy, or fruity, depending on the milk and fermentation conditions. For example, the fermentation of sheep milk with kefir cultures can lead to a significant increase in FFAs, which in turn affects the sensory properties of the final product (Wszolek et al., 2007). The temperature and duration of fermentation also influence the levels of FFAs and other volatile compounds, such as acetaldehyde and diacetyl, which further contribute to kefir's aroma and flavor (Cais-Sokolińska et al., 2008).

It was discovered that camel milk kefir had a lower ratio of polyunsaturated fatty acids than cow milk kefir, which correlated with a greater Lactobacillus ssp. concentration in the cow milk kefir (Kavas, 2015). The peptide bacteriocin, whose antibacterial activity is still unknown, is thought to be the reason for the low number of microorganisms in camel milk.

Milk kefir with 2.83% fat was generated by inoculating kefir grains with 3% (w/v) of cow's milk, according to (Dinkçi, 2015). Similarly, a 10% (w/v) inoculum utilized by Gamba et al., (2020) yielded 1.34% fat content of fermented cow's milk (Dinkçi, 2015) whereas Otles,

(2003) and Shen et al., (2018) demonstrated that a 100 g cow's milk kefir sample included 3.5g of fat. (Abdolmaleki et al., 2015) achieved a similar conclusion, finding that the fat content of kefir beverages was strongly dependent on the substrate. For example, soy milk (2.30%) has less fat than cow's milk (3.71%), which explains why soymilk kefir has less fat than cow's milk kefir.

### 2.5.4 Carbohydrate content

According to the United States Department of Agriculture's (USDA) nutritional information database, whole milk has a total sugar content of 12.3% (Collard & McCormick, 2021), but whole milk kefir has nearly half of the total sugar content (6%) after fermentation. The hydrolysis of lactose into glucose and galactose during fermentation by lactic acid bacteria may be the cause of the reduction in carbohydrate content. The galactosidase enzyme hydrolyzes around 30% of milk lactose into glucose and galactose (Barreca et al., 2020).

Farag et al., (2020) found that normal kefir included 6.0% of total available carbohydrates. However, Azizkhani et al., (2021) found a carbohydrate content of 3.95% in cow's milk kefir. For those who are lactose intolerant in this situation, kefir is an appropriate substitute because its microorganisms also convert lactose to lactic acid (Rosa, Dias, & ŁM, 2017).The findings of this investigation were consistent with a report by (Gamba et al., 2020), who found lactose to be the primary sugar utilized by microorganisms in cow's milk kefir, with levels decreasing from 4703 to 3314 mg per 100 mL during fermentation. Proximate and High-Performance Anion Exchange (HPAE) chromatography studies revealed that the sugar level in fermented cow's milk dropped by 1.06% and 1.36%, respectively (Gamba et al., 2020). On the other hand, research on the primary sugars utilized in the manufacturing of almond milk kefir is still lacking.

### 2.5.5 Vitamin content

Kefir is a great source of minerals, vital amino acids, and vitamins $B_1$, $B_2$, $B_5$, and C. These nutrients can help with healing, homeostasis, and physical fitness. The type of milk and microbial flora employed in the manufacturing of kefir have an impact on the vitamin content. A greater amount of vitamin B6 was supported by *Propionibacterium Shermanii subsp. Freudenreichii*, while *Propionibacterium peterssoni* and *Propionibacterium pituitosum* generated vitamin B12 (Sarkar, 2007).

### 2.5.6 Ash content

According to Gamba et al., (2015)., there is 0.75% ash in cow's milk kefir. Even though the kefir produced in Poland and Scotland is made from diverse milk sources from mammals with varying ranges of ash level (0.7–1.11%), the study's ash content for cow's milk kefir (0.73%) compared favorably with the findings of (Wszolek et al., 2001). Another finding by Gamba et al., (2015) showed that unfermented and fermented cow's milk had equal ash content (0.75%), while unfermented soy milk (0.44%) had comparable ash content to fermented soy milk (0.40%). This could indicate that fermentation in either kefir sample had no significant effect on the ash content.

### 2.5.7 Minerals in kefir

Calcium, magnesium, potassium, and salt are macro-elements that are enhanced in kefir and help with the use of proteins, lipids, and carbohydrates for energy production, maintenance, and cell growth. Kefir includes micro-elements, including iron, zinc, and copper, which are of exceptional significance in cellular metabolism and blood synthesis (Bakircioglu et al., 2018).

### 2.5.8 Moisture content

According to Wszolek et al., (2001), kefirs' moisture content varied from 85.1% to 89.4%. Similarly, the moisture content of their cow's milk kefir was found to be 87.5% by (Otles, 2003). Arslan, (2015) reported 89–90% moisture content. Milk blends with increasing total solids showed gradually reduced moisture content (Kundu et al., 2018).

### 2.5.9 Total dietary fiber

It is imperative to emphasize that kefiran (Magalhães et al., 2011; Rodrigues et al., 2005) consist of a water-soluble glucogalactan polysaccharide matrix encircling the bacteria that are inherently present in the kefir grains (MyFCD, 1997). According to MyFCD (1997), there was no dietary fiber in cow's milk. Therefore, the 6.97% of dietary fiber in the kefir made from cow's milk came from the kefir grains that were added to the milk during the fermentation process.

### 2.5.10 Volatile content

The volatile kefir flavor components were monitored using the headspace solid-phase micro-extraction (HS-SPME) method while skim milk powder was fermented with kefir starting culture. The analysis revealed the presence of eight volatile flavor compounds: acetone (3.6%), 3-hydroxy-2-butanone (acetoin, 3.3%), ethanol (39.3%), 2-butanone (31.6%), ethyl acetate (8.9%), ethyl butyrate (5.5%), and acetaldehyde (1.7%) (Aghlara et al., 2009). These compounds are representative of the alcohol, ketone, ester, and aldehyde classes, respectively. Furthermore, acetone, diacetyl, ethanol, acetaldehyde, and ethyl acetate levels increased throughout fermentation (Aghlara et al., 2009).

Yeast *(S. cerevisiae)* produces fragrance in kefir, along with other volatile esters such as isopentyl acetate, ethyl hexanoate, ethyl octanoate, phenethyl acetate, and ethyl decanoate (Hu et al., 2014; Magalhães et al., 2011). Esters are known for their characteristic aroma in many herbs, and they appear to be responsible for the prominent odors in kefir. *Lactobacillaceae, Streptococcaceae,* and *Leuconostocaceae* are the families that distinguish Tibetan kefir (W. Gao & Zhang, 2019).

In contrast to ethanol, 2-butanone was found to be volatile, whose content remained steady during fermentation during the kefir production process. Dependent on pH, acetoin levels were seen to drop significantly between pH values 4.6 and 5 (Aghlara et al., 2009; Arslan, 2015). These findings show that measuring acetoin and alcohol may offer a more accurate readout of kefir manufacturing conditions than monitoring only 2-butanone.

## 2.6 Shelf life of kefir

Kefir's microbiological shelf life is determined by several factors that include milk type, starter culture employed, formulation of kefir, processing conditions, and storage duration. To maintain the quality and safety of kefir during storage it is important to maintain the viability of microorganisms such as lactic acid bacteria, acetic acid bacteria, and yeasts. Concerning microbial viability over time in storage, the inclusion of fruit within formulation can improve this situation leading to an increase in the shelf-life of Kefir (Ozcan et al., 2018). Furthermore, variables specific to manufacturers like milk type and processing conditions determine its microbiological properties as well as shelf life. Store-bought kefir, if unopened and refrigerated between 0°C & 4°C can stay fresh for one to two weeks past its printed date but should be preferably consumed within two weeks once opened. The shelf

life for homemade kefir when refrigerated ranges between two and four weeks depending on environmental factors like ingredients used (Ozcan et al., 2018). Airtight containment with original sealed container while fermentation occurs using stainless steel equipment will keep out contaminants thereby retain quality. To sustain good quality and safety standards for kefir effective refrigeration must be adhered to strictly (Ozcan et al., 2018).

## 2.7 Health benefits of kefir

### 2.7.1 Anti-carcinogenic properties and inhibition of tumor growth

Tumors are divided into carcinomas and sarcomas. Tissues including cartilage, fat, and bone are sources of support for sarcoma tumors (Kuby, 1994). Furthermore, Liu (2003) investigated the effects of freeze-dried kefir, made from soy milk and cows' milk and enriched with kefir grains, on tumor formation in mice. Before the experiment's feeding phase began, mice received weekly injections of Sarcoma 180 cells. Up to 30 days were expected for the tumor's growth (volume). A 30-day estimate of the tumor's growth (volume) was made. In comparison to mice in the positive control group, soymilk kefir (70.9%) and cows' milk kefir (64.8%) significantly suppressed tumor growth.

In a study on mice given induced breast cancer, Moreno et al., (2006) found that after 27 days of two-day cycle feeding with both kefir and a cell-free portion of kefir, the tumor growth was inhibited and the number of IgA(+) cells increased in the mice. They further proposed that the harmful compounds generated during tumor formation might be bound by IgA (+) cells, highlighting the significance of non-microbial components released during milk fermentation. Furthermore, kefir extracts have been proven to inhibit the growth of breast cancer cells in vitro.

Moreover, the ability of kefir extracts and bacterial isolates to diminish the likelihood of malignant growths occurring in vitro or in animal models, or to halt their progression entirely (Rattray & Connell, 2011).

### 2.7.2 Cholesterol lowering effect

According to several studies, kefir supplementation in rats on a high cholesterol diet reduced total serum cholesterol and phospholipids while leaving HDL and serum triglycerides unaltered (Rattray & Connell, 2011). In contrast, Liu et al., (2005) discovered

that soymilk kefir and milk both decreased the total cholesterol and serum triacylglycerol in hamsters. This finding raises the possibility that the hypocholesterolemic substances that soymilk kefir contains are what give soymilk kefir its greater ability to lower cholesterol.

Other researchers Brashears et al., (1998) & Tamai et al., (1996) claimed that kefir's serum cholesterol reduction could be attributed to the deconjugation of bile acids by Lactobacillus spp. Reynier et al., (1981) reinforced this by showing that deconjugation of bile acids could lower blood cholesterol by requiring the production of new bile acids, hence improving cholesterol metabolism and lowering serum levels.

### 2.7.3 Improving lactose tolerance

Lactose maldigestion, the inability to fully digest lactose found in nearly all mammalian milks, affects 75% of adults globally and typically results from a genetically programmed decline in intestinal lactase activity after ages 3 to 5 (Sahi, 1994; Swaggerty et al., 2002). Hertzler & Clancy, (2003) found that commercial kefir, made from a starter culture containing six bacteria and one yeast (excluding L. acidophilus), was as effective as yogurt in reducing breath hydrogen in lactose maldigestion. Fermented milk products, which have shorter transit times than regular milk, may further aid lactose digestion(Labayen et al., 2001; Vesa et al., 1996). Rattray & Connell (2011) noted that kefir's diverse microbial population provides some β-galactosidase activity, converting lactose into easily digestible glucose and galactose. However, Steven et al., (2003) pointed out that despite the potential for kefir to improve lactose digestion similarly to yogurt, research is limited. This study was the first to demonstrate that plain kefir improved lactose digestion as effectively as plain yogurt.

### 2.7.4 Wound healing properties

Atalan et al., (2003) and Koutinas et al., (2007) have reported that kefir, a probiotic mixture comprising a variety of bacteria and yeasts (Witthuhn et al., 2005) can elicit an increase in innate immunity against infections. Polysaccharides in kefir extract have anti-inflammatory qualities that aid in wound healing (Chena et al., 2008; Kyoung et al., 2007). Lactic acid, acetic acid, polysaccharides, and other compounds in kefir are essential to its wound-healing qualities, according to research by Huseini et al., (2012). Kamila et al., (2005) found that a simple kefir formulation produced from dry grains promoted wound healing in rats

more than a clostebol-neomycin emulsion. Similarly, Rodrigues et al., (2005) discovered that rats treated with 70% kefir gel saw faster wound healing than rats treated with the same emulsion. Kefir proved to be more effective than traditional silver sulfadiazine treatment for treating heat burns (Huseini et al., 2012).

### 2.7.5 Probiotic and prebiotic properties

Kefir is a complex microbial system that offers nutritional benefits and inhibits various food-borne pathogens and spoilage microorganisms (Paucean & Carmen, 2008). Unlike many probiotic products that contain a limited number of bacteria, kefir's microbiological and chemical compositions indicate a more intricate probiotic. The long association between yeasts and bacteria in kefir grains results in a microbial population with similar characteristics, complicating the isolation and identification of individual species, which are now being identified using advanced molecular techniques (Edward, 2006). Kefir exemplifies the coevolution of a microbial consortium, demonstrating strong resistance against various microorganisms and enhancing mammalian immunity. It is considered a natural drug with the potential to alleviate various illnesses (Jose et al., 2015). Santos et al., (2003) found that Lactobacillus strains from kefir adhered well to Caco-2 cells, were resistant to low pH and bile acid, and exhibited antimicrobial activity against common enteropathogens. Additionally, kefir produces prebiotics, such as lactacin, bacteriocins, and kefiran, which improve nutritional status and provide health benefits like protection against carcinogenesis, mutagenesis, free radical damage, and gastrointestinal resistance (Barbosa et al., 2011; J & D, 2004).

### 2.7.6 Benefits of kefir for pregnant and nursing women

Better nutritional absorption, immune system support, help with hormone regulation, and protection against diseases are all benefits of kefir consumption (E, 2013).

### 2.7.7 Anti allergenic effects

According to Shokryazdan et al., (2014) the incidence of allergic diseases, including food allergies and asthma, has been rising in developed countries for several years. Most food allergies manifest early in children, usually in the first two years of life, however other food allergies have a lifelong course. Asthma and food allergies are among the allergic illnesses

for which the complexity and diversity of the gut microbiota are important risk factors, according to recent studies (Kirjavainen et al., 1998).

### 2.7.8 Control of plasma glucose by kefir

Regular consumption of probiotics has the potential to enhance blood sugar regulation, primarily by positively influencing the composition of gut bacteria. This modulation reduces intestinal permeability, oxidative stress, and inflammation (Gomes et al., 2014). Similar benefits are observed with regular kefir consumption. Hadisaputro et al., (2012) studied the impact of kefir consumption for 30 days in managing blood sugar levels in Wistar rats with induced diabetes. Kefir supplementation notably lowered plasma glucose compared to the control group. In clinical trials, diabetic adults who consumed 600 ml/day of kefir for 8 weeks experienced significant reductions in fasting glucose levels and glycosylated hemoglobin compared to baseline. Moreover, these parameters were notably lower in kefir consumers compared to those who consumed conventional fermented milk (Alireza Ostadrahimi et al., 2015). Regular probiotic intake can also decrease Gram-negative bacteria and lipopolysaccharide (LPS) in the gut, improving intestinal

barrier function and reducing inflammation. Lower LPS levels may aid in controlling blood glucose levels and restoring insulin receptor function, suggesting kefir's potential in diabetes prevention, though further studies are warranted to confirm these effects.

### 2.7.9 Anti-hypertensive effect of kefir

Studies have shown anti-hypertensive effects in both clinical and experimental settings, indicating that probiotic bacteria or their fermented products may help control blood pressure (Parvez et al., 2006). Bioactive peptides produced from casein during milk fermentation are the mechanism by which kefir lowers the activity of the angiotensin-converting enzyme (ACE), as found by (Quirós et al., 2005). Kefir's antihypertensive properties were ascribed by (Maeda et al., 2004) to kefiran's capacity to block ACE. Blood pressure is raised when angiotensin I and aldosterone are produced in excess, which is blocked by these ACE-inhibitory peptides. Furthermore, they block the breakdown of bradykinin, a vasodilator, which helps to lower blood pressure (Hernández-Ledesma et al., 2011). A lack of clinical research has been done on milk kefir's ability to lower blood pressure, and it is yet unknown which ACE-inhibitory peptides are present in milk kefir.

### 2.7.10 Antioxidative activity of kefir

The body uses a variety of antioxidant defense methods to combat dangerous reactive oxygen species, including dietary substances and enzyme activity. When carbon tetrachloride ($CCl_4$) toxicity was introduced to mice, a study by Guven & Gulmez (2003) revealed that kefir had a greater antioxidant effect than vitamin E. Additionally, in mice experiencing oxidative stress brought on by lead (Pb), Ozcan et al., (2018) discovered that kefir supplementation raised glutathione peroxidase and lowered malondialdehyde levels. Kefir may be helpful in controlling oxidative stress, according to these findings. Kefir made from goat and cow milk has potent antioxidant qualities because it scavenges radicals and prevents peroxidation (Liu et al., 2005). This antioxidant activity could lessen DNA oxidation and possibly enhance its anticarcinogenic qualities.

### 2.7.11 Healing action of kefir

Probiotics offer a variety of advantages beyond gut health that have recently come to light, such as better skin, relief from dermatitis, scar healing, and tissue rejuvenation (Lew & Liong, 2013). In their study, Rodrigues et al., (2005) examined the potential of a 70% kefir and kefiran gel to promote healing in rats with infected skin wounds. By comparison with conventional topical emulsions, the therapy improved tissue healing substantially. In contrast to traditional creams, kefir showed potential in treating burns infected with *Pseudomonas aeruginosa* by lowering inflammation and encouraging epithelialization and healing (Huseini et al., 2012). Beyond its benefits for the gastrointestinal system, kefir may also have potential in wound treatment due to its antibacterial and anti-inflammatory qualities.

## 2.8 Gut microbiota

The 'gut microbiota' is a collection of bacteria, archaea, and eukarya that colonizes the GI tract and has co-evolved with the host over thousands of years to develop an intricate and mutually beneficial interaction (Backhed, 2005; Neish, 2009). More than 1014 different types of bacteria exist within the human gut; moving from the stomach to colon enhances the diversity as well as the density of these microbes (Xu et al., 2013).The gut microbiota is made up of varied ecologies of bacteria, archaea, fungi, viruses, and bacteriophages (Manrique et al., 2016)

Most people think that the microbiota develops from birth, although a few studies have shown that microorganisms can be found in womb tissues like the placenta, casting doubt on this belief (Aagaard et al., 2014; Rodriguez, 2015). Chemical, nutritional, and immunological gradients along the gut have an impact on the density and makeup of the microbiota. These characteristics restrict the growth of bacteria, leaving only facultative anaerobes that can cling to mucus or epithelia and multiply quickly. The microbiota of distinct colorectal mucosal areas varies, in contrast to the various GI organs diverse microbial compositions (Donaldson et al., 2015).

Diseases ranging from allergies in childhood to inflammatory bowel disease (IBD) in young adults are caused by aberrant alterations in gut microbiota (Harmsen & Goffau, 2016). Dysbiosis is an adverse condition caused by an imbalance or disruption of the intestinal microbiota. Pathological problems including malnourishment and obesity can result from dysbiosis (Kau et al., 2011). Chronic inflammatory illnesses like inflammatory bowel disease (IBD) and Crohn's disease have been linked to altered gut microbiota (Frank et al., 2007; Matsuoka & Kanai, 2015). According to Kalliomaki (2008), levels of *Staphylococcus aureus* increased whereas *bifidobacteria* reduced during gut dysbiosis.

## 2.9 Probiotics

Probiotics, which translate to "life" in Greek, are a type of microbial dietary adjuvant that regulates systemic and mucosal immunity to positively alter host physiology. They also support a healthy and well-balanced microbial ecology in the intestinal tract (Naidu et al., 1999).

In the food industry, the term is described as "live microbial feed compounds which benefit the health" (Clancy et al., 2003), yet the most widely accepted definition of probiotics states that "live microorganisms (bacteria/yeasts), which when consumed or locally applied in adequate amounts imparts one or more specific demonstrated health benefits in the host body" (FAO/WHO, 2001).

Nowadays, the dairy food business is the key area in which probiotics are a viable market for food makers, with great growth potential. The oldest techniques for food preservation are fermentation-based techniques. Prior to the discovery of microbes, people used fermented foods like kefir, cheese, curd, and koumiss. Eli Metchnikoff, a Nobel Prize

winner, pioneered the notion of probiotics by suggesting that consuming fermented foods could help you live longer. His opinion was that the lactic acid bacteria found in fermented milk products, which replaced toxic gut microbes, were responsible for Bulgarian farmers' longevity (J. Gao et al., 2021). He has also shown that L. bulgaricus eliminates putrefactive intestinal bacteria, which improves the host's overall health (Metchnikoff, 1907). Furthermore Lilly & Stillwell (1965), described probiotics as beneficial microbes that promote the growth of other microorganisms, while Parker (1974) expanded on probiotics as bacteria that support the microbiota of the host organism.

Probiotics are also defined by (Fuller, 1989) as live microbial food supplements that enhance the host organism's gut microbiota and promote better health. Identifying and describing a prospective probiotic strain requires careful research on the effectiveness of the strains in human subjects.

### 2.9.1 Lactic acid bacteria

According to Alvarez-Sieiro et al., (2016), LAB are spore-forming and they can survive in environments deficient in oxygen, withstand acid, and have a rigid preference for either homo- or heterofermentation. LAB, a widely spread genus of bacteria, is used in a variety of industries. They are Gram-positive bacteria that do not generate spores and have rod or cocci forms. LAB ferment carbohydrates to create lactic acid as the primary product (Andrabi et al., 2016). *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* are key genera with shared metabolic features (Andrabi et al., 2016). Probiotic screening focuses on strain specificity, while certain processes are similar at the sub-species, species, or genus level (Sanders & Ebner, 2014). They colonize a variety of environments, including the gastrointestinal tract (GIT) of humans and animals, dairy products, seafood, soil, and plant surfaces, with significant contributions from sources such as poultry guts, infant feces, and various host anatomical regions (Andrabi et al., 2016; Razdan et al., 2012). Due to variables including age, location, health, diet, and manner of delivery, the species composition of LAB in the gut varies greatly (Hemarajata & Versalovic, 2013).

For Lactobacillus, notable strains include *Lactobacillus acidophilus* (e.g., NCFM, La-14),

*Lactobacillus rhamnosus* (e.g., GG, GR1), *Lactobacillus casei* (e.g., Shirota), *Lactobacillus plantarum* (e.g., LP299V), and *Lactobacillus fermentum* (e.g., ME-3). *Bifidobacterium* strains with proven benefits include *Bifidobacterium bifidum* (e.g., PRL2010), *Bifidobacterium lactis* (e.g., BB-12, DN-173 010), *Bifidobacterium longum* (e.g., BB536), and *Bifidobacterium breve* (e.g., M-16V). Additionally, *Saccharomyces boulardii* (e.g., CNCM I-745) and *Streptococcus thermophilus* (e.g., TH-4) are recognized for their probiotic properties. These strains have been extensively studied for their benefits in improving gut health, supporting immune function, and overall well-being. It is essential to select probiotic strains based on their proven efficacy for the specific health benefits desired (Barreca et al., 2020; Collard & McCormick, 2021)

Particularly in the GIT environment, these bacteria are responsive to food components, pH, stress, and salinity, among other nutritional and environmental cues (Wang et al., 2017). LAB's carbohydrate fermentation pattern separates them into hetero-fermentative groups that produce equimolar amounts of $CO_2$, lactate, and ethanol/acetate through the phosphoketolase pathway, and homo-fermentative groups that primarily produce lactic acid using the Embden-Meyerhof-Parnas pathway (Alvarez-Sieiro et al., 2016).

Probiotics may be a useful treatment option for both preventing and treating osteoporosis, as several human and animal research have shown that gut microbiota is linked to several disorders, including osteoporosis (Schepper et al., 2017). Because they are common in food and help to maintain healthy mucosal surfaces, the genera *Streptococcus, Leuconostoc, Pediococcus*, and *Lactobacillus* are categorized as Generally Recognized as Safe (GRAS).

### 2.9.2 Yeast

Yeasts are frequently employed in the food industry for both animal and human consumption, especially *Saccharomyces, Candida*, and *Kluyveromyces.* Even though lactic acid bacteria and bifidobacteria continue to be the primary focus of probiotic research, *Saccharomyces cerevisiae var. boulardii* is gaining popularity for its therapeutic benefits in treating gut disorders and normalizing intestinal flora(Fleet & Balia, 2006; Saad et al., 2013; Szajewska et al., 2007; Zanello et al., 2009). Probiotic milk products should have at least $10^7$ CFUs per mL, with yeast counts not less than $10^4$ CFU/mL (Rosa et al., 2017). Diosma et al., (2013), observed that four species of yeast *Saccharomyces cerevisiae, Saccharomyces*

*unisporus, Issatchenkia occidentalis,* and *Kluyveromyces marxianus* were identified from 34 yeast strains recovered from kefir grains using standard microbiological and molecular approaches. Cassanego et al., (2017) discovered that kefir samples from Santa Maria, RS were microbiologically safe for eating, having passed testing for *Salmonella sp., coliforms, Staphylococcus*, and yeast and lactic bacteria counts. Nineteen yeast strains were recovered, and PCR analysis revealed three species: *Saccharomyces cerevisiae, Hanseniospora uvarum*, and Kasachstania unispora. Kefir, made by the symbiotic fermentation of several microorganisms, primarily contains yeasts from the *Kluyveromyces* and *Saccharomyces* genera, with *Saccharomyces boulardii* being the only commercially available probiotic yeast.

Although bacteria dominate kefir grains, yeasts play an important role in maintaining microbiological equilibrium and generating the final product's physical, chemical, and sensory properties. According to Farnworth, (2005), yeasts are necessary in kefir preparation because they provide nutrients to acetic acid bacteria and release metabolites such as ethanol and CO2, which improve the flavor and texture of the kefir.

Yeasts are of great economic significance due to their multiple applications in biotechnology; these include pharmaceuticals, enzymes as well as fermented foods and beverages being a few of them(Yarrow, 1998). Kefir is preferred because of its typical yeast flavor and health benefits, making it an attractive fermented probiotic drink (Ahmed et al., 2013; Farnworth, 2005). For kefir fermentation, lactic acid bacteria and yeasts on kefir grains form a complex microbial community composed of various types of organisms(Garrote et al., 2010; Magalhães et al., 2011; Miguel et al., 2010) leading to many different health promoting properties such as antibacterial activity, immunomodulation activity as well as antioxidant effects among others (Ahmed et al., 2013; Farnworth, 2005).

### 2.9.3 Mechanism of probiotics

It is believed that probiotic microbes enhance host health. But according to Holzapfel et al., (1998), the support mechanisms are not explained. Probiotics mechanisms of action have been studied. Numerous mechanisms are being proposed by these studies to explain how probiotics could shield the body from internal illnesses. Following is a list of these (Çakir, 2003, 2003; Castagliuoluolo et al., 1999; Salminen et al., 1998). .

- Manufacturing of compounds that inhibit pathogenic bacteria: Certain organic acids, hydrogen peroxide, and bacteriocins are produced. These substances inhibit both gram-positive and gram-negative bacteria.
- Inhibition of adhesion sites: Pathogenic bacteria and probiotics compete with one other. The infections' ability to adhere to the intestinal epithelial surfaces is inhibited by probiotics through the inhibition of adhesion sites.
- Nutrient competition: By taking up the resources that the bacteria require, probiotics suppress the pathogen.
- Immunity stimulation: Probiotics may safeguard people against interior illnesses by stimulating both specific and nonspecific immunity. Although the exact mechanism is unknown, it is believed that certain cell wall elements or cell layers may function as adjuvants and boost humoral immune response.
- Toxin receptor degradation: It has been demonstrated that *Saccharomyces boulardii* shields the body against intestinal illnesses caused by *Clostridium difficile* due to the degradation of toxin receptor on the intestinal mucosa. *Saccharomyces boulardii* was demonstrated to protect the host against *Clostridium difficile* intestinal illnesses. Other mechanisms proposed include toxin suppression, gut pH decrease, and virulence attenuation (Fooks et al., 1999).

### 2.9.4 Health benefits of probiotics

#### 2.9.4.1 Bowel diseases and the immune system

Intestinal immunity and microbiota are out of balance in both Crohn's disease and ulcerative colitis, two bowel illnesses that are impacted by the genetic predisposition and environmental variables of the gut microbiome (Khor et al., 2011). The etiology of these illnesses is partly explained by changes in pro- and anti-inflammatory cytokine levels, which are mediated by Th1 and Th2 cells (D, 2009). The ability of probiotics to modify immune responses has led to their identification as a potential treatment and prevention of intestinal illnesses. Research on *L. salivarius CECT* 5713 in rats given colitis-induced diets revealed noteworthy anti-inflammatory benefits backed by decreased expression of inflammatory markers and histological observations (Peran et al., 2005). In tests using human cells, similar anti-inflammatory properties were discovered, especially with *L. salivarius Ls-33*. In animal models and in vitro tests, several probiotic strains such as *L.*

*plantarum 91* and *L. kefiranofaciens M1* showed anti-colitis effects: they strengthened intestinal barriers and improved cytokine balance (Chen et al., 2012)

### 2.9.4.2 Hypocholesterolemic effect

Probiotics are known for their hypocholesterolemic effects achieved through various mechanisms such as cholesterol assimilation, binding to cell surfaces, co-precipitation, interference with micelle formation, and bile salt hydrolase (BSH) activity (Lye et al., 2010; Zhang et al., 2008). BSH, present in lactobacilli and bifidobacteria, plays a key role by deconjugating bile acids, reducing their solubility and leading to excretion as free bile acid in feces, thus lowering serum cholesterol (Nguyen et al., 2007). Bile presence enhances cholesterol removal by aiding its attachment to bacterial cell membranes, a process influenced by bacterial growth (Lye et al., 2010). However, detailed investigations into the optimal treatment efficacy of each probiotic strain regarding dosage and duration are yet to be explored.

### 2.9.4.3 Dermal health

Improvements in atopic eczema, wound and scar healing, and skin regeneration are just a few of the encouraging effects of probiotics on skin health. Oral and topical probiotic treatments have been studied; however the results are still unclear because of differences in probiotic dosage, strain, duration, and follow-up times (Lye et al., 2016).

### 2.9.4.4 Oral health

Researchers have recently focused on the possible use of probiotics to improve oral health in response to the rise of bacteria resistant to antibiotics. Probiotics may help prevent oral illnesses such dental caries, periodontal infections, and halitosis (K et al., 2009; Masdea et al., 2012; Shimauchi et al., 2008).

### 2.9.4.5 Periodontal disease

Due to their virulent characteristics, primary pathogenic agents such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* can infiltrate subgingival tissues, compromise the host's immune system, and cause tissue damage. Common species found in the oral cavity, such as *Bifidobacterium, L. fermentum, L. salivarius*, and *L. gasseri*, greatly

contribute to the ecological balance of the oral cavity (Hojo et al., 2007). Research conducted by Krasse et al., (2006) and N et al., (2007) demonstrated that *L. brevi* and *L. reuteri* have anti-inflammatory and plaque-reducing properties in patients with periodontitis and gingivitis.

### 2.9.4.6 Halitosis

Halitosis, characterized by oral malodor due to volatile sulfur compounds (VSC) produced by anaerobic bacteria, involves bacteria like *F. nucleatum, P. gingivitis, P. intermedia*, and T*. denticola. Weisella cibaria's* hydrogen peroxide production inhibited *F. nucleatum* growth, reducing VSC production. Additionally, *S. salivarius* produces bacteriocins that suppress VSC-producing species. Despite promising preliminary data, more clinical studies are needed to establish probiotics' potential for oral health, including identifying safe and effective strains, optimal dosage, and mode of administration. Mechanisms of immunomodulation and long-term effects require further clarification through well-designed trials to confirm probiotics' effectiveness in oral disease treatment (S et al., 2006).

## 2.10 Probiotics characterization of lactic acid bacteria

To identify superior probiotics, isolates exhibiting the largest inhibition zone (> 15 mm), resistance to low acid and bile salts, and potential survival rate (> 65%) were considered in addition to their antimicrobial efficacy, bacteriocin production, and ability to survive in simulated gastrointestinal conditions (Amenu & Bacha, 2023). LABs should survive gastrointestinal circumstances and make it to the small intestine, where they colonize and provide health benefits to the host, and gastric and intestinal juice resistance is an important consideration when selecting possible probiotics (Larsen et al., 2018).

### 2.10.1 Bile salt tolerance

Bile salt tolerance is a crucial criterion for probiotic candidates because bile salts, as strong surfactants, are highly toxic to bacteria in the digestive tract, challenging their survival and activity (Ruiz et al., 2013). Probiotics must endure the gastrointestinal tract's complex conditions, including bile's antimicrobial properties, which play a significant role in the body's defense system (Long et al., 2017). High bile salt resistance facilitates the colonization of the host's digestive tract (Zhang et al., 2019), making it essential to assess

probiotics growth in the presence of bile salts. Bile salt tolerance is essential for probiotics due to bile's antibacterial effect in the small intestine, which can damage bacterial cell membranes (Dhanya Raj et al., 2023). LAB species exhibit complex bile response mechanisms, including active efflux of bile salts, bile salt hydrolysis, and changes in cell membrane and wall composition (Ruiz et al., 2013). The recommended bile salt concentration for testing probiotics ranges from 0.15% to 0.5%, aligning with physiological GIT levels (Papadimitriou et al., 2015). Studies show significant variability in bile resistance among strains, indicating that bile tolerance is strain-dependent rather than universal across species (Jacobsen et al., 1999; Pennacchia et al., 2004).

The growth of microorganisms can be inhibited by bile salts in multiple ways. As a fat emulsifier, bile salts can disrupt the phospholipid structures of organelles and bacterial cell membranes. According to Foley et al., (2019), the primary cause of resistance to bile salts is the existence of bile salt hydrolase (BSH). This enzyme acts as a defensive mechanism against intracellular acidity generated by conjugated bile salts. BSH hydrolyses and deconjugates glycine or taurine from bile acid cholesterol cores (Parasar & Chang, 2022). Deconjugated bile salts are changed into free bile salts (FBS) by BSH, according to (Bustos et al., 2018). In numerous metabolic processes, such as the control of dietary lipid absorption, cholesterol metabolism, energy balance, and inflammation, FBS functions as a signaling (Martoni et al., 2015). FBSs have the potential to contribute to the development of homeostatic conditions in bacterial membranes by modulating the fluctuations of nitrogen bases, lipids, and amino acid biosynthesis. These fluctuations can impact fat alteration, ultimately leading to the synthesis of exopolysaccharides (EPSs) (Harnentis et al., 2020).

Hidalgo-Cantabranaa et al., (2014) state that bacteria can employ EPS to defend themselves from the hostile gastrointestinal tract environment, which lengthens their survival in the host organism. According to Nambiar et al., (2018), EPS makes bacteria more resilient to bile salts and low pH. EPS protects against bile salt concentrations of 0.15% to 0.3% (Lebeer et al., 2018). Lactic acid bacteria (LAB) with good bile salt resistance can survive in the digestive tract, showing high probiotic potential. The digestive tracts bile salt concentration ranges from approximately 0.2% to 0.3% consumed (Hu et al., 2018), with 0.3% considered a critical limit for selecting probiotics. In another study, LAB isolates were

tested for resistance to 0.3% and 0.5% oxgall salt concentrations. Bacteria are deemed bile salt-resistant if they survive above 50% at certain concentrations ( Liu et al., 2013). LAB isolates in another research demonstrated over 50% resistance to low pH gastric juice and 0.3% bile salt concentration after 4 hours of incubation (Kaewarsar et al., 2023) classifying them as good probiotic candidates.

### 2.10.2 pH tolerance

Probiotic lactic acid bacteria (LAB) must withstand the acidic environment of the gastrointestinal tract (GIT) to survive and function effectively. LAB isolates have shown resistance to pH 2.5 for 3 hours, with over 50% survival (Mulaw et al., 2019). LAB growth lowers pH due to lactic acid production, aiding in their survival in gastric juice by tolerating low pH conditions, a key probiotic characteristic (Aswathy et al., 2008). Gastric juice with a pH of 2.0 is hostile to most pathogens, killing them upon ingestion (Amenu & Bacha, 2023). Strains with high survival rates in such conditions are ideal for crossing the human intestinal barrier. Their survival depends not only on their intrinsic resistance but also on the ingestion vector and food contents, with high-fat and certain proteins (Monteagudo-Mera et al., 2012).

Gastric juice's low pH (approximately 2) can reduce microorganism colonies, necessitating protection systems for survival during digestion. Lactic acid bacteria (LAB) tolerate acidic pH (Reuben et al., 2019) through three main defense mechanisms: the H+-ATPase proton pump, arginine deaminase, and glutamate decarboxylase systems (Wang et al., 2020). Additionally, microorganisms can defend against low pH via proton extrusion, decarboxylase-mediated proton consumption, alkaline substance production, cell membrane modification, macromolecule repair, and damage avoidance, and metabolic pathway reconstruction (Guo et al., 2019). LAB isolates is characterized as gram-positive bacteria, possess thick cell walls with peptidoglycan and teichoic acids (TA), which provide mechanical strength and aid in surface attachment. TA can be wall-bound or membrane-bound and contributes up to 50% of cell wall mass, maintaining cation homeostasis and preserving cell wall shape in low pH (Harnentis et al., 2020; Jeong et al., 2023; Pasquina-Lemonche et al., 2020).

The study conducted by Pennacchia et al., (2004) used a pH of 2.5 to select potential

probiotic strains, ensuring the isolation of very acid-tolerant strains.

### 2.10.3 Cell autoaggregation

Auto-aggregation, the clustering of bacterial cells of the same strain, is vital for the adhesion of lactic acid bacteria (LAB) to the oral cavity and gastrointestinal tract, playing a significant role in biofilm formation (Agaliya & Jeevaratnam, 2012; Nikolic et al., 2012). This adhesion is essential for creating a barrier against pathogens and for immunomodulation (Nallala & Jeevaratnam, 2015). Auto-aggregation indicates cell surface properties and correlates with the ability of probiotics to adhere to epithelial cells, a prerequisite for colonization and infection prevention (Collado et al., 2007; Gupta & Bajaj, 2017). It leads to bacterial clump formation, which precipitates from the microbial suspension, marking the initial step in adhesion to intestinal cells (Mobili et al., 2010). While various mechanisms for studying auto-aggregation have been proposed, molecular studies are limited (Malik et al., 2013). Nevertheless, the auto-aggregation of probiotic strains is linked to their colonization potential in the gastrointestinal tract through adhesion to intestinal epithelial cells (Kaktcham et al., 2017).

### 2.10.4 Cell coaggregation

Co-aggregation, the aggregation of different bacterial cell types, allows close interaction between probiotic and pathogenic bacteria (Kumar et al., 2012). This process, defined as the attachment of genetically distinct bacteria via surface-specific molecules (Rickard et al., 2003), is critical for both food preservation and therapeutic impacts on gut microbiota (Collado et al., 2007). The ability of probiotics to co-aggregate with pathogens is strain-specific and dependent on time and incubation conditions. This phenomenon can be used to screen for probiotics with desirable properties for food, human, or animal use (Collado et al., 2007; Rickard et al., 2003).

Due to the rise of multidrug-resistant pathogenic bacteria, alternative methods such as probiotic intervention are being explored to combat infectious diseases (Lehri et al., 2017). Co-aggregation helps eliminate pathogens from the gut by creating a barrier to colonization. Probiotics can indirectly compete with pathogens for cell surface receptors or directly bind to them, preventing their attachment and colonization in the gut (Abushelaibi et al., 2017). The interaction is facilitated by surface binding proteins on LAB

and pathogens (Pellegrino et al., 2018), leading to the clumping and precipitation of probiotic cells in their medium, useful for selecting the best probiotic strains (Kaktcham et al., 2017).

### 2.10.5 Cell surface hydrophobicity

Surface characteristics of bacteria play a crucial role in understanding their probiotic potential, especially concerning adhesion and colonization abilities. Hydrophobicity, a key surface property, influences bacterial attachment to various substrates, including mucosal cells, and is linked to adhesion strength (Otero et al., 2004; Rijnaarts et al., 1993). Higher hydrophobicity correlates with increased adhesion levels, particularly for hydrophobic microorganisms (Gilbert et al., 1991). Cell surface hydrophobicity represents a nonspecific interaction with the host and is essential for bacterial adhesion and colonization in the digestive tract (Agaliya & Jeevaratnam, 2012). Evaluating hydrophobicity, often using toluene or chloroform, serves as an initial assessment of a bacterium's ability to bind epithelial cells, which is crucial for probiotic strains (Falah et al., 2019; Zommara et al., 2023). This hydrophobic feature enhances the initial contact between host cells and probiotic strains, facilitating pathogen reduction and promoting health benefits (Monteagudo-Mera et al., 2019).

Cell surface hydrophobicity also influences intestinal colonization, indicating the potential for adhesion and persistence in the gut environment (Farid et al., 2021). Bacterial isolates with higher hydrophobicity exhibit stronger interactions with mucosal cells, which vary depending on the cell's composition and structure, including molecules like TA, lipoteichoic acid, lipopolysaccharide, and surface proteins (Lebeer et al., 2010). Additionally, EPS plays a role in bacterial adhesion to host cells, although adhesion mechanisms are species- or strain-dependent cells (Han et al., 2021).

### 2.10.6 Bile salt hydrolase assay

Bile salt hydrolase (BSH) plays a crucial role in maintaining gut bacteria balance and reducing blood cholesterol levels (Agaliya & Jeevaratnam, 2012). Studies have consistently shown that probiotic strains with BSH activity contribute to hypocholesterolemic effects (Ooi & Liong, 2010; Pavlović et al., 2012). These effects occur through deconjugation, reducing bile salt solubility and reabsorption, leading to increased excretion of free bile

acids in feces. This process may lower serum cholesterol levels by enhancing fecal bile acid loss or reducing cholesterol solubility after intestinal absorption (Pavlović et al., 2012). Additionally, microbial BSH activity aids in bile salt detoxification, enhances intestinal survival of producing strains, and potentially yields beneficial effects associated with these strains (Begley et al., 2006). However, further research is needed to fully understand BSH activity and its mechanisms to prevent potential risks, such as sepsis or colon cancer from secondary bile salts produced (Ishimwe et al., 2015).

### 2.10.7 NaCl tolerance

Lactic acid bacteria are known for their ability to tolerate high salt concentrations, a trait that enables them to initiate metabolism and produce acid, effectively inhibiting the growth of unwanted organisms (Aswathy et al., 2008). When these bacteria are cultivated in a saline environment, their turgor pressure diminishes, impacting their metabolism, enzymes, and water activity. To counteract this, the cells regulate internal and external pressures by inducing osmolytes like glycine betaine, adapting to higher osmotic potentials (Mohd Adnan & Tan, 2007).

### 2.10.8 Phenol tolerance

Probiotics' tolerance to phenol is a critical trait, as phenols can arise in the intestines through bacterial deamination of aromatic amino acids from dietary proteins or endogenous proteins (Gilliland & Walker, 1990). This process can inhibit the growth of lactic acid bacteria (LAB). Thus, probiotics' ability to resist phenol is vital for their survival in the gastrointestinal tract (Xanthopoulos et al., 1997).

### 2.10.9 Growth at different temperature

The heat tolerance of LAB isolates was tested at 42°C, which is similar to the temperature in the chicken body and digestive tract (Mhone et al., 2022; Y. Yang et al., 2014). Probiotic bacterial strains are likely to survive and grow at these temperatures. Microbes face a variety of environmental conditions, including temperature variations, that influence their survival capacity.

Microbes are categorized into three types based on their temperature tolerances: psychrotrophs (2°C-7°C), mesophiles (10°C-40°C), and thermophiles (43°C-66°C) (Pellissery

et al., 2020). Alternatively, some authors classify mesophiles and thermophiles as thriving at 23°C-45°C and 45°C-65°C, respectively (Syahid et al., 2020).

### 2.10.10 Antibiotic susceptibility test

Antibiotic resistance in the food industry is a pressing issue due to the risks of foodborne illnesses and the proliferation of multidrug-resistant pathogens. While bacteria's intrinsic resistance poses minimal horizontal spread risk, acquired resistance is more concerning. However, probiotic LAB are generally considered safe (GRAS) because their acquired resistance genes are not associated with foodborne pathogens (Kim & Ahn, 2022). Evaluating their antipathogen activities based on inhibition zone diameters categorized them into strong, moderate, and weak (Lim, 2010).

To ensure the safety of bacteria as starter or adjunct cultures in food systems, antibiotic susceptibility is a crucial requirement. Concerns have emerged regarding probiotics with antibiotic-resistant strains potentially transferring genes to intestinal pathogens. However, intrinsic antibiotic resistance in *Lactobacillus* strains is chromosomally encoded and not transmissible, making it a safer option (Danielsen & Wind, 2003). While opinions vary, specific antibiotic resistance in probiotics can be beneficial for conditions like antibiotic-induced diarrhea (Temmerman et al., 2003). Yet, safety requires that resistance be chromosomally encoded and not transferable, as acknowledged by EFSA (2008), to minimize horizontal dissemination risks (Bernardeau et al., 2008).

### 2.10.11 Antimicrobial activity

According to Winastri et al., (2015), the probiotic bacteria's ability to suppress infections can be categorized as weak (≤ 5 mm), moderate (6-10 mm), strong (11-20 mm), or extremely powerful (≥ 21 mm. By producing antimicrobial substances including organic acids, short-chain fatty acids, and bacteriocins, probiotic bacteria demonstrate this inhibitory ability (Argyri et al., 2013).

Monteagudo-Mera et al., (2012) warn that probiotic behavior in vivo may not be reliably predicted by relying exclusively on in vitro antimicrobial substance synthesis, even while these antimicrobial qualities help to improve the function of the intestinal barrier and balance intestinal microbiota. Chauhan & Singh, (2019) state that when choosing probiotic

strains, it is important to consider their antibacterial capacity against infections, which is essential for protecting host digestive systems. This antimicrobial activity not only inhibits infections but also helps to prevent diseases in the host by diminishing harmful bacterial colonies on enterocyte walls (Shang et al., 2018).

LAB produce cell surface proteins that help in attaching to intestinal cells, activating immunoregulation, and preventing pathogen colonization (Sirisopapong et al., 2023). LAB also produce organic acids such as lactic acid, which provide a hostile environment for pathogenic microbes, inhibiting their growth and causing their demise (Dittoe et al., 2018; Nair et al., 2017; C. Wang et al., 2015). The use of probiotics, alone or in interaction with bile acids, is a modern strategy in the prevention and treatment of hypercholesterolemia. Numerous mechanisms for hypocholesterolemic effect of probiotics have been hypothesized, based mostly on in vitro evidence. Interaction with bile acids through reaction of deconjugation catalyzed by bile salt hydrolase enzymes (BSH) is considered as the main mechanism of cholesterol-lowering effects of probiotic bacteria, but it has been reported that microbial BSH activity could be potentially detrimental to the human host. There are several approaches for prevention of possible side effects associated with BSH activity, which at the same time increase the viability of probiotics in the intestines and in food matrices. Our study aimed to summarize present knowledge of probiotics—bile acids interactions, with special reference to cholesterol-lowering mechanisms of probiotics, and to report novel biotechnological approaches for increasing the pharmacological benefits of probiotics (Pavlović et al., 2012).

### 2.10.12 Biofilm assay

Biofilms are complex structures formed by bacterial communities, particularly lactic acid bacteria, offering them resilience against environmental stressors like low pH, osmotic stress, and antimicrobials (Mobili et al., 2010; Rendueles & Ghigo, 2012; Shetty et al., 2016). These biofilms consist of microcolonies surrounded by protective extracellular substances, aiding in bacterial survival and resistance to antimicrobial agents (Yang et al., 2012).

Biofilm development involves attachment, microcolony formation, and maturation, enhancing bacterial survivability up to 1000 times against antimicrobials like antibiotics

(Shahandashti et al., 2016). Probiotics adherence and colonization, facilitated by biofilm formation, are crucial for their immunoregulatory function and anti-inflammatory properties (Rieu et al., 2014). However, biofilm formation can be influenced by physiological stress factors and gastrointestinal conditions, impacting probiotics resilience and biofilm characteristics (Aoudia et al., 2016). Nonetheless, biofilm-forming lactic acid bacteria in the gastrointestinal tract hold promise for protective roles and improved probiotic functionality, as assessed by biofilm-forming potential assays (Aoudia et al., 2016).

## 2.11 Safety assay

### 2.11.1 Hemolytic test

The FAO guidelines advise using microbial strains as probiotics with no hemolytic activity, ensuring safety (Bajagai. Yadav S, 2016). The findings revealed that none of the probiotics exhibited α- or β-hemolytic activity when cultured on Columbia blood agar; instead, they showed γ-hemolytic activity, consistent with previous studies (Argyri et al., 2013; G Pavli et al., 2016; Maragkoudakis et al., 2006; Oyewole et al., 2018; Pisano et al., 2014). This suggests that most LAB isolates are non-hemolytic, making them suitable and safe probiotic candidates (Zommara et al., 2023).

Ensuring the absence of hemolytic activity in Lactobacillus isolates is crucial for their safe use as starter or adjunct cultures in food systems (Zommara et al., 2023). Hemolysis, a virulence factor linked to pathogenic microorganisms, can disrupt red blood cells, release hemoglobin, and potentially harm host cells (Seker, 2010; Shin et al., 2012). Non-hemolytic (γ-hemolytic) strains are generally considered safe for hosts, while hemolytic strains are associated with pathogenicity. Beta hemolytic activity, in particular, indicates the presence of cytotoxic phospholipase in microorganisms (Sorokulova et al., 2008).

### 2.11.2 Arginine test

The arginine test for lactic acid bacteria assesses their ability to hydrolyze arginine, resulting in the production of ammonia (Shilpakar et al., 2022). This test is performed by inoculating a loopful of bacterial culture into an arginine broth containing Nessler's reagent. A positive result is indicated by a brown coloration of the medium. Lactic acid

bacteria that possess the enzyme arginine deiminase can hydrolyze arginine, producing ammonia as a byproduct. The presence of ammonia changes the color of the medium, signaling a positive result for arginine hydrolysis. The appearance of a brown color confirms the presence of ammonia, indicating the hydrolysis of arginine by the lactic acid bacteria (Lakshmi & Vijaya Gopal, 2021). The arginine hydrolysis test is a crucial step in the characterization of probiotic bacteria. The arginine hydrolysis test helps identify bacteria that can produce ammonia from arginine, which is a potential safety concern for human consumption. Probiotics should not produce ammonia to ensure their safety for human consumption. It helps determine whether a strain is safe for use as a probiotic, as it can identify strains that may pose a risk to human health(Soltan et al., 2017).

# CHAPTER 3. MATERIALS AND METHODS

## 3.1 Materials used

All the instruments, culture media, chemicals and various reagents used during the project and their preparations have been attached in Appendix I, II,III & IV.

## 3.2 Kefir production

In each batch 500 mL of raw bovine milk was pasteurized at 95℃ for 10 mins and then 15g of Kefir grain (Milk kefir grain, Mr. Kefir Active, Chennai, India) was inoculated into pasteurized milk and incubated for 24 hours at room temperature (27-30$^{o}$C) so that fermentation takes place. When the milk had coagulated, the milk was strained using a plastic strainer to get the kefir grains back (Witthuhn et al., 2005).

## 3.3 Isolation of lactic acid bacteria and yeast from kefir

### 3.3.1 Cultivation of lactic acid bacteria and yeast from kefir

The kefir samples underwent a series of dilutions using autoclaved distilled water. Following dilution, 100 μL aliquots from each dilution were inoculated onto MRS agar (Lactobacillus MRS Agar, Hi-media, USA) plates for cultivation of lactic acid bacteria and simultaneously and in YEGA for cultivation of yeast. These plates were then incubated anaerobically at 37℃ for LAB and aerobically at 27℃ for yeast for a period of two days and 4-5 day respectively. After the incubation period, a comprehensive examination was conducted to confirm the presence of bacterial colonies. Colonies displaying various morphologies were then transferred onto respective fresh agar plates for purification ( Xu et al., 2023). Utilizing a sterile loop, individual bacterial colonies and yeast colonies were meticulously isolated from each respective plate and systematically streaked onto freshly prepared MRS agar plates for LAB and YEGA for yeast using the Quadrant streaking technique. The plated cultures were subsequently incubated at 37°C for 48 hours and 27oC for 5 days respectively to monitor bacterial and yeast colony growth. To obtain pure colonies, individual colonies were selected from each plate and re-streaked onto new MRS plates and YEGA respectively.

### 3.3.2 Morphological and cultural characterization of isolates

Following purification, a thorough examination was conducted on the bacterial and yeast colonies focusing on colony morphology, Gram staining, lactophenol test, and catalase test in accordance with the methodology outlined by Goyal et al., (2013). Specifically, Gram-positive rods, identified as lactobacillus, displaying negative catalase activity were selected for further investigation. Additionally, yeast colonies exhibiting positive staining with lactophenol cotton blue were chosen for further identification. Continuous cultures were maintained on agar slants at 4°C, and sub-culturing was performed every four weeks to facilitate ongoing analysis (Goyal et al., 2013).

### 3.3.3 Gram Staining

First, a thin smear of the isolates was meticulously prepared and allowed to undergo air drying. After this, the smears underwent heat fixation. Following the fixation process, the smears were inundated with crystal violet, functioning as the primary stain, for a duration of 1 minute. Post-staining, the slides were gently rinsed with tap water. Gram's iodine solution(1%), serving as a mordant, was subsequently administered to the smears for 1 minute, followed by rinsing off the iodine solution with 95% ethanol for a period of 30 seconds. Following this step, the slides were gently rinsed once more. Safranin, employed as the counterstain, was then applied to the slides for 1 minute. Ultimately, the prepared slides were examined under a microscope at a magnification of 100x using immersion oil (Manandhar & Sharma, 2017).

### 3.3.4 Catalase Test

To assess catalase activity, a slide method was employed. Specifically, an isolated colony was selected and placed onto a clean, grease-free glass slide. A drop of 3% hydrogen peroxide solution was then added to the culture, and the reaction was closely monitored for the evolution of bubbles. The presence of bubbles indicated a positive catalase reaction, thereby providing evidence for the presence or absence of the catalase enzyme. (Manandhar & Sharma, 2017).

### 3.3.5 Lactophenol cotton blue staining

A Lactophenol Blue droplet was placed on a clean microscope slide, and a small yeast colony fragment was carefully removed with an inoculating needle and added to the droplet. A coverslip was gently lowered over the sample, avoiding air bubble entrapment. The slide was then examined under a microscope, allowing for detailed observation and analysis of the yeast morphological characteristics (Manandhar & Sharma, 2017).

## 3.4 Molecular analysis of isolated colony

### 3.4.1 Colony PCR

A small colony of yeast & bacteria was selected using a toothpick within a laminar hood. The toothpick was then immersed in 20 µL of TE buffer pH contained within a PCR tube. Subsequently, a PCR reaction was conducted with the following parameters: incubation at 95℃ for 3 minutes followed by cooling to 12℃ for 2 minutes. From this tube, regular PCR was performed using only 1 µL of mixture (Pereira et al., 2023).

### 3.4.2 PCR amplification

PCR amplification of bacterial and yeast isolated colonies were carried out by using its specific universal primers i.e. 16S rRNA for bacteria and 18S rRNA for yeast. The forward and reverse primer sequences of 16S rRNA were 27F (5'-AGAGTT TGATYM TGGCTCAG-3') & 515R (5'-TTACCGCGGCKGCTGGCA C-3') and 18S rRNA primer were 20F (5'-GTAGTCATATGCTTGTCTC-3') & 516R (5'-ACCAGACTTGCCCTCC-3') (Pereira et al., 2023).

**Table 3.1:** PCR Components used for lactic acid bacteria

| Component | Volume |
|---|---|
| Master mix | 12.5 µL |
| Forward primer | 1 µL |
| Reverse primer | 1 µL |
| Nuclease free water | 8.5 µL |
| Template | 2 µL |
| Total | 25 µL |

**Table 3.2:** PCR condition for amplification of lactic acid bacteria colony by 16SrRNA

| **Steps** | **Temperature/time** | |
|---|---|---|
| Initial denaturation | 94℃ for 3 minutes | |
| Denaturation | 94℃ for 30 seconds | |
| Annealing | 60℃ for 40 seconds | 28 cycles |
| Extension | 72℃ for 1 minute | |
| Final extension | 72℃ for 5 minutes | |

**Table 3.3:** PCR component used for yeast

| Component | Volume |
|---|---|
| Master mix | 12.5 µL |
| Forward primer | 1 µL |
| Reverse primer | 1 µL |
| Nuclease free water | 9.5 µL |
| Template | 1 µL |
| Total | 25 µL |

**Table 3.4:** PCR condition for amplification of yeast colony by 18S rRNA

| **Steps** | **Temperature/time** | |
|---|---|---|
| Initial denaturation | 94℃ for 5 minutes | |
| Denaturation | 94℃ for 30 seconds | |
| Annealing | 55℃ for 40 seconds | 35 cycles |
| Extension | 70℃ for 1.5 minute | |
| Final extension | 72℃ for 5 minutes | |

### 3.4.3 Gel electrophoresis:

Agarose gel electrophoresis was done to check the success of PCR amplification and amplicon size with reference to the DNA ladder (Bio-rad, USA). Aliquots of 5.0 µL of PCR amplifies products and a 100-bp & 1kb both ladders were loaded onto 1.5% agarose

electrophoresis gel (Sigma Chemical, USA) and run at 50V for 1h with 1x tris-acetate EDTA buffer and 1mM EDTA was used to stain the gel. After that, it was examined using a UV transilluminator.

### 3.4.4 Sanger sequencing

The isolate's PCR product was forwarded to Nepal Academy of Science and Technology(NAST) to be sequenced. Sequencer 4.1.4 (Bio-Rad Laboratories, USA) was used to modify the sequences obtained. NCBI Blast was used to compare sequences. Finally, the MEGA11 software was used to align the sequences and create a phylogenetic tree using neighbor-joining approach.

## 3.5 Probiotic characterization of lactic acid bacteria

### 3.5.1 Acid tolerance test

To assess the tolerance capacity of lactic acid bacteria (LAB) under acidic conditions, the pH of the MRS broth was adjusted to 3.0, 4.6, and 5.6 utilizing 1 M HCl. An aliquot of 100 µl from an overnight culture of LAB was inoculated into 5 ml of MRS medium, which had been adjusted to a mentioned pH level, and then incubated anaerobically for 0 hour, 3hours and 24 hours at 37°C. The growth kinetics of the LAB were monitored by measuring the optical density at 600 nm (OD600nm) at each incubation time interval. These experiments were conducted in triplicate for each bacterial strain to ensure the reliability and reproducibility of the results (Li et al., 2020).

### 3.5.2 Bile tolerance test

The MRS medium was formulated in four variants: each supplemented with 0.3%,0.5%, & 1% bile salt and the other devoid of bile salt. All media were inoculated with a 1% culture solution and subsequently incubated anaerobically for 0 hour, 3 hours, and 24 hours at 37 degrees Celsius. The optical density at 600 nm (OD600nm) was determined using a Spectrometer (Shimadzu Corp). (Nami et al., 2019).

### 3.5.3 Bile salt hydrolase assay

For this test, the isolated strains were cultivated in a medium enriched with 0.5% (w/v) bile salt. The culture medium was then subjected to incubation to promote dissolution, a procedure spanning 48 to 72 hours at a temperature of 37°C under anaerobic conditions.

After the incubation interval, thorough examination of the plates was carried out to identify the existence of white precipitates. These precipitates functioned as an indicator of bile salt hydrolysis (Hoque et al., 2010).

### 3.5.4 Cell auto aggregation

Initially, cultures were cultivated in MRS broth for a period ranging from 16 to 18 hours and subsequently collected through centrifugation. The harvested cells underwent a dual washing process with PBS, were resuspended in PBS, and their concentration was adjusted to achieve an optical density (OD) of 0.5 at 600 nm, which served as the initial OD. Following another centrifugation, the cells were resuspended in the same volume of MRS broth that was initially removed. This suspension was then incubated at 37°C for 2 hours. To measure the absorbance at 600 nm (final OD), 1 ml of the upper phase of the suspension was meticulously extracted (Tomas & Nader, 2005).

$$\text{Percent autoaggregation} = \frac{\text{Initial OD} - \text{final OD}}{\text{Initial OD}} \times 100$$

### 3.5.5 Co aggregation assay

This capability facilitates a close interaction between probiotic and pathogenic bacteria, crucial for the context of this study where E. coli served as the indicator organism to assess co-aggregation with selected Lactobacillus isolates. In the experimental procedure, cultures of Lactobacillus, grown overnight for 16-18 hours, and E. coli were subjected to centrifugation at 10,000 rpm for 15 minutes. The resultant pellets were then washed twice using a phosphate-buffered saline (PBS) solution with a pH of 6.0, before being resuspended in the same PBS solution. Following vortexing, the suspension's absorbance was adjusted to 0.5 at 600 nm. Subsequently, 500µl of the Lactobacillus culture was mixed with 500µl of the E. coli pathogen, and the mixture's optical density (OD) was measured at 600 nm. This mixture was then incubated at 37˚C for 2 hours. The upper phase of the mixture was carefully removed, and the absorbance was again measured at 600nm. A decrease in absorbance was indicative of cell co-aggregation, serving as a measure of the interaction between the probiotic Lactobacillus and pathogenic *E. coli* bacteria. (Kumar et al., 2012).

Percent coaggregation $= \frac{(OD1 + OD2) - 2(OD3)}{(OD1 + OD2)} \times 100$

Where, $OD_1$: optical density of Lactobacillus isolates, $OD_2$: optical density of E. coli & $OD_3$: optical density of mixture.

### 3.5.6 Tolerance to NaCl and phenol

The investigation into the NaCl and phenol tolerance of isolated bacterial cultures involved utilizing MRS broth containing NaCl concentrations of 2%, 5%, 7%, and 8% and 0.4% phenol respectively. A newly prepared culture was subjected to anaerobic incubation for a period of 48 hours at 37°C, with turbidity measurements taken at 48-hours by measuring the absorbance at 600nm. Notably, the negative control, consisting solely of the media without any bacterial inoculation, exhibited no observable growth throughout the experimental period (Chen et al., 2022).

### 3.5.7 Cell surface hydrophobicity

To investigate cell surface hydrophobicity, the strains were assessed for their ability to bind to hydrocarbons. Initially, bacterial cultures grown overnight in MRS broth underwent centrifugation at 8,000 rpm for 10 minutes. Subsequently, the harvested cells were subjected to two washes with PBS and were then resuspended in a PBS buffer solution. Absorbance ($A_0$) at 600 nm was measured for these suspensions. A solution of approximately 3.0 ml containing the cells was then mixed with 1.0 ml of hydrocarbon (hexane) and incubated at 37°C for 1 hour to allow separation between the aqueous and organic phases. After the incubation period, 1 ml of the aqueous phase was carefully removed, and the absorbance ($A_1$) was determined using a spectrophotometer at 600 nm. The percentage of hydrophobicity was subsequently calculated using the obtained absorbance values. The percentage hydrophobicity was calculated based on the reduction in absorbance(Somashekaraiah et al., 2019).

Cell surface hydrophobicity% = $\frac{A0 - A1}{A1} \times 100$

Where, $A_{0=}$ Initial absorbance & $A_{1=}$ Final absorbance

### 3.5.8 Growth at different temperatures

Actively growing bacteria isolated in their respective medium broths were transferred from 1.0 mL to 10 mL of their respective broth and incubated at temperatures of 15℃, 25℃, 37℃ & 45℃ for 2 days under optimal conditions. After incubation, each sample was spread onto individual agar plates and then incubated at 15℃, 25℃, 37℃ & 45℃ to assess for growth. The number of colonies were observed for good and excellent growth.

### 3.5.9 Antibiotic susceptibility test

The Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA)-recommended disc diffusion method, was used to determine the antibiotic resistance/susceptibility pattern for the isolates. *Lactobacillus* isolates' antibiotic susceptibility was evaluated on MRS plates using the antibiotic disc diffusion method. MRS plates were made by pouring MRS medium and allowing it to cool to room temperature before solidifying. Freshly developed bacterial cultures (100 µL) were placed on MHA medium plates. On top of these plates, antibiotic discs were incubated for two days at 37°C. The diameter of the zone of inhibition was determined using an antibiotic zone scale. The collected data were categorized as susceptibility, moderate susceptibility, or resistance. The acquired data were compared with the interpretation zone diameters as indicated by Performance standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007).Antibiotic susceptibility pattern of isolates was assessed using Cefotaxime (10µg), Bacitracin (10 µg), Cloxacillin (10 µg), Piperacillin (100 µg) & Ertapenem (10 µg).

### 3.5.10 Antimicrobial activity test

The isolate's antimicrobial activity against pathogenic strains was determined using the agar well diffusion method (Ridwan et al. 2008). The lactic acid bacteria were inoculated into MRS broth and incubated for 24 hours. After 24 h, the LAB cultures were centrifuged at 8000×g for 10 minutes, the cell free supernatant (each LAB isolate) was collected for antimicrobial test. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* were the bacteria tested. A 100 µl pathogen sample was added to soft agar, stirred, and overlaid on Muller Hinton Agar. Wells were made on MHA plates using borer. A 100 µl of cell free supernatant poured into a well on MHA plates. Plates were allowed to dry and incubated for 24-48 h at 37°C. Plates were observed for inhibition zone

and the diameter of the zone of inhibition was measured.

### 3.5.11 Biofilm assay

Congo red agar plates were made by adding Congo red in MRS agar. These plates are used to identify the biofilm assay of lactic acid bacteria. Identified lactic acid bacteria were streaked onto Congo red plates and incubated at 37℃ for 2 days to see the color change in colonies.

## 3.6 Safety assay

### 3.6.1 Arginine hydrolysis

The Arginine Test is used to detect bacteria that can convert arginine into putrescine. The test involves replacing ammonium citrate with 0.3% arginine in MRS broth, then inoculating 100 µL of overnight activated cultures into autoclaved 5 mL of arginine broth with a pH of 7.0. The mixture is incubated at 37°C for 24-48 hours. After incubation, a few drops of Nessler reagent are added to the broth, and a brown color indicates positive results, signifying that arginine has been hydrolyzed. This color disappears upon heating, confirming the presence of bacteria capable of converting arginine into putrescine.

### 3.6.2 Hemolysis

The hemolysis test is a crucial step in probiotic characterization to assess the safety of the bacteria. The test involves streaking overnight cultures of the isolated bacteria onto 5% defibrinated mammal's blood agar plates and incubating them at 37°C for 48 hours. The resulting zones around the colonies are observed to determine the hemolytic activity: α-hemolysis (dark and greenish zones), β-hemolysis (lightened –yellow or transparent zones), and γ-hemolysis (no change or no zones). This test ensures that the bacteria do not produce hemolysins, which could harm the host cells and indicate potential pathogenicity.

## 3.7 Physiochemical analysis of kefir

### 3.7.1 Acidity measurement

The titratable acidity of kefir samples was determined using a standardized titration method. Briefly, 10 mL of kefir was diluted with 10 mL of distilled water. The mixture was

then titrated with 0.1 N NaOH using phenolphthalein as an indicator until a faint pink color persisted. The titratable acidity was calculated as a percentage of lactic acid (AOAC, 2000).

$$\text{Acidity\%} = \frac{\text{VT}\times 0.009\times 100}{\text{VS}\times \text{d}}$$

Where, VS =Volume of sample, VT = Volume of 0.1N NaOH consumed & d = Kefir density equivalent to milk (1.0284 g/ml)

### 3.7.2 pH measurement

The pH of the kefir samples was measured using a digital pH meter (Hanna instruments, India). The pH meter was calibrated with standard buffer solutions at pH 4.0 and 7.0 before use. A 10 mL sample of kefir was placed in a beaker, and the electrode was immersed in the sample. The pH value was recorded once it stabilized. Regular calibration of the pH meter was performed to ensure accuracy.

### 3.7.3 Total soluble solids by brix refractometer

The total soluble solids (TSS) content of the kefir samples was determined using a refractometer. A few drops of the kefir sample were placed on the refractometer prism, and the Brix value was read directly from the scale. The refractometer was calibrated with distilled water before measurements. The Brix value represents the percentage of total soluble solids, which primarily consist of sugars (AOAC, 2000).

### 3.7.4 Organic acid content analysis by HPLC

The organic acid content in kefir was analyzed using High-Performance Liquid Chromatography (HPLC). Organic acids such as lactic acid and acetic acid, ethanol and carbohydrate such as glucose & lactose were identified and quantified by comparing the retention times and peak areas with those of known standards. All standards are taken in four different concentrations: 1, 10, 50 & 100 mg/ml for lactic acid, acetic acid, lactose, glucose and 1, 10, 20 & 50 mg/ml for ethanol due to presence of little concentration of ethanol in kefir. Thus, prepared series of calibration standard compound were filtered through 0.2 mm syringe filter and taken in HPLC vial. Kefir samples were taken in eppendorf tube and centrifuged to 10000rpm for 10 min & then supernatant was filter using 0.2mm syringe filter and 200 µL aliquot kept in HPLC vial. The Aminex HPX 87H(250×4mm) column

was used for product analysis equipped with a Silica column and a RID detector. The mobile phase consisted of 0.01 N sulfuric acid (eluent) with a flow rate of 0.6 ml/min. Colum temperature was 50 and detected based on RID with sample injection volume 10 (Bhatt et al., 2024).

### 3.8 Statistical analysis

Statistical analyses for all figures in the results section were performed using MS Excel. The comparison of multiple groups was carried out by one-way ANOVA, followed by Ducan's or Tukey's post-test for correction of multiple comparisons as indicated in the figures legend. The data probability value of $P < 0.05$ was considered significant.

# CHAPTER 4. RESULTS

## 4.1 Isolation of lactic acid bacteria and yeast from kefir

The results of the isolates on MRS and YEGA plates revealed distinct characteristics of bacterial and yeast species. The bacterial isolates, including isolates 1-4, exhibited Gram-positive and rod-shaped morphology, with varying colony morphologies such as pinhead, white, rough, and circular shapes. The catalase test was negative for all bacterial isolates, indicating the absence of catalase enzyme. In contrast, the yeast isolates, including isolates 1 and 2, showed typical yeast morphology with budding cells and were positive for Lactophenol staining, indicating the presence of chitin in their cell walls. The yeast isolates also exhibited mucoid and rough colony morphologies, respectively. Both yeast isolates tested positive for the catalase test, suggesting they can produce the catalase enzyme which is responsible for breaking down hydrogen peroxide into water and oxygen. These results suggest that the bacterial isolates are likely to be Gram-positive, rod-shaped bacteria, while the yeast isolates are likely to be yeast species with typical yeast morphology and chitin-containing cell walls.

**Table 4.1:** Isolation & cultural characteristics of Lactic acid bacteria and yeast from kefir

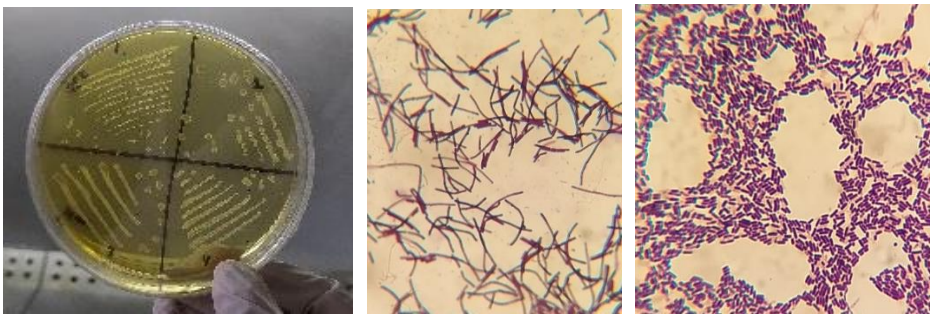
| Isolates | Colonies Morphology | Staining characteristics | Catalase test |
|---|---|---|---|
| Bacterial isolate 1 on MRS plate | Pin head & small, circular, white creaming | Gram positive and rod shaped | - |
| Bacterial isolate 2 on MRS plate | White, rough, round | Gram positive and rod shaped | - |
| Bacterial isolate 3 on MRS plate | Brownish white, rough, round | Gram positive and rod shaped | - |
| Bacterial isolate 4 on MRS plate | Circular, small, white | Gram positive and rod shaped | - |
| Yeast isolate 1 on YEGA plate | Round, white, mucoid | Lactophenol positive and typical yeast morphology with | + |

| | | | |
|---|---|---|---|
| | | budding cells | |
| Yeast isolate 2 on YEGA plate | Round, white, rough | Lactophenol positive and typical yeast morphology with budding cells | + |

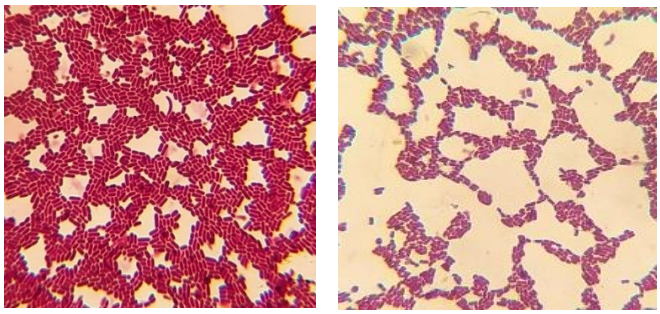
(a) (b) (c)


(e)

**Figure 4.1 :** Production of kefir. (a) Kefir grain, (b) Inoculation of kefir grain into pasteurized milk, (c) straining of kefir grain after fermentation & (e) Final product (kefir)

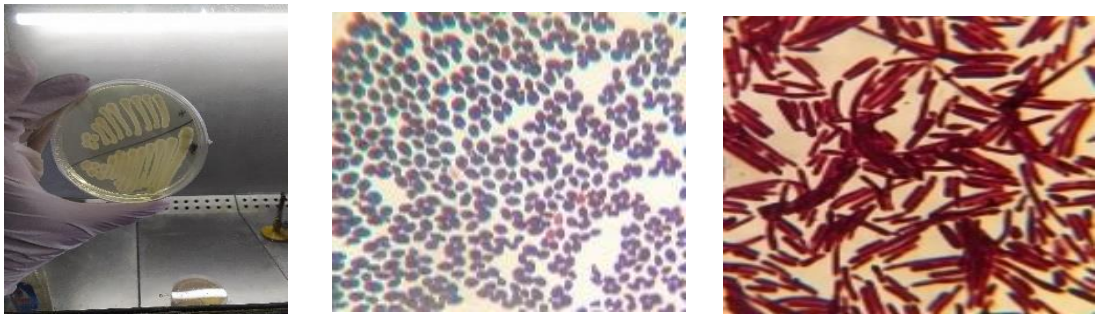


(a) (b) (c)



(d) (e)

**Figure 4.2:** Microbiological characteristics of bacterial isolates. (a) colonies on MRS agar, (b) Isolate 1, (c) Isolate 2, (d) Isolate 3 & (e) Isolate 4.



(a) (b) (c)

**Figure 4.3:** Microbiological characteristics of yeast isolates. (a) Yeast colonies on YEG agar, (b) Yeast isolate 1 & (c) Yeast isolate 2

## 4.2 Molecular analysis of isolated colony

### 4.2.1 Colony PCR of bacterial isolates

The 16S rRNA gene is a highly conserved region among bacteria and is commonly used for bacterial identification and phylogenetic analysis. The amplification of the 16S rRNA gene from individual colonies of lactic acid bacteria using 16S rDNA colony PCR involves the application of universal primers tailored to anneal to conserved regions of the gene. Gel electrophoresis is then employed to determine the size of the resulting DNA fragments from the PCR products. A distinct band within the 500 to 600 bp range signifies successful gene amplification. This technique is a common and effective means of identifying and characterizing lactic acid bacteria, providing an expedient and accurate method for confirming their presence in diverse environmental samples (Tilahun et al., 2018). The gel electrophoresis image shows the results of a 16S rDNA colony PCR performed on four lactic acid bacteria isolates selected based on their subcultural survival rates. The PCR products from these bacterial isolates were loaded in wells 1 to 4, while wells 5 serve as reference ladders containing a 100bp ladder. The PCR products in wells 1 to 4 each display a single prominent band, located between the 500bp and 600bp markers of the reference ladders, indicating that the amplified 16S rDNA fragments from these lactic acid bacteria isolates are within the 500 to 600 base pair (bp) range. This uniformity in band size suggests successful and specific amplification of the 16S rDNA region, which is a conserved region commonly used for bacterial identification and phylogenetic studies.

The consistency of the 500-600bp band size in the 16S rDNA colony PCR products of lactic acid bacteria isolates has a significant impact on bacterial identification. This uniformity in band size suggests successful and specific amplification of the 16S rDNA region, which is a conserved region commonly used for bacterial identification and phylogenetic studies (Tilahun et al., 2018). The consistency in band size across different isolates indicates that the PCR products are likely to be of high quality and free from contamination or non-specific binding. This is crucial for accurate bacterial identification, as it ensures that the amplified DNA sequences are representative of the target organism and not influenced by extraneous DNA (Hang et al., 2014).

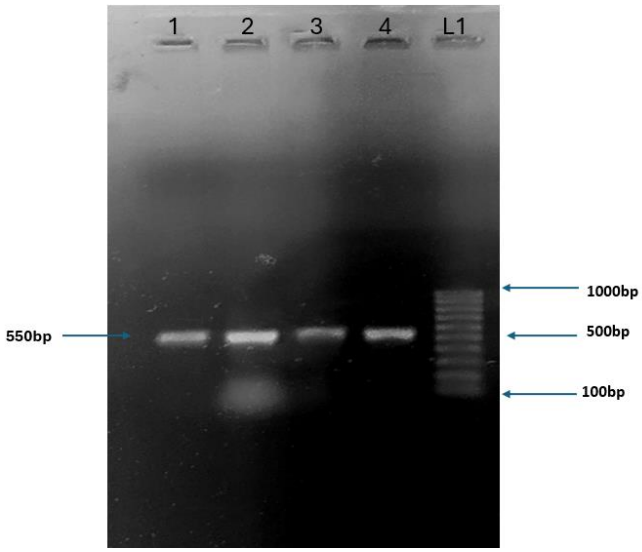



Figure 4.4 : Gel electrophoresis results showing DNA fragments from wells 1-4 (containing IS1-IS4 respectively), all around 550 bp. Lane L1 contains a DNA ladder(100bp) used for size reference.

### 4.2.2 Colony PCR of yeast isolates

The 18S rRNA gene serves as a molecular marker and is more suitable for yeast biodiversity studies compared to other genes due to its highly conserved nature within species. This conservation allows for the use of universal primers that can target the 18S rRNA gene across different yeast species, making it a reliable marker for identifying and characterizing yeast isolates. The 18S rRNA gene is a component of the small eukaryotic ribosomal subunit (40S) and is essential for all eukaryotic cells. The gene is highly conserved within species, with similarities reaching nearly 100%, making it an ideal marker for species-level analysis (Halim et al., 2024).

The gel electrophoresis image shows the outcomes of the 18S rDNA colony PCR for yeast isolates Y1 and Y2. The PCR products from these samples are placed in wells 1 and 2, with reference ladders in wells 3 and 4. The appearance of a 510bp band in wells 1 and 2 signifies a successful amplification of the 18S rDNA gene from the yeast isolates. This gene is a highly preserved area commonly utilized for yeast identification and phylogenetic research. The coherence and precision of the band indicate that the PCR amplification was successful in specifically targeting the correct region of the yeast genome. The consistent band size of 510bp in both yeast isolates indicates they could be of the same species or closely related species. Nonetheless, additional sequencing or a more thorough analysis would be necessary to validate identification at the species level. The size of the 510bp band is in the anticipated range for yeast 18S rDNA amplicons, which usually vary in length from 350bp

to 880bp. This validates that the PCR primers and conditions were suitable for amplifying the target region, showing a successful experimental configuration.The existence of a single, prominent 510bp band for each isolate indicates that the PCR reaction was precise and did not have any non-specific amplification or contamination. This particularity is essential for accurate recognition and additional genetic examination. The reference ladders found in wells 3 and 4 serve as a standard for determining the size and purity of the PCR products. Confirm that the bands in wells 1 and 2 are exactly 510bp by comparing them to the reference ladder. The DNA ladder acts as a visual guide, strengthening the precision of sizing the PCR products.

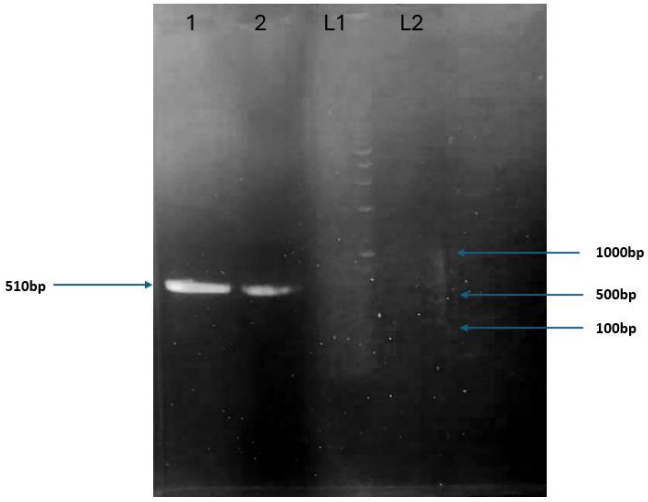


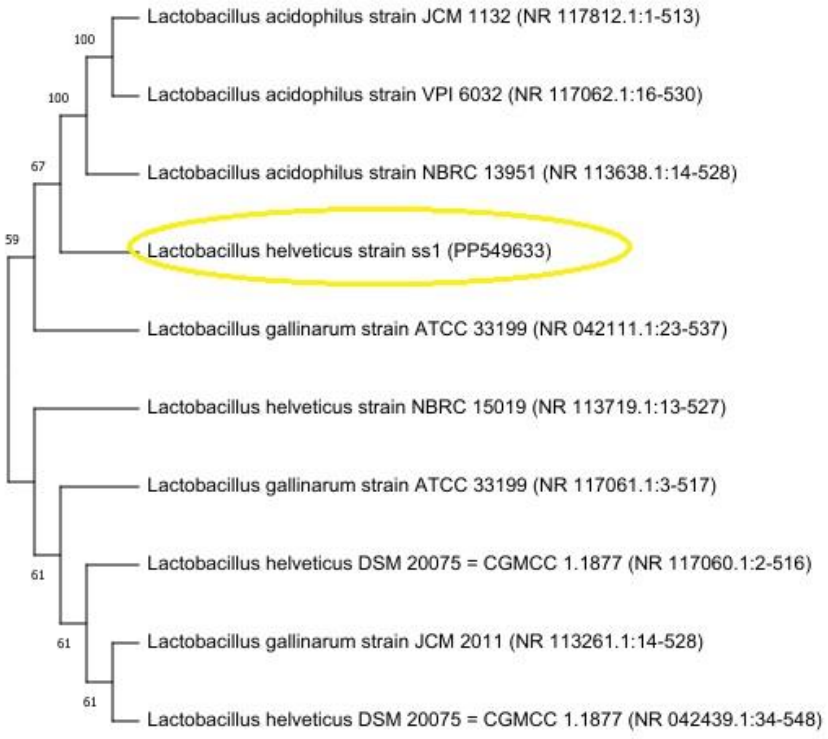

Figure 4.5 : Gel electrophoresis results showing colony PCR amplification products. Lanes 1 and 2 display clear bands of Y1 & Y2 isolates at approximately 510 bp respectively. L1 is the 1kb DNA ladder, and L2 is 100bp ladder.

### 4.2.3 Sequencing and phylogenetic tree of lactic acid bacteria and yeast

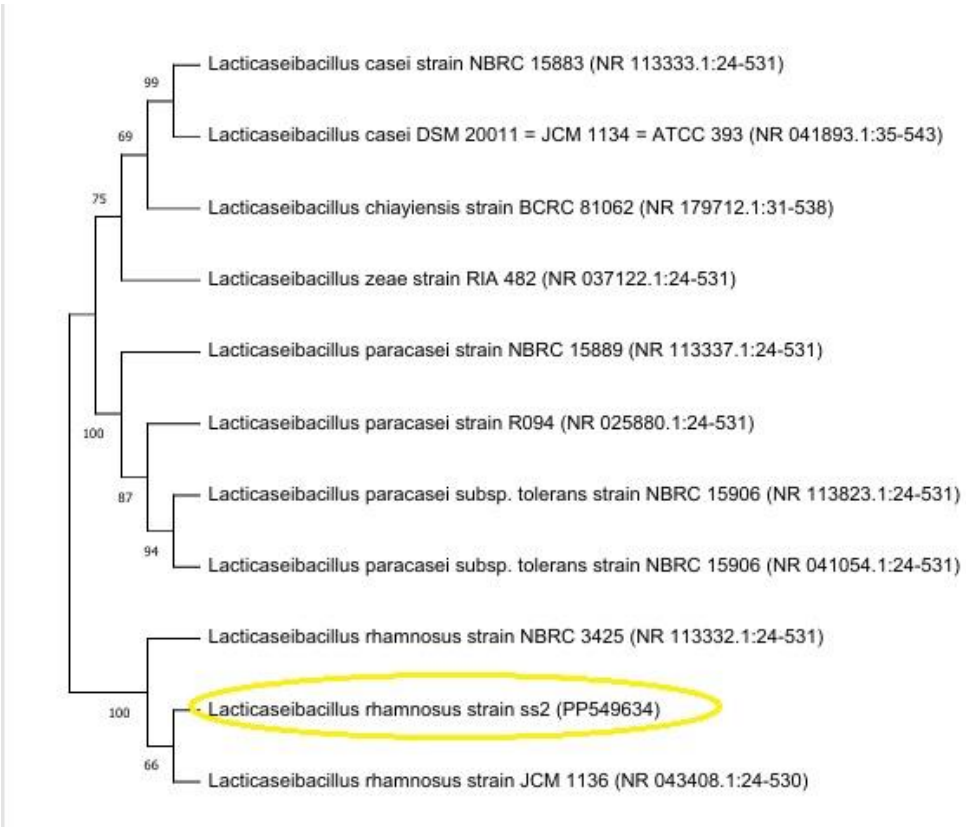
The lactic acid bacteria and yeast were analyzed through sequencing their 16S rRNA and 18S rRNA, respectively, to conduct phylogenetic analysis. The NAST organization conducted Sanger sequencing to obtain these sequences. The sequences generated were subsequently examined with the BLAST (Basic Local Alignment Search Tool) to match and contrast them with existing sequences in the database. Later, evolutionary relationships between the isolates and their closest relatives were clarified by generating phylogenetic trees using the Mega11 software as shown in figures below. This thorough method made it easier to correctly identify and categorize the lactic acid bacteria and yeast samples, offering knowledge about their genetic variation and evolutionary background.

**Table 4.2 :** Accession numbers of lactic acid bacteria and yeast after sequence submission in GenBank.

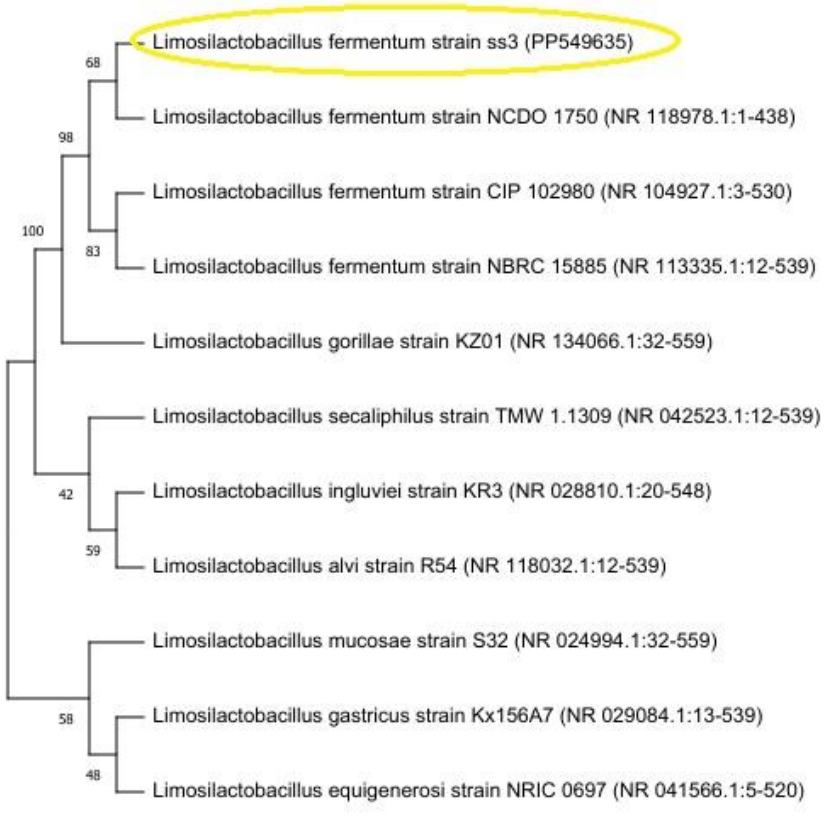
| Isolates code | Strains name | Accession numbers |
|---|---|---|
| ss1 | *Lactobacillus helveticus* | PP549633 |
| ss2 | *Lacticasiebacillus rhamnosus* | PP549634 |
| ss3 | *Limosilactobacillus fermentum* | PP549635 |
| ss4 | *Kazachstania martinaie* | PP551228 |
| ss5 | *Pichia chibdodasensis* | PP551229 |



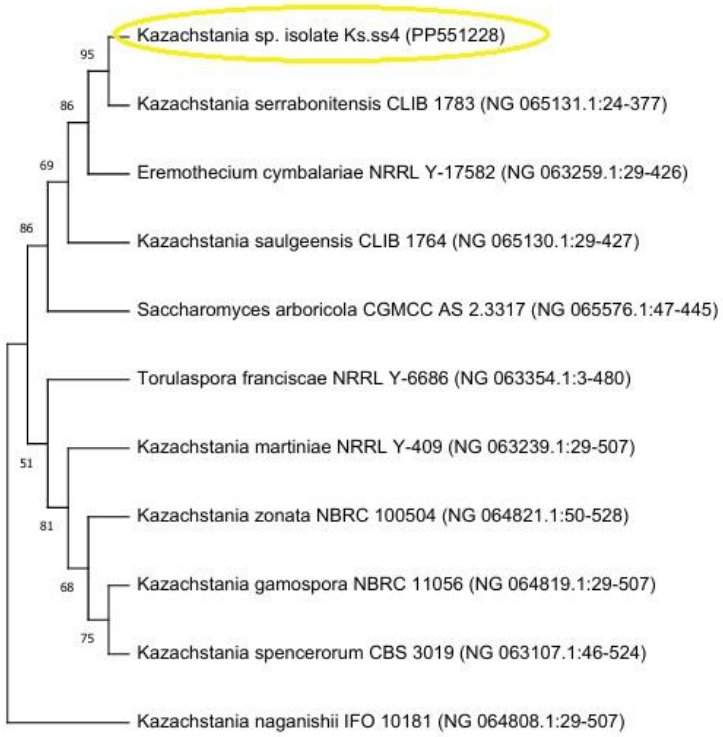

(a)


Lacticaseibacillus casei strain NBRC 15883 (NR 113333.1:24-531)
Lacticaseibacillus casei DSM 20011 = JCM 1134 = ATCC 393 (NR 041893.1:35-543)
Lacticaseibacillus chiayiensis strain BCRC 81062 (NR 179712.1:31-538)
Lacticaseibacillus zeae strain RIA 482 (NR 037122.1:24-531)
Lacticaseibacillus paracasei strain NBRC 15889 (NR 113337.1:24-531)
Lacticaseibacillus paracasei strain R094 (NR 025880.1:24-531)
Lacticaseibacillus paracasei subsp. tolerans strain NBRC 15906 (NR 113823.1:24-531)
Lacticaseibacillus paracasei subsp. tolerans strain NBRC 15906 (NR 041054.1:24-531)
Lacticaseibacillus rhamnosus strain NBRC 3425 (NR 113332.1:24-531)
Lacticaseibacillus rhamnosus strain ss2 (PP549634)
Lacticaseibacillus rhamnosus strain JCM 1136 (NR 043408.1:24-530)
99
69
75
100
87
94
100
66

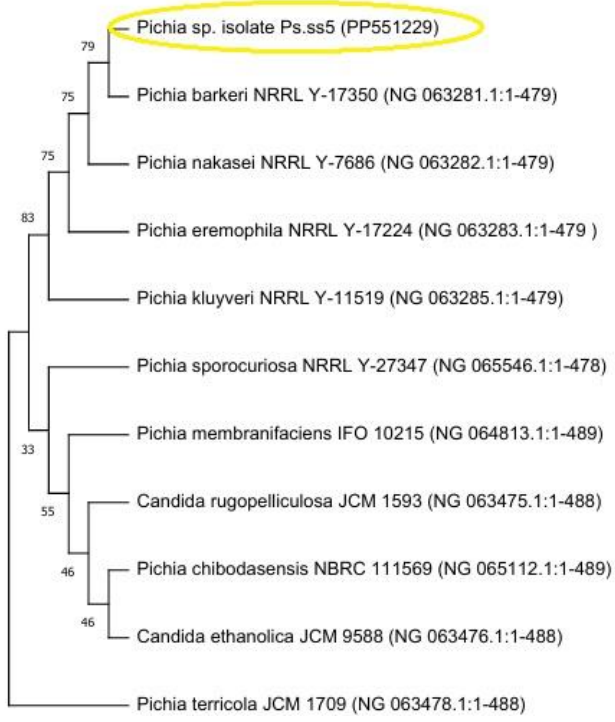
(b)


Limosilactobacillus fermentum strain ss3 (PP549635)
Limosilactobacillus fermentum strain NCDO 1750 (NR 118978.1:1-438)
Limosilactobacillus fermentum strain CIP 102980 (NR 104927.1:3-530)
Limosilactobacillus fermentum strain NBRC 15885 (NR 113335.1:12-539)
Limosilactobacillus gorillae strain KZ01 (NR 134066.1:32-559)
Limosilactobacillus secaliphilus strain TMW 1.1309 (NR 042523.1:12-539)
Limosilactobacillus ingluviei strain KR3 (NR 028810.1:20-548)
Limosilactobacillus alvi strain R54 (NR 118032.1:12-539)
Limosilactobacillus mucosae strain S32 (NR 024994.1:32-559)
Limosilactobacillus gastricus strain Kx156A7 (NR 029084.1:13-539)
Limosilactobacillus equigenerosi strain NRIC 0697 (NR 041566.1:5-520)
68
98
100
83
42
59
58
48

(c)




(d)




(e)

Figure 4.6: Phylogenetic tree of bacterial and yeast isolates. (*a) Lactobacillus helveticus, (b) Lacticasiebacillus rhamnosus, (c) Limosilactobacillus fermentum, (d) Kazachstania martinaie & (e) Pichia chibdodasensis*

## 4.3 Probiotic characterization of Lactic acid bacteria

### 4.3.1 Acid tolerance test

The graph illustrates the results of an acid tolerance test conducted on identified three types of lactic acid bacteria i.e. *Lactobacillus helveticus, Lactobacillus rhamnosus,* and *Lactobacillus fermentum*, measuring the optical density (OD) at 600 nm to indicate bacterial growth under different pH conditions and time periods (3 hours and 24 hours). At pH 2.5, all strains exhibit low growth, with *L. helveticus* showing slightly higher OD values at both time points. At pH 3, growth increases for all strains, with *L. helveticus* displaying the highest OD, followed by *L. rhamnosus* and *L. fermentum*, both at 3 and 24 hours. At pH 4.6, growth continues to increase, with *L. helveticus* showing the highest OD, while *L. rhamnosus* and *L. fermentum* have similar growth levels. At pH 5.6 (control), significant growth is observed for all strains, with *L. helveticus* having the highest OD at 3 hours, and *L. rhamnosus* leading at 24 hours, followed by *L. helveticus* and *L. fermentum*. This indicates that *L. helveticus* is more resilient across a range of pH conditions, especially at the control pH, making it a promising candidate for probiotic applications where pH stability is crucial (Hassan et al., 2020a; Taverniti & Guglielmetti, 2012). It also indicate that all three strains can tolerate a range of acidic environments, with *L. helveticus* being the most acid-tolerant, particularly at lower pH levels (2.5 and 3), and *L. rhamnosus* performing best in mildly acidic to neutral conditions (pH 5.6). *L. helveticus* and *L. rhamnosus* appear to have better acid tolerance than *L. fermentum*, particularly at lower pH levels. The data analysis was done by Two-Way ANOVA which revealed that both pH and time significantly affected bacterial absorbance for *L. helveticus, L. rhamnosus,* and *L. fermentum.* Absorbance increased with pH, from 0.1910 at pH 2.5 to 0.5914 at pH 5.6 after 3 hours, and from 0.1202 to 1.3378 at 24 hours. The model, explaining 97.3% of the variance ($R^2 = 0.973$), showed significant effects for pH ($p < 0.05$), time ($p < 0.05$), and their interaction ($p < 0.05$). The error bars, which reflect variability within the triplicate data, further support the statistical significance of these differences.

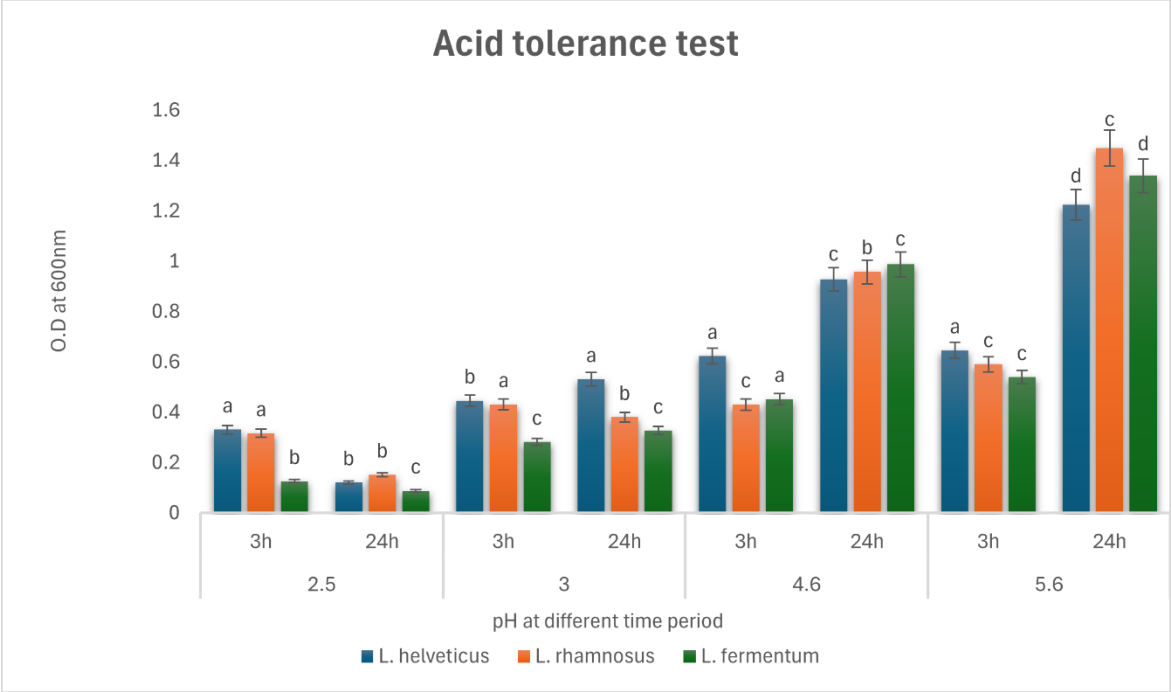



**Figure 4.7:** The data is statistically significant, as determined by two way ANOVA ($p < 0.05$). Tukey's HSD post hoc test was used to identify significant differences between groups, which are indicated by alphabetic superscripts in the error bars of the graph. *L. helveticus* demonstrates the highest acid tolerance, especially at pH 3, 4.6, and 5.6, while *L. fermentum* shows the lowest. 'a': not significantly different, 'b': different from 'a', not from 'c', 'c': significantly different.

### 4.3.2 Bile salt tolerane test

The data shows the growth of *Lactobacillus helveticus, Lactobacillus rhamnosus,* and *Lactobacillus fermentum* at different bile salt concentrations (0.3%, 0.5%, and 1%) after 3 and 24 hours, compared to the control (no bile salts) to assess their probiotic potential. The optical density (OD) at 600 nm was measured. At 0.3% bile salt, *L. helveticus* showed the highest growth at both time points, followed by *L. rhamnosus* and *L. fermentum*. At 0.5% bile salt, the growth pattern was similar, with *L. helveticus* maintaining the highest OD. At 1% bile salt, growth decreased for all strains, but *L. helveticus* still exhibited the highest OD. In the control condition, all strains exhibited significant growth, with *L. helveticus* leading, followed by *L. rhamnosus* and *L. fermentum*. These results indicate that all three strains can tolerate bile salts to some extent, with *L. helveticus* demonstrating the highest tolerance across all conditions, making it the most promising candidate for probiotic applications requiring bile salt tolerance. *L. rhamnosus* shows moderate tolerance, performing best in the control and lower bile salt conditions, while *L. fermentum* exhibits the least tolerance but still shows significant growth in the absence of bile salts.

Compared to the control, all three species showed reduced growth as the bile salt concentration increased, with *L. helveticus* being the most tolerant, followed by *L. rhamnosus* and *L. fermentum* (Hassan et al., 2020a).

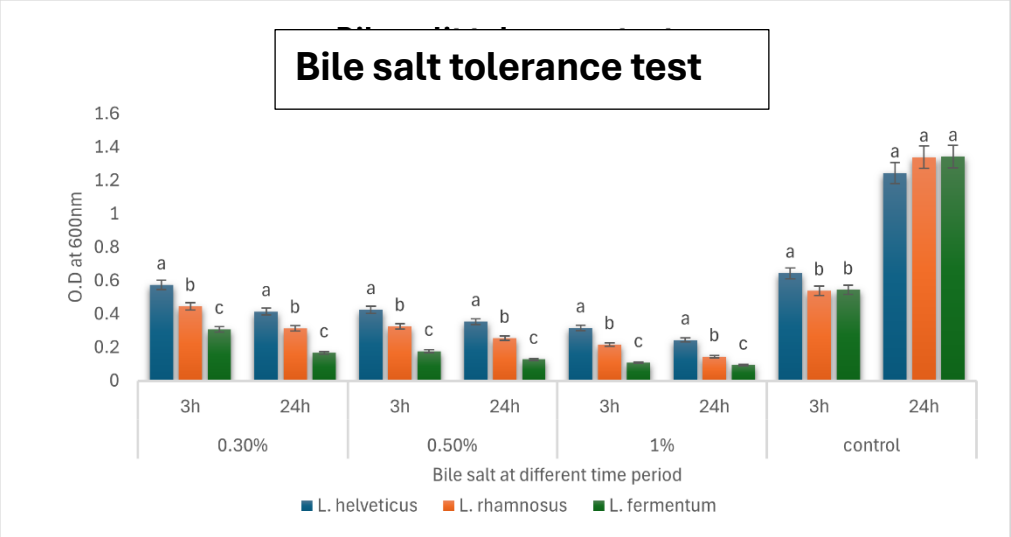



**Figure 4.8:** The graph illustrates the bile salt tolerance of *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* at various concentrations over 3 and 24 hours. Statistically significant differences ($p < 0.05$) in optical density (OD) were observed by two-way ANOVA followed by post hoc test. *L. helveticus* showing the highest tolerance. The control group had significantly higher OD values, indicating that bile salts inhibit growth, especially with prolonged exposure. 'a': not significantly different, 'b': different from 'a', not from 'c', 'c': significantly different.

### 4.3.3 Cell autoaggregation test

Cell autoaggregation test conducted on identified three types of lactic acid bacteria i.e. *Lactobacillus helveticus, Lactobacillus rhamnosus,* and *Lactobacillus fermentum* to assess their potential for probiotic characterization as results shown in the bar graph illustrated below. The test measures the percentage of cell autoaggregation, an important characteristic for colonization and persistence in the gastrointestinal tract. *Lactobacillus helveticus* exhibits the highest autoaggregation percentage at around 65%, indicating a strong tendency to aggregate, followed by *Lactobacillus rhamnosus* at close to 60%, also demonstrating good aggregation properties. *Lactobacillus fermentum* has the lowest autoaggregation percentage, approximately 45%, suggesting a weaker aggregation capability compared to the other two strains. These results highlight *Lactobacillus helveticus* and *Lactobacillus rhamnosus* as promising candidates for probiotic applications based on their superior cell autoaggregation characteristics, which enhance their ability to adhere to the intestinal mucosa and form biofilms, beneficial traits for probiotics.

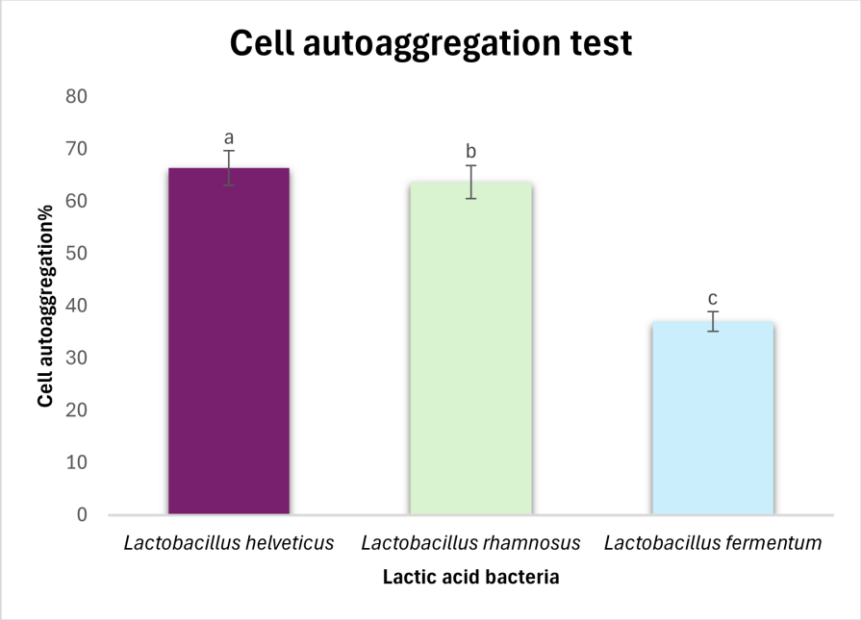



Figure 4.9: The graph shows the cell autoaggregation percentages for *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum*, analyzed by one-way ANOVA and Tukey's post-hoc test, indicates significant differences ($p < 0.05$) between all three bacterial strains, differences are marked using distinct letters. *L. helveticus* has the highest autoaggregation ability, followed by *L. rhamnosus* and *L. fermentum*.

### 4.3.4 Cell coaggregation test

The results of a study on the coaggregation abilities of *Lactobacillus helveticus, Lactobacillus rhamnosus*, and *Lactobacillus fermentum* with *E. coli* are shown in the bar graph below. The percentage of cell coaggregation is depicted on the y-axis, with the three different LAB strains listed on the x-axis. The data presented demonstrates that *Lactobacillus helveticus* exhibits the highest coaggregation rate, standing at about 28%, with *Lactobacillus rhamnosus* trailing at approximately 20%, and *Lactobacillus fermentum* at around 15%. The findings indicate that *Lactobacillus helveticus* has the highest effectiveness in coaggregating with *E. coli* compared to *Lactobacillus rhamnosus* and *Lactobacillus fermentum*. This suggests that *Lactobacillus helveticus* might be a more effective probiotic because of its better coaggregation ability, which can aid in preventing the presence and multiplication of harmful bacteria such as E. coli in the intestines.

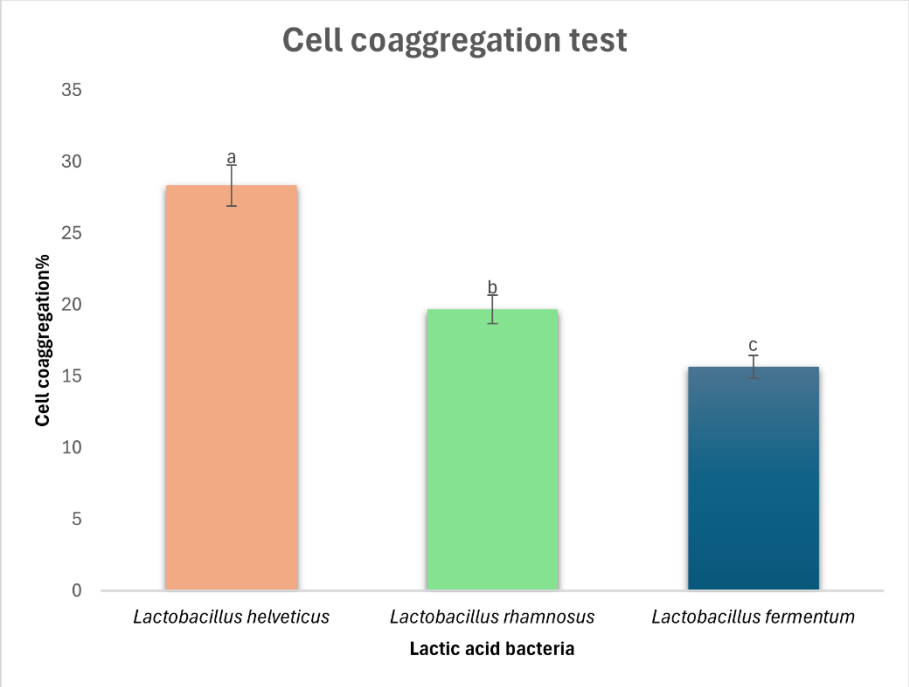



**Figure 4.10:** The graph shows the cell coaggregation percentages for *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum*, analyzed by one-way ANOVA and Tukey's post-hoc test, indicates significant differences ($p < 0.05$) between all three bacterial strains, differences are marked using distinct letters. *L. helveticus* has the highest autoaggregation ability, followed by *L. rhamnosus* and *L. fermentum*.

### 4.3.5 Cell surface hydrophobicity

Cell surface hydrophobicity test is important for evaluating probiotic characteristics, as hydrophobicity can impact the bacteria's ability to bind to intestinal epithelial cells. It was examined by subjecting all three identified strains of LAB to hexane, which is a type of hydrocarbon. The findings demonstrate that *Lactobacillus helveticus* exhibits the greatest level of hydrophobicity, around 40%, suggesting a significant attraction towards hexane. This is then followed by *Lactobacillus rhamnosus*, which has a hydrophobicity level of approximately 30%, and *Lactobacillus fermentum*, which has a level of about 25%. The results indicate that *Lactobacillus helveticus* has the highest cell surface hydrophobicity among the strains tested, which could improve its ability to stick to the intestinal lining and withstand being washed away. Therefore, *Lactobacillus helveticus* is likely the top choice for probiotic use because of its exceptional adhesive properties, with *Lactobacillus rhamnosus* and *Lactobacillus fermentum* following closely behind.

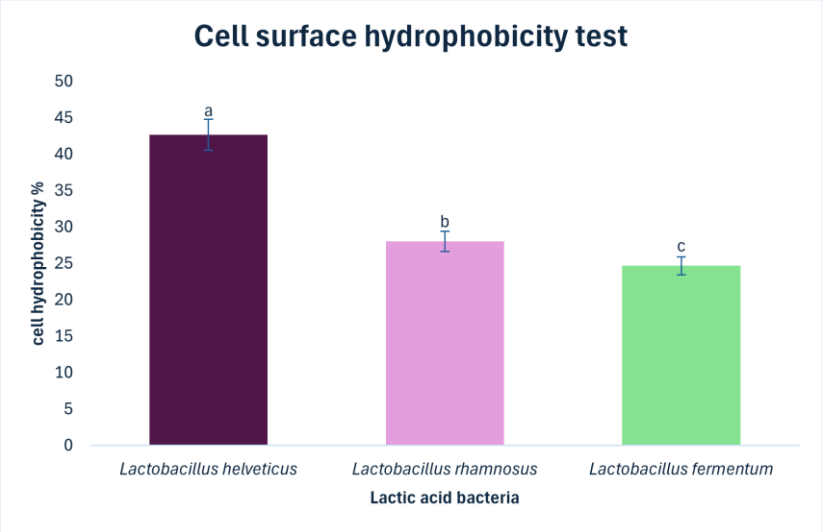



**Figure 4.11:** The cell surface hydrophobicity of three *Lactobacillus* species, with *Lactobacillus helveticus* exhibiting the highest hydrophobicity. The differences between these strains appear to be statistically significant, as indicated by the minimal overlap in error bars, and confirmed by one-way ANOVA and Tukey's post-hoc test, indicates significant differences ($p < 0.05$) between all three bacterial strains, differences are marked using distinct letters.

### 4.3.6 NaCl tolerance test

This test assesses the growth and tolerance of LAB to varying concentrations of NaCl (2%, 5%, 7%, and 9%) which is crucial for probiotic characterization. *Lactobacillus helveticus* exhibits the highest O.D. in the control and at 2% NaCl (approximately 1.2), decreasing gradually to around 0.8 at 9% NaCl. Similarly, *Lactobacillus rhamnosus* peaks in the control (about 1.3), slightly lower at 2% NaCl, and declines to approximately 0.7 at 9% NaCl. *Lactobacillus fermentum* shows peak O.D. in the control (around 1.2), slightly reduced at 2% NaCl, and drops to about 0.6 at 9% NaCl. Overall, all three strains demonstrate reduced growth as NaCl concentration increases, indicating inhibition by higher salt levels. However, they exhibit good tolerance at lower NaCl concentrations (2% and 5%), with *Lactobacillus rhamnosus* demonstrating the highest overall tolerance. These results suggest *Lactobacillus rhamnosus* may be the most resilient strain in environments with varied salt levels, followed closely by *Lactobacillus helveticus* and *Lactobacillus fermentum*. These findings are significant for probiotic applications, highlighting the importance of salt tolerance for survival and effectiveness in diverse food products and gastrointestinal conditions.

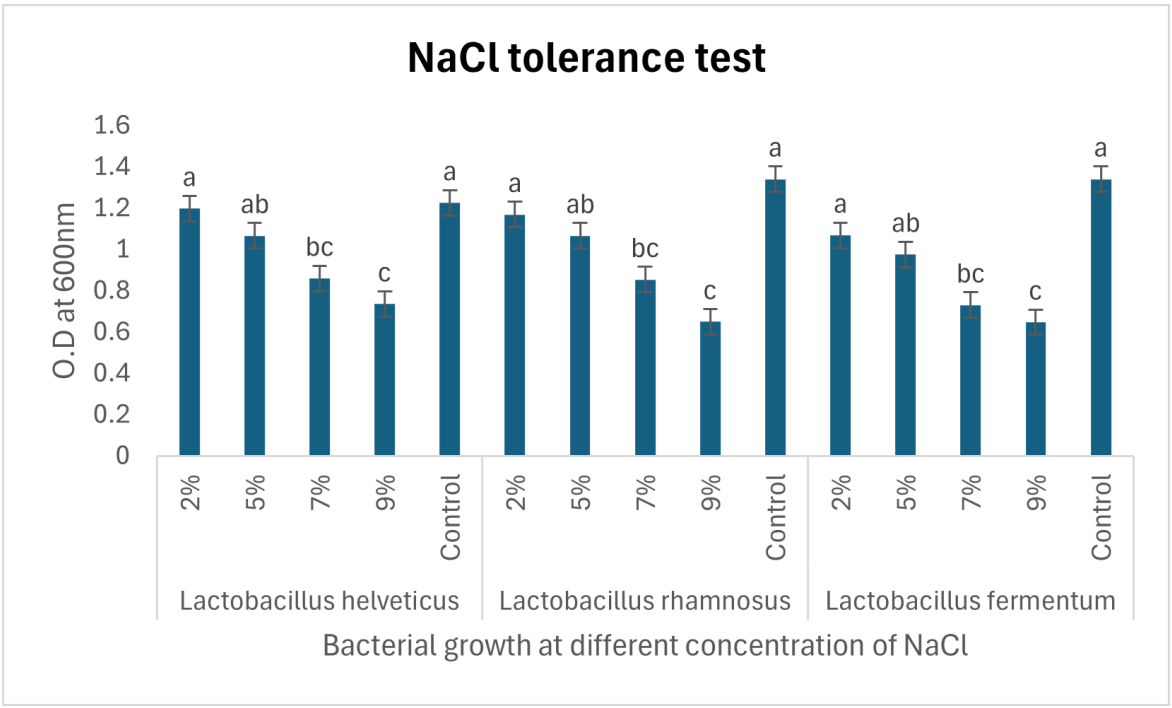



**Figure 4.12:** Significant differences between bacterial growth at different concentrations of NaCl are indicated by different alphabetic superscripts (a, b, c). Different concentrations of NaCl sharing the same superscript are not significantly different from each other ($p > 0.05$), while different concentration of NaCl with different superscripts are significantly different ($p < 0.05$), as determined by Tukey's HSD post hoc test following a significant one-way ANOVA result. The analysis reveals that higher NaCl concentrations (7% and 9%) significantly reduce the growth of all three Lactobacillus species compared to lower concentrations (2% and 5%) and the control group.

### 4.3.7 Phenol tolerance test

The phenol tolerance test results for the lactic acid bacteria (LAB) strains *Lactobacillus helveticus, Lactobacillus rhamnosus*, and *Lactobacillus fermentum* provide insights into their ability to survive in the presence of phenol, a compound toxic to many microorganisms. The optical density (O.D.) at 600 nm was measured to determine bacterial growth under two conditions: in the presence of 0.4% phenol and in a control without phenol. *Lactobacillus helveticus* showed a significant reduction in growth, with the O.D. dropping from 1.343 (control) to 0.406 in phenol, indicating moderate tolerance. *Lactobacillus rhamnosus* also exhibited reduced growth, with the O.D. decreasing from 1.202 (control) to 0.425 in phenol, demonstrating moderate tolerance but a significant impact. *Lactobacillus fermentum* displayed the highest phenol tolerance, with the O.D. reducing from 1.345 (control) to 0.557 in phenol, indicating a relatively better ability to withstand phenol's inhibitory effects. Overall, all three strains show reduced growth in the presence of phenol, with *Lactobacillus fermentum* being the most resilient, suggesting its

potential as a robust probiotic in environments with phenolic compounds, while *Lactobacillus helveticus* and *Lactobacillus rhamnosus*, although somewhat tolerant, are more affected.

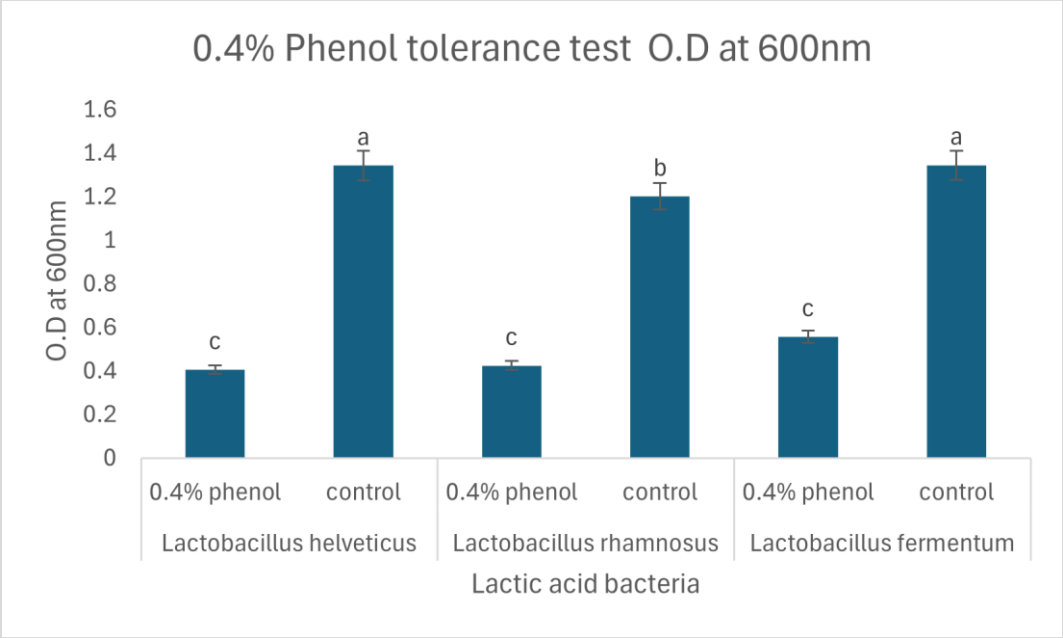



**Figure 4.13:** Observation of phenol tolerance of three *Lactobacillus* species at 0.4% phenol vs control with no phenol. The differences between these strains appear to be statistically significant, as determined by one-way ANOVA and Tukey's HSD post hoc test $p<0.05$. 'a': not significantly different (*L. helveticus*, *L. fermentum* control), 'b': different from 'a', not from 'c' (*L. rhamnosus* control), 'c': significantly different (0.4% phenol for all species).

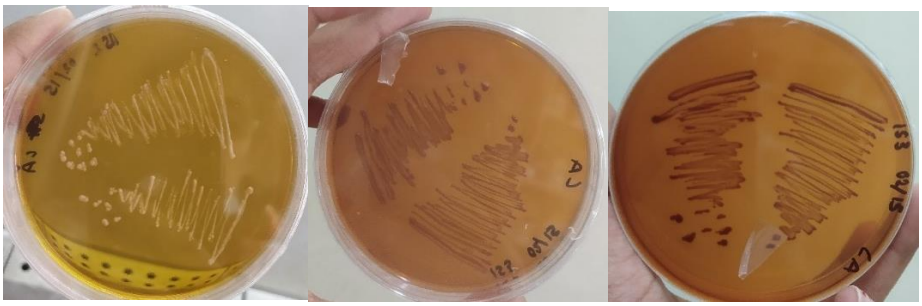
### 4.3.8 Biofilm formation test

The biofilm activity of lactic acid bacteria (LAB) can be studied using the Congo Red Agar (CRA) method, which involves the use of CRA containing Congo Red stain to visualize biofilm formation on the agar surface. This method is based on the principle that Congo Red binds to EPS produced by LAB, resulting in black colonies that indicate biofilm formation, while the absence of black colonies indicates no biofilm formation(Mekky et al., 2022). The results from this test indicate that *L. rhamnosus* and *L. fermentum* colonies changes into black color indicating biofilm activity, while *L. helveticus* does not, suggesting *L. helveticus* may be less effective in forming biofilms, which are important for its probiotic potential. Biofilms are surface-adhering communities of microorganisms that produce extracellular polymeric substances (EPS) and can grow on any surface, providing protection and support for bacteria, allowing them to survive in stressful environments with high levels of antimicrobial agents.

**Table 4.3:** Biofilm formation test of lactic acid bacteria

| Species | Biofilm activity on Congo Agar plates |
|---|---|
| *Lactobacillus helveticus* | - |
| *Lactobacillus rhamnosus* | + |
| *Lactobacillus fermentum* | + |

+: colonies change in black color, -: no changes in colonies



(a) (b) (c)

Figure 4.14: Lactic acid bacteria on CA plate showing absence of black colonies (a)*Lactobacillus helveticus,* (b) *Lactobacillus rhamnosus &* (c) *Lactobacillus fermentum*

### 4.3.9 Bile salt hydrolase test

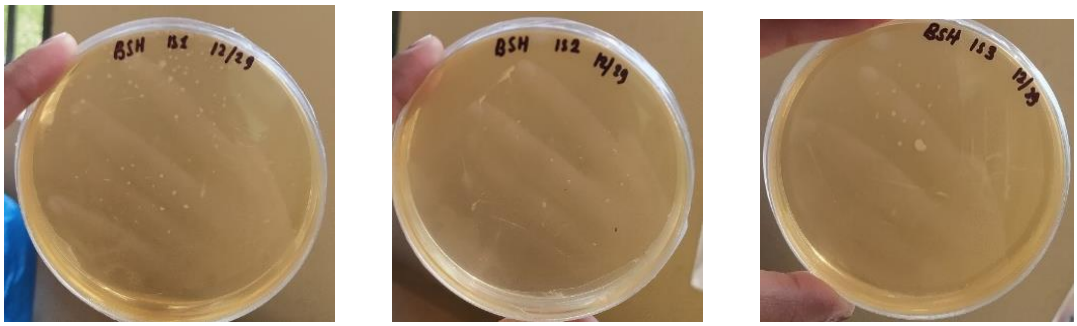
In the BSH assay, a mixture of conjugated bile salts, such as taurocholic acid, taurochenodeoxycholic acid, and glycocholic acid, is used to assess bile salt hydrolase (BSH) activity. The BSH enzyme hydrolyzes these bile salts into deconjugated bile salts and free amino acids, resulting in a white or yellowish precipitate around the bacterial colonies. The size and intensity of this precipitation zone indicate the level of BSH activity, with larger and more intense zones reflecting higher activity(Ghosh et al., 2008; Kim & Lee, 2005; Tanaka et al., 1999). In this test, *Lactobacillus helveticus* showed the highest BSH activity with intense precipitation (+++), indicating a strong capacity to deconjugate bile salts. *Lactobacillus rhamnosus* and *lactobacillus fermentum* exhibited moderate activity with slight precipitation (++). These results suggest that *L. helveticus* has the most robust ability to hydrolyze bile salts, which is beneficial for its probiotic potential, as BSH activity enhances bacterial survival in the gastrointestinal tract, inhibits pathogenic bacteria, and

improves gut health.

**Table 4.4:** Bile salt hydrolase test of lactic acid bacteria

| Species | Bile salt hydrolase activity |
|---|---|
| *Lactobacillus helveticus* | +++ |
| *Lactobacillus rhamnosus* | ++ |
| *Lactobacillus fermentum* | ++ |

-: no growth, +: growth only, ++: slight precipitation, +++: intense precipitation,




(a) (b) (c)

Figure 4.15: Lactic acid bacteria on BSH plate showing intense precipitation (a)*Lactobacillus helveticus,* (b) *Lactobacillus rhamnosus &* (c) *Lactobacillus fermentum.*

### 4.3.10 Antibiotic susceptibility test

The data presented in the table shows the antibiotic susceptibility of three lactic acid bacteria (LAB) strains: *Lactobacillus helveticus, Lactobacillus rhamnosus,* and *Lactobacillus fermentum*. The strains were tested against six different antibiotics: Bacitracin, Cefotaxime, Entrapenem, Piperacillin, Cloxacillin, and Vancomycin. The results show that *Lactobacillus helveticus* is sensitive to Bacitracin, Cefotaxime, and Piperacillin, but resistant to Entrapenem and Cloxacillin. *Lactobacillus rhamnosus* is sensitive to Bacitracin, Cefotaxime, and Piperacillin, but resistant to Entrapenem and Cloxacillin. *Lactobacillus fermentum* is sensitive to Bacitracin, Cefotaxime, and Piperacillin, but resistant to Entrapenem and Cloxacillin. The antibiotic susceptibility of these strains is important for understanding their potential as probiotics. Probiotics are live microorganisms that are intended to confer health benefits when administered in adequate amounts. The ability of these strains to resist certain antibiotics may impact their ability to survive and thrive in the gastrointestinal

tract, where they are intended to provide health benefits.

**Table 4.5:** Antibiotic susceptibility test of lactic acid bacteria

| Antibiotic discs | Lactic acid bacteria | | |
|---|---|---|---|
| | *Lactobacillus helveticus* | *Lactobacillus rhamnosus* | *Lactobacillus fermentum* |
| Bacitracin | S | I | S |
| Cefotaxime | S | S | S |
| Entrapenem | R | R | R |
| Piperacillin | R | R | R |
| Cloxacillin | R | R | R |

R: Resistant (Zone size≤14mm); I: Intermediate (Zone size=15–19mm); S: Sensitive (Zone size≥20mm).

### 4.3.11 Antimicrobial activity test

The analysis of the antimicrobial activity of different *Lactobacillus* strains against various pathogens revealed significant differences in their inhibitory effects, as measured by the zone of inhibition (ZOI) in millimeters. For *E. coli, Lactobacillus helveticus* exhibited a significantly larger ZOI (14.4 ± 0.3 mm) compared to *Lactobacillus fermentum* (12.1 ± 0.7 mm), with no data available for *Lactobacillus rhamnosus*. In the case of *Staphylococcus*, *Lactobacillus helveticus* again showed the highest inhibitory effect (27.9 ± 0.8 mm), significantly different from both *Lactobacillus rhamnosus* (24.7 ± 0.3 mm) and *Lactobacillus fermentum* (23.8 ± 1.5 mm), which did not differ significantly from each other. Against *Pseudomonas, Lactobacillus rhamnosus* demonstrated the greatest inhibition (30.8 ± 0.8 mm), significantly different from both Lactobacillus helveticus (26.1 ± 0.5 mm) and *Lactobacillus fermentum* (25.5 ± 0.1 mm), which were not significantly different from each

other. There were no zones of inhibition recorded for *Klebsiella* for any of the tested strains. The results indicate that *Lactobacillus helveticus* generally has superior antimicrobial activity compared to the other strains, especially against *E. coli* and *Staphylococcus,* while *Lactobacillus rhamnosus* is most effective against Pseudomonas.

**Table 4.6 :** Antimicrobial sensitivity test of lactic acid bacteria

| Tested Organisms (ATCC) | Lactic acid bacteria | | |
|---|---|---|---|
| | Lactobacillus helveticus (mean ± SD, SE) | Lactobacillus rhamnosus (mean ± SD, SE) | Lactobacillus fermentum (mean ± SD, SE) |
| *E. coli* 25922 | 14.4 ± 0.3 (0.2)[a] | - | 12.1 ± 0.7 (0.4)[b] |
| *Staphylococcus aureus* 43300 | 27.9 ± 0.8 (0.5)[a] | 24.7 ± 0.3 (0.2)[b] | 23.8 ± 1.5 (0.9)[b] |
| *Pseudomonas aeroginosa* 49619 | 26.1 ± 0.5 (0.3)[b] | 30.8 ± 0.8 (0.5)[a] | 25.5 ± 0.1 (0.1)[b] |
| *Klebsiella pneumoniea* 700603 | - | - | - |

ZOI= Zone of inhibition, the value represents the mean zone of inhibition with a standard deviation of three replicates in mm, including a 6.5mm diameter of the well (symbol '–' shows no zone of inhibition). Statistical analysis (One-way ANOVA and Duncan's multiple range tests) identifies significant differences ($p \leq 0.05$) marked by superscripts (a–b).

(a) (b) (c) (d)

Figure 4.16: Antimicrobial activity test of lactic acid bacteria against pathogenic bacteria (a) *E. coli* 25922, (b) *Staphylococcus aureus* 43300, (c) *Pseudomonas aeroginosa* 49619 & (d) *Klebsiella pneumoniea* 700603

### 4.3.12 Growth at different temperatures

The growth of three different species of lactic acid bacteria, namely *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum*, was evaluated at four different temperatures: 15℃, 25℃, 37℃, and 45℃. At 15℃, all three species exhibited growth, indicated by a single "+" symbol. At 25 ℃, *Lactobacillus helveticus* and *Lactobacillus rhamnosus* continued to show growth ("+"), while *Lactobacillus fermentum* showed an increase in growth performance ("++"). At 37℃, *Lactobacillus helveticus* and *Lactobacillus fermentum* demonstrated excellent growth ("++"), whereas *Lactobacillus rhamnosus* maintained a steady growth ("+"). Similarly, at 45℃, both *Lactobacillus helveticus* and *Lactobacillus fermentum* exhibited excellent growth ("++"), while *Lactobacillus rhamnosus* showed consistent growth ("+"). Overall, *Lactobacillus helveticus* and *Lactobacillus fermentum* displayed optimal growth at higher temperatures (37℃ and 45℃), whereas *Lactobacillus rhamnosus* showed consistent but moderate growth across all tested temperatures.

**Table 4.7:** Growth at different temperature of lactic acid bacteria

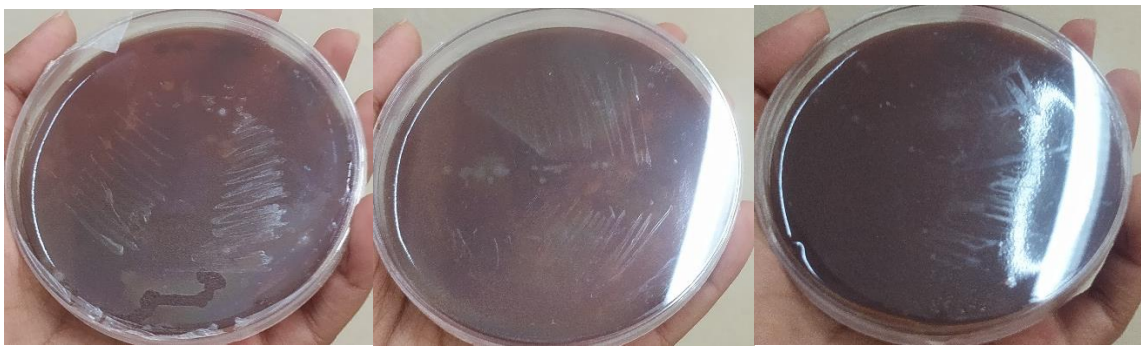
| Temperatures | Lactic acid bacteria | | |
|---|---|---|---|
| | *Lactobacillus helveticus* | *Lactobacillus rhamnosus* | *Lactobacillus fermentum* |
| 15℃ | + | + | + |
| 25℃ | + | + | ++ |
| 37℃ | ++ | + | ++ |
| 45℃ | ++ | + | ++ |

-: no growth, +: growth only, ++: excellent growth.

## 4.4 Safety assay

### 4.4.1 Hemolysis test

The hemolysis test, which assesses the ability of lactic acid bacteria (LAB) to lyse red blood cells, showed negative results for all three strains of LAB: Lactobacillus helveticus, Lactobacillus rhamnosus, and Lactobacillus fermentum. This means that none of these strains exhibited hemolytic activity, as indicated by the absence of clear zones on sheep blood agar plates. A negative result for hemolysis is considered a safety requirement when selecting probiotic strains, as it ensures that the bacteria are not virulent and do not produce hemolysins, which can have cytolytic effects on host cells (Golnari et al., 2024).

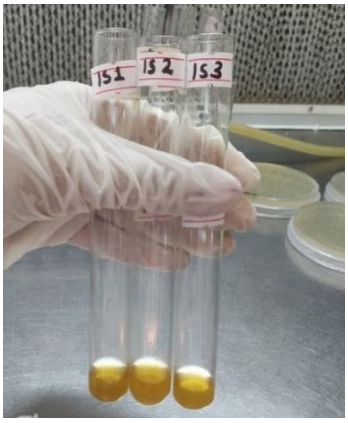


(a) (b) (c)

Figure 4.17: Hemolysis test of lactic acid bacteria showing negative results (a)*Lactobacillus helveticus,* (b) *Lactobacillus rhamnosus* & (c) *Lactobacillus fermentum*

### 4.4.2 Arginine test

The arginine test was negative for all the lactic acid bacteria strains tested, including *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum.* A negative arginine test indicates that these bacteria are unable to hydrolyze arginine to produce ammonia and carbon dioxide, which is a desirable characteristic for probiotics as it ensures their safety for human consumption. In contrast, a positive result would indicate the presence of arginine hydrolysis, which could lead to adverse effects on human health. The negative result for these LAB strains suggests that they are safe for use as probiotics, as they do not possess the ability to produce ammonia from arginine and cause harm (Soltan et al., 2017).




(a) (b) (c)

Figure 4.18: Arginine test of lactic acid bacteria showing negative results (a)*Lactobacillus helveticus,* (b) *Lactobacillus rhamnosus* & (c) *Lactobacillus fermentum*

## 4.5 Physiochemical analysis of kefir

The study results indicate notable differences between buffalo milk kefir and cow dairy milk kefir in terms of pH, acidity, Degree Brix, and the concentrations of organic acids and sugars as measured by HPLC. Buffalo milk kefir exhibited a higher pH (4.7 ± 0.082) and lower acidity (1.37 ± 0.024%) compared to cow dairy milk kefir, which had a pH of 4.5 ± 0.082 and an acidity of 1.96 ± 0.033%. Degree Brix was also higher in buffalo milk kefir (6 ± 0.163) than in cow dairy milk kefir (5 ± 0.082), indicating a greater sugar content.

HPLC analysis revealed that buffalo milk kefir had a significantly higher lactose concentration (32.92 ± 0.088 mg/ml) compared to cow dairy milk kefir (18.02 ± 0.061 mg/ml), while glucose levels were similar in both (47.00 ± 0.09 mg/ml for buffalo and 45.00 ± 0.09 mg/ml for cow dairy). Lactic acid concentration was much higher in cow dairy milk kefir (115.76 ± 0.204 mg/ml) than in buffalo milk kefir (45.30 ± 0.163 mg/ml), contributing to the higher overall acidity in cow dairy milk kefir. Acetic acid concentrations were slightly higher in cow dairy milk kefir (5.53 ± 0.021 mg/ml) compared to buffalo milk kefir (5.18 ± 0.028 mg/ml). Lastly, ethanol levels were marginally higher in buffalo milk kefir (0.625 ± 0.004 mg/ml) than in cow dairy milk kefir (0.477 ± 0.002 mg/ml).

These findings suggest that the fermentation process in buffalo milk kefir results in lower lactic acid production and higher residual lactose, contributing to its higher pH and lower acidity. The higher Degree Brix and lactose content in buffalo milk kefir indicate a sweeter taste profile compared to cow dairy milk kefir. The HPLC method effectively quantified these differences, providing insights into the distinct biochemical compositions and fermentation dynamics between the two types of kefirs.

**Table 4.8:** Physiochemical properties of Kefir

| Fresh Milk Kefir | pH (mean ± SD) | Acidity (%) (mean ± SD) | Degree Brix (mean ± SD) |
|---|---|---|---|
| Buffalo milk kefir | 4.7 ± 0.082 | 1.37 ± 0.024 | 6 ± 0.163 |
| Cow dairy milk kefir | 4.5 ± 0.082 | 1.96 ± 0.033 | 5 ± 0.082 |

**Table 4.9:** Operation conditions in HPLC for Quantification of organic acids & sugars.

| Chromatograph | Operation conditions |
|---|---|
| Colum temperature | 50℃ |
| Mobile phase | $H_2SO_4$ |
| Stationary phase | Silica |
| Washing Solvent | Acetonitrile & Water |
| Pressure limits | 0-400 bar |
| Injected volume | 10µL |
| Stop Time | 25 min |

**Table 4.10 :** Retention time of some standards, monitored in chromatographic column, under operational conditions.

| Component | Retention time |
|---|---|
| Glucose | 7.5 |
| Lactose | 8.5 |
| Lactic acid | 12.5 |
| Acetic acid | 14.5 |
| Ethanol | 19.5 |

**Table 4.11:** Concentration of different organic acids and sugars determined by HPLC analysis.

| Organic acids and sugars | Organic acids and sugar concentrations(mg/ml) | |
|---|---|---|
| | Buffalo milk kefir (mean ± SD) | Cow dairy milk kefir (mean ± SD) |
| Lactose | 32.92 ± 0.088 mg/ml | 18.02 ± 0.061 mg/ml |
| Glucose | 47.00 ± 0.09 mg/ml | 45.00 ± 0.09 mg/ml |
| Lactic acid | 45.30 ± 0.163 mg/ml | 115.76 ± 0.204 mg/ml |
| Acetic acid | 5.18 ± 0.028 mg/ml | 5.53 ± 0.021 mg/ml |
| Ethanol | 0.625 ± 0.004 mg/ml | 0.477 ± 0.002 mg/ml |

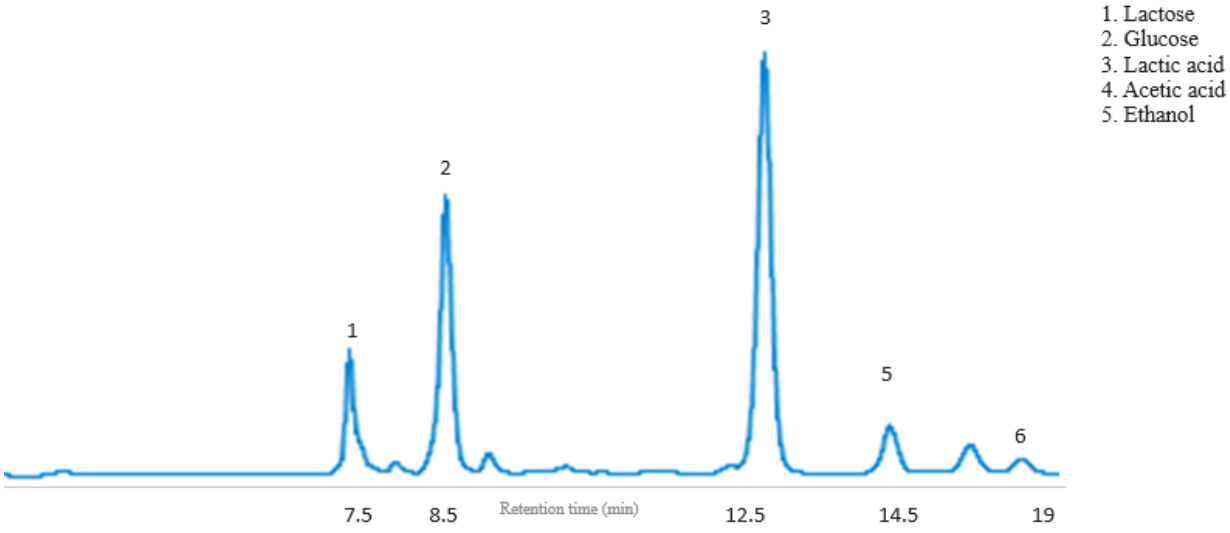



**Figure 4.19:** Chromatogram of Buffalo milk kefir using RI detector

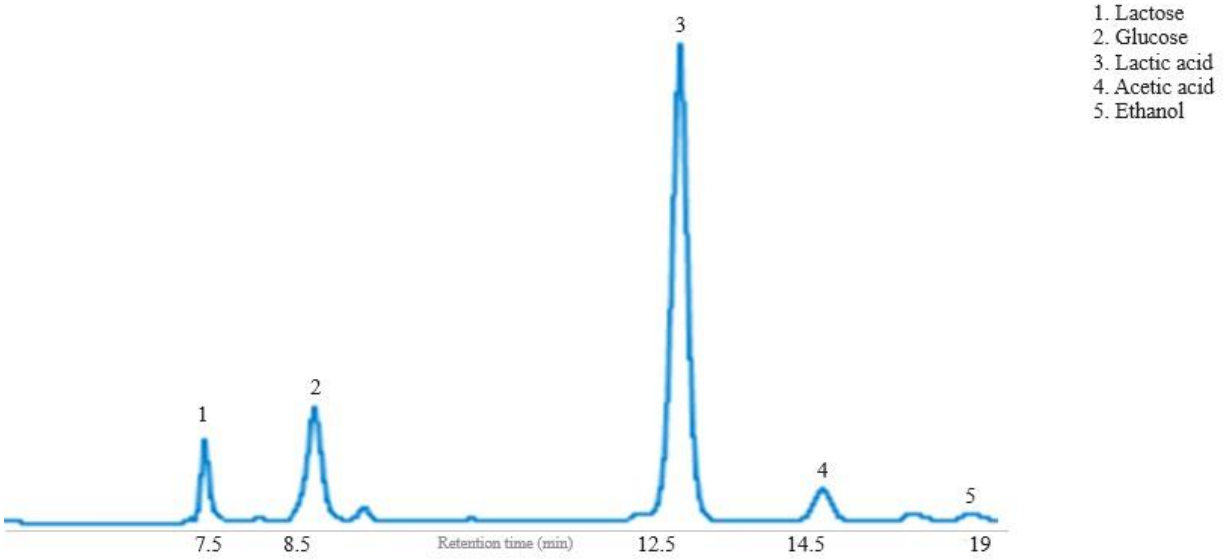



**Figure 4.20:** Chromatogram of  Dairy Cow dairy milk kefir using RI detector

# CHAPTER 5: DISCUSSION

Three lactic acid bacteria: *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* were identified based on their phenotypic and genotypic characteristics. *Lactobacillus helveticus*, a thermophilic bacterium from the *Lactobacillus delbrueckii* group, is known for its high lactic acid production in milk, making it valuable in the dairy industry as a starter culture (Zago et al., 2021). It enhances cheese flavor, reduces bitterness, and shortens ripening time due to its robust proteolytic system. Additionally, *L. helveticus* offers numerous probiotic benefits, including immunomodulation, antimicrobial activity, pathogen antagonism, gastrointestinal infection prevention, gut microbiota improvement, enhanced nutrient bioavailability, cholesterol reduction, and antihypertensive effects (Zago et al., 2021). *Lactobacillus rhamnosus* is noted for its probiotic properties, particularly in pathogen control. It survives gastrointestinal transit, modulates the immune response, and produces biopeptides in milk. Its antimicrobial properties are effective against *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus pneumoniae.* Extensively studied, *L. rhamnosus* adheres to epithelial cells, produces antimicrobial compounds, and influences the gut microbiome. It is also used in functional foods, exhibits antioxidant activity, and holds potential for bioengineering for therapeutic purposes. Further research is recommended to enhance its beneficial attributes and applications in pathogen control (Mathipa-Mdakane & Thantsha, 2022). *Lactobacillus fermentum*, a Gram-positive bacterium, has significant potential in food preservation and biomedical applications. It boosts the immune response and helps prevent gastrointestinal and respiratory infections. *L. fermentum* produces antimicrobial peptides called fermenticins, which can serve as food preservatives or antibiotic alternatives. Its health benefits include lowering blood cholesterol, preventing alcoholic liver disease, and reducing colorectal cancer risk. In the food industry, it is essential for sourdough technology and the creation of fortified and functional foods, contributing to improved flavor, texture, and health-promoting properties (Naghmouchi et al., 2020).

The pH in the stomach ranges from 1 during fasting to 4.5 after a meal, with food ingestion taking up to 3 hours. *Lactobacillus* strains generally survive well at pH 4.6, prompting studies to test their resilience at lower pH levels. Most examined strains withstand pH 3 for 3 hours but lose viability at pH 1 or 2 within an hour. These findings are consistent with earlier research showing Lactobacillus strains maintain viability at pH 2.5 to 4.0 but not at

lower pH levels(Bujnakova et al., 2014; De Angelis et al., 2006; C. Y. Wang et al., 2010). Interestingly, some strains that did not survive pH 2 in vitro still reached the colon alive and effective in vivo, suggesting factors like food's buffering capacity and encapsulated delivery systems enhance their survival through the stomach (Huang & Adams, 2004). Probiotic strains also need to withstand bile salts in the small intestine, typically at concentrations of 0.10 to 0.30% (w/v). This context led to selecting pH 2.5 for testing Lactobacillus survival in our experiments (Bujnakova et al., 2014; Sieladie et al., 2011).

The data also shows that *L. helveticus* has better tolerance to acidic conditions compared to *L. rhamnosus* and *L. fermentum*, as evidenced by its higher growth at pH 3. This is in line with the findings of Hassan et al., (2020) who reported that *L. helveticus* isolates exhibited significant antimicrobial activity against test foodborne microorganisms. At pH 4.6 and 5.6, all three species showed good growth, with *L. rhamnosus* having the highest OD values at 24 hours. This suggests that *L. rhamnosus* may be more suitable for applications in foods with slightly acidic to neutral pH, such as cheeses, juices, and fermented milk, as mentioned by Melo et al., (2017) for *L. fermentum* TCUESC01. The bile salt tolerance test results for *Lactobacillus helveticus, Lactobacillus rhamnosus*, and *Lactobacillus fermentum* demonstrate that all three strains can tolerate bile salts to some extent, with L. helveticus exhibiting the highest tolerance across all conditions. This is consistent with previous studies on the bile salt tolerance of these bacteria.

For *Lactobacillus helveticus*, a study by Hassan et al., (2020) found that it can tolerate bile salts up to 1% concentration, which is in line with the results of the bile salt tolerance test. The study also noted that L. helveticus can survive in the gastrointestinal tract due to its ability to tolerate bile salts and other harsh conditions. Regarding *Lactobacillus rhamnosus*, a study by Zhang et al., (2019) found that it can tolerate bile salts up to 0.5% concentration, which is consistent with the bile salt tolerance test results. For *Lactobacillus fermentum*, a study by Naghmouchi et al., (2020) found that it can tolerate bile salts up to 0.3% concentration, which is consistent with the bile salt tolerance test results. The study also noted that *L. fermentum* can survive in the gastrointestinal tract and has been used as a probiotic to improve gut health.

The high autoaggregation percentage of around 65% exhibited by *Lactobacillus helveticus* aligns with findings by Tareb et al., (2013), who reported a similar rate of 65.4%. This strong aggregation ability is desirable for probiotic bacteria as it enhances their capacity to adhere

to the intestinal mucosa and form biofilms, improving persistence and colonization in the gastrointestinal tract (Kos et al., 2003). *Lactobacillus rhamnosus*, with an autoaggregation percentage close to 60%, is also supported by Gueimonde et al., (2007) who reported levels ranging from 50% to 70%. This characteristic facilitates the formation of bacterial aggregates and biofilms, enhancing survival in the gastrointestinal environment. In contrast, *Lactobacillus fermentum* shows a lower autoaggregation percentage of approximately 45%, consistent with Collado et al., (2008) who reported around 40%. While recognized for its probiotic potential, *L. fermentum's* lower aggregation capacity suggests a weaker ability to adhere to the intestinal mucosa and form biofilms, potentially impacting its persistence and colonization (Kos et al., 2003).

The results of the cell coaggregation test demonstrate that *Lactobacillus helveticus* exhibits the highest coaggregation rate with *E. coli*, at approximately 28%, followed by *Lactobacillus rhamnosus* at around 20%, and *Lactobacillus fermentum* at around 15%. This suggests that *Lactobacillus helveticus* might be a more effective probiotic due to its better coaggregation ability, which can aid in preventing the presence and multiplication of harmful bacteria such as *E. coli* in the intestines (Pessoa et al., 2017).This finding is consistent with the results of a study on the coaggregation patterns and surface characteristics of *Lactobacillus rhamnosus GG(LGG)* under varying nutrient conditions. The study found that *LGG* cultured in BeYG medium exhibited the highest coaggregation scores, indicating that different nutrient conditions can enhance the ability of *LGG* to colonize the human gut microbiome and act as a successful probiotic(Rielinger, 2019). The high coaggregation ability of Lactobacillus helveticus can be attributed to its ability to adhere to epithelial cells and antagonize pathogens, which is a common probiotic property of *Lactobacillus* species (Taverniti & Guglielmetti, 2012). This property can aid in preventing the colonization of the gut by harmful bacteria such as E. coli, thereby promoting a healthy gut microbiota.

The cell surface hydrophobicity test shows that *Lactobacillus helveticus* has the highest hydrophobicity at around 40%, followed by *Lactobacillus rhamnosus* at 30%, and *Lactobacillus fermentum* at 25%, indicating that *L. helveticus* may be the most effective probiotic due to its superior adhesion to the intestinal lining. This finding is consistent with research on *Lactobacillus rhamnosus*, where the strain PEN, lacking exopolysaccharides (EPS) but having specific surface proteins, demonstrated higher adhesion and aggregation compared to the EPS-producing strain E/N. Additionally, studies on *Lactobacillus* strains

with and without surface layer proteins (SLP) show that the presence of SLP, such as in *Lactobacillus acidophilus* ATCC 4356, enhances hydrophobicity in low-ionic-strength solutions, thereby influencing their adhesive properties and supporting the observed hydrophobicity differences among the strains (Vadillo-Rodríguez et al., 2004).

Furthermore, the study on the dynamic cell surface hydrophobicity of *Lactobacillus* strains with and without surface layer proteins (SLP) supports the observed differences in hydrophobicity among the three *Lactobacillus* strains. The study found that *Lactobacillus acidophilus* ATCC 4356, which has an SLP, exhibited a higher contact angle (more hydrophobic) in low-ionic-strength solutions compared to high-ionic-strength solutions (Vadillo-Rodríguez et al., 2004). This suggests that the presence of SLP can contribute to the dynamic hydrophobicity of Lactobacillus cell surfaces, which can influence their adhesive properties.

*Lactobacillus helveticus's* sensitivity to NaCl was also noted by Carvalho et al., (2018), who observed that while *Lactobacillus helveticus* showed high tolerance to bile salts and acidity, its growth was more sensitive to sodium chloride. Similarly, Fontana et al., (2019) highlighted the probiotic potential of *Lactobacillus helveticus*, noting its resilience in diverse conditions, including varying NaCl concentrations. The findings from the NaCl tolerance test align with existing literature. For instance, Rocha-Ramírez et al., (2021) found that Lactobacillus rhamnosus showed significant NaCl tolerance and exhibited probiotic properties including hydrophobicity, auto-aggregation, and pathogen inhibition Similarly, Reale et al., (2015) reported that *Lactobacillus rhamnosus* and other *Lactobacillus* strains demonstrated varied tolerance to NaCl, with some strains enduring high osmolarity conditions, which supports their use in food processing and gastrointestinal environments. Regarding *Lactobacillus fermentum*, Mishra et al., (2018) found that this strain efficiently survived in environments with 0.5% bile salts and exhibited high cell surface hydrophobicity, which correlates with its tolerance to lower NaCl concentrations observed in the test.

The phenol tolerance test results indicate that all three strains of lactic acid bacteria (LAB) showed reduced growth in the presence of phenol, with *Lactobacillus fermentum* displaying the highest tolerance, followed by *Lactobacillus rhamnosus* and *Lactobacillus helveticus*. This aligns with studies that highlight the varying tolerance of different *Lactobacillus* strains to phenolic compounds. For instance, (Rocha-Ramírez et al., 2021)

demonstrated that *L. rhamnosus* exhibited significant tolerance to various stress conditions, including the presence of toxic compounds like phenol. Similarly, another study found that *Lactobacillus fermentum* was the most tolerant to 0.4% phenol, with 7.89 log CFU/mL viable counts. This suggests that *Lactobacillus fermentum* may have evolved to develop higher tolerance to phenolic compounds, which could be beneficial for its survival in environments where such compounds are present (Kumari V. B. et al., 2022).The tolerance of *Lactobacillus fermentum* to phenol is likely due to its ability to adapt to different environments and stress conditions. This adaptation is crucial for the survival and effectiveness of probiotic bacteria in various applications, such as fermented foods and gastrointestinal health (Jena et al., 2013).

The biofilm formation test revealed that *L. rhamnosus* and *L. fermentum* could form biofilms, indicated by black colonies on Congo Red Agar, whereas *L. helveticus* was not. This suggests that *L. helveticus* may be less effective in forming biofilms, which are crucial for probiotic potential and survival in hostile environments. Mishra et al. (2018) also reported that L. fermentum exhibited high cell surface hydrophobicity and biofilm formation capabilities. However, high cell surface hydrophobicity does not necessarily translate to biofilm formation. Biofilm development involves multiple factors, including the ability to produce extracellular polymeric substances (EPS), autoaggregation capabilities, and specific interactions with surfaces. *L. helveticus* strains may lack efficient EPS production or the genetic machinery required for robust biofilm formation. In the study by Dhewa et al., (2009), although *L. helveticus* showed high hydrophobicity, it did not demonstrate significant biofilm formation, possibly due to inadequate EPS production or other biofilm-associated genes. Moreover, biofilm formation is often enhanced under stress conditions such as the presence of bile or specific nutrients, which might not have been optimized in the biofilm formation assays. Ambalam et al., (2012) reported that bile acids could enhance biofilm formation in some *Lactobacillus* strains, suggesting that the environmental conditions play a crucial role in biofilm development.

The BSH test showed that L. helveticus had the highest BSH activity, followed by *L. rhamnosus* and *L. fermentum*. This is consistent with studies such as Fontana et al., (2019) which found *L. helveticus* strains to possess significant BSH activity, beneficial for probiotic functionality and gut health.

All three LAB strains showed sensitivity to Bacitracin, Cefotaxime, and Piperacillin but

resistance to Entrapenem and Cloxacillin. The antibiotic resistance profiles of these strains are crucial for their use as probiotics. Mogha & Prajapati, (2017) also observed similar antibiotic susceptibility patterns in *L. helveticus* and *L. rhamnosus* strains, emphasizing the importance of understanding these profiles for probiotic applications.

The antimicrobial activity test revealed that *L. helveticus* had superior inhibitory effects against *E. coli* and *Staphylococcus*, while *L. rhamnosus* was most effective against *Pseudomonas.* These results are supported by studies showing the potent antimicrobial activity of *L. helveticus* against various pathogens due to its production of organic acids and bacteriocins (Bian et al., 2016).

The growth performance at different temperatures showed that *L. helveticus* and *L. fermentum* had optimal growth at higher temperatures (37℃ and 45℃), whereas *L. rhamnosus* showed consistent but moderate growth across all temperatures. This aligns with the findings of Yao-hui, (2008), who reported that *L. rhamnosus* strains are capable of growing under a wide range of temperature conditions, which is beneficial for their use in various probiotic formulations.

The comparison of fresh milk kefir made from buffalo milk and cow dairy milk shows distinct differences in pH, acidity, and degree Brix. Buffalo milk kefir has a higher pH of 4.7 and lower acidity (1.37%) compared to cow milk kefir, which has a pH of 4.5 and higher acidity (1.96%). This indicates that cow milk kefir is more acidic, potentially due to a higher concentration of lactic acid bacteria which accelerates the fermentation process. According to Guzel-Seydim et al., (2000), higher acidity in cow milk kefir can be attributed to the more active fermentation by lactic acid bacteria. The degree Brix, which measures the sugar content, is higher in buffalo milk kefir (6) compared to cow milk kefir (5). This suggests that buffalo milk kefir retains more sugars post-fermentation, potentially due to different microbial compositions and fermentation efficiencies. Leite et al., (2013) noted that the microbial composition, including yeast and bacteria, significantly influences the sugar content in kefir.

The comparison of organic acids and sugar concentrations in buffalo milk kefir and cow milk kefir reveals distinct differences, reflecting variations in their fermentation processes and microbial activity. Buffalo milk kefir has a significantly higher lactose content (32.92 ± 0.088 mg/ml) than cow milk kefir (18.02 ± 0.061 mg/ml), indicating less lactose fermentation by the microorganisms, consistent with findings by Gul et al., (2015) who

noted higher lactose in buffalo kefir due to less microbial breakdown. Both kefirs have similar glucose levels, suggesting comparable initial fermentation activity. However, the lactic acid content is significantly higher in cow milk kefir (115.76 ± 0.204 mg/ml) compared to buffalo milk kefir (45.30 ± 0.163 mg/ml), which may be due to more robust lactic acid bacteria activity in cow milk kefir, as supported by Guzel-Seydim et al., (2000) who found higher lactic acid levels in cow milk kefir. Acetic acid levels are relatively similar between the two kefirs, suggesting similar acetic acid bacteria activity. The ethanol content is slightly higher in buffalo milk kefir (0.625 ± 0.004 mg/ml) compared to cow milk kefir (0.477 ± 0.002 mg/ml), possibly due to differences in yeast strains used in fermentation, as indicated by Ghasabnezhad et al., (2019) who found varying ethanol levels based on yeast activity.

# CHAPTER 6: CONCLUSION

In conclusion, *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* demonstrate distinct probiotic potential and resilience under various environmental conditions. *L. helveticus* exhibits superior lactic acid production, antimicrobial activity, and bile salt tolerance, making it valuable in dairy applications and for health benefits like immunomodulation and cholesterol reduction (Zago et al., 2021). *L. rhamnosus* is notable for its robust pathogen control, gastrointestinal survival, and potential therapeutic uses, supported by its high aggregation and biofilm formation capabilities (Mathipa-Mdakane & Thantsha, 2022). *L. fermentum* shows significant potential in food preservation and biomedical applications, demonstrating high resilience to bile salts and phenol, alongside producing health-promoting fermenticins (Naghmouchi et al., 2020). While all three strains exhibit beneficial properties, *L. helveticus* and *L. rhamnosus* show greater potential for use in diverse food and health applications due to their higher tolerance to harsh conditions and strong probiotic attributes (M. U. Hassan et al., 2020b; J. Zhang et al., 2019). These findings emphasize the importance of selecting specific *Lactobacillus* strains based on their targeted application to maximize probiotic efficacy and consumer health benefits.

Overall, these findings support the multifaceted benefits of kefir as a probiotic drink, highlighting the unique contributions of each *Lactobacillus* strain to health and industry. Despite these promising results, limitations include variability in in vitro and in vivo strain survival under gastrointestinal conditions and the need for more extensive testing on the efficacy and safety of these strains in human trials. Future research should focus on optimizing the delivery systems to enhance strain survival through the gastrointestinal tract, exploring the genetic basis for the observed probiotic properties, and conducting long-term clinical studies to validate health benefits and safety profiles. Additionally, Kefir nutritional profiling will provide deeper insights into their therapeutic benefits.

# CHAPTER 7: RECOMMENDATION

To further enhance the study, it is recommended to isolate additional probiotic lactic acid bacteria and yeast from kefir using strain-specific culture media. Subsequent in vitro probiotic tests should be performed to confirm the probiotic characteristics of the isolated bacteria and yeast. Additionally, evaluating their antimicrobial activity against common food pathogens would provide valuable insights into their potential health benefits and food safety applications.

Nutritional profiling of kefir is also essential to ascertain its nutrient content comprehensively. This analysis should include macro and micronutrients, vitamins, and minerals to provide a complete nutritional profile. Sensory evaluation tests are crucial to understanding consumer preferences and acceptance, which can guide product development and marketing strategies.

Additionally, exploring the effects of different fermentation conditions on the microbial composition and properties of kefir can optimize its production and quality.

## Limitations:

I. The composition of kefir can change based on milk type and fermentation, making results less consistent.
II. The study may not cover all the microorganisms in kefir, missing some important ones.
III. The study predicts probiotic characterization only, but actual health effects need further testing in clinical trials.

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# APPENDICES

## APPENDIX I: LIST OF CHEMICAL MATERIALS AND EQUIPMENTS

### A. Reagents/Chemical materials

1. Gram stain
2. Lactophenol cotton blue
3. Hydrogen peroxide
4. TE buffer
5. Nuclease free water
6. Master mix
7. DNA ladder
8. Loading dye
9. Tris acetate-EDTA buffer
10. EDTA
11. DNA primers
12. Distilled water
13. Hydrochloric acid
14. Bile salts
15. PBS(phosphate buffer saline)
16. Nacl
17. Phenol
18. Hexane
19. Antibiotic discs
20. Congo red
21. Nessler reagent
22. Arginine
23. NaOH
24. Phenolphthalein indicator
25. Lactic acid
26. Acetic acid
27. Ethanol
28. Lactose
29. Glucose

## B. Equipments

1. pH meter(Hanna instruments, India)
2. UV Spectrometer(Thermo Scientific, UK)
3. Electrophoretic apparatus (Biobase, China)
4. Gel documentation system(Syngene, UK)
5. Thermal cycler(Bioer, China)
6. HPLC(Agilent, USA)
7. Centrifuge
8. Laminar airflow
9. Vortex
10. Refrigerator
11. Sequencer
12. Incubators
13. Sonicator
14. Brix Refractometer
15. Microscope

## APPENDIX II: MEDIA COMPOSITION AND PREPARATIONS

### 1. MRS broth

| Ingredients | Gms/litre |
|---|---|
| Dextrose(Glucose) | 20.000 |
| Proteose peptone | 10.000 |
| Yeast extract4sodium acetate | 5.000 |
| 2-phenyl ethanol | 3.000 |
| Ammonium citrate | 2.000 |
| Dipotassium hydrogen phosphate | 2.000 |
| Magnesium sulphate | 0.100 |
| Manganese sulphate | 0.050 |
| Bromocresol green | 0.040 |
| Cycloheximide | 0.004 |
| Final pH (at 25℃) | 4.3±0.2 |

### 2. MRS agar

| Ingredients | Gms/litre |
|---|---|
| Peptone | 10.00 |
| HB peptone B# | 8.00 |
| Yeast extract | 5.00 |
| Ammonium citrate | 2.00 |
| sodium acetate | 5.00 |
| Magnesium sulphate heptahydrate | 0.20 |
| Manganese sulphate tetrahydrate | 0.05 |
| Dipotassium hydrogen phosphate | 2.00 |
| Dextrose | 20.00 |
| Polysorbate 80 (Tween 80) | 1.00 |
| Agar | 12.00 |
| Final pH (at 25℃) | 5.7±0.2 |

### 3. YEGA agar

| Ingredients | Gms/litre |
|---|---|
| Yeast extract | 5.00 |
| Glucose | 20.00 |
| Agar agar | 15.00 |

### 4. MHA agar

| Ingredients | Gms/litre |
|---|---|
| Beef Extract | 2.00 |
| Acid Hydrolysate of Casein | 17.50 |
| Starch | 1.50 |
| Agar | 17.00 |

### 5. Blood agar

| Ingredients | Gms/litre |
|---|---|
| Peptic Digest of Animal Tissue | 5.0 |
| Heart Extract | 3.0 |
| Yeast Extract | 2.0 |
| Sodium Chloride | 5.0 |
| Agar | 15.0 |

### 6. Congo red agar

| Ingredients | Gms/litre |
|---|---|
| Congo red powder | 0.8 |
| MRS agar | 65.13 |

### 7. Nutrient agar

| Ingredients | Gms/litre |
|---|---|
| Peptone | 5.000 |
| Sodium chloride | 5.000 |
| Peptone | 1.500 |
| Yeast extract | 1.500 |
| Agar | 15.000 |
| Final pH (at 25°C) | 7.4±0.2 |

8. Nutrient broth

| Ingredients | Gms/litre |
|---|---|
| Peptone | 10.000 |
| Beef extract | 10.000 |
| Sodium chloride | 5.000 |
| pH after sterilization | 7.3±0.1 |

## APPENDIX III: STANDARD CURVES FOR HPLC

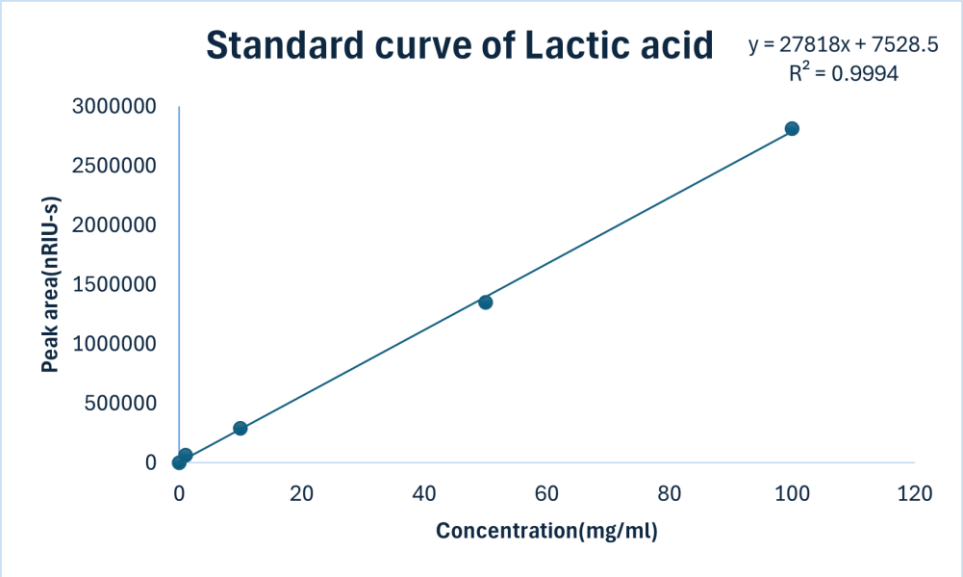



Fig 1: Standard curve for lactic acid production assay

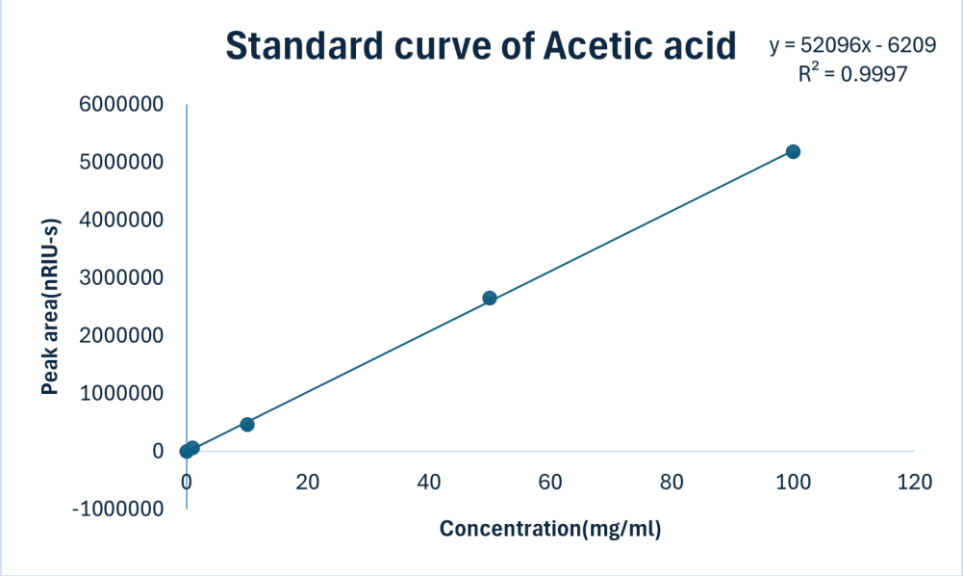



Fig 2: Standard curve for acetic acid production assay

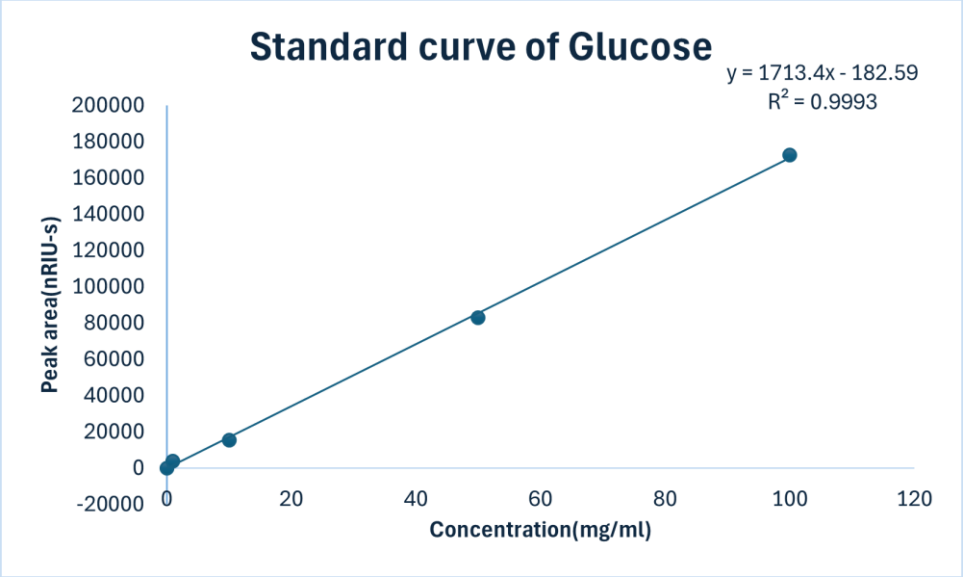



Fig 3: Standard curve of glucose production assay

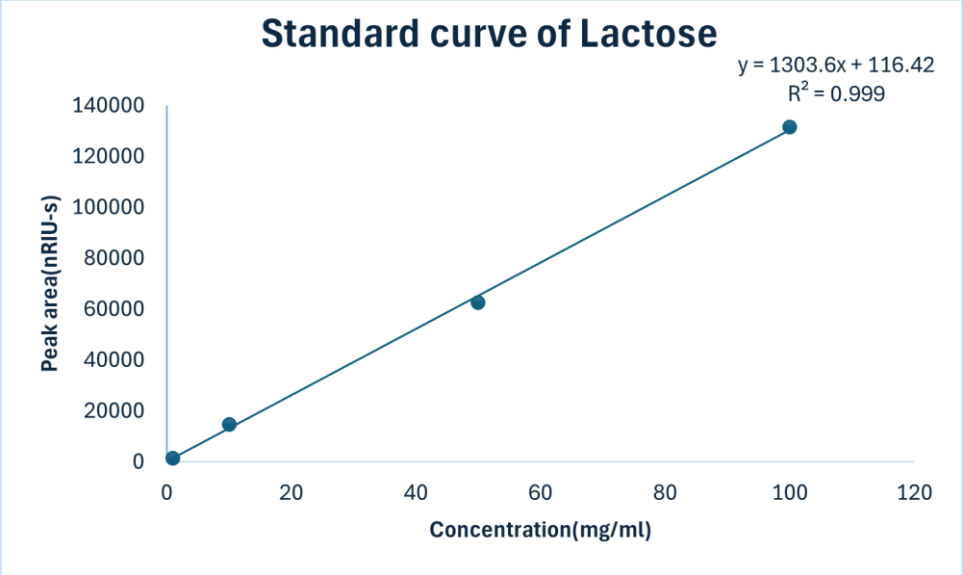



Fig 4: Standard curve of lactose production assay

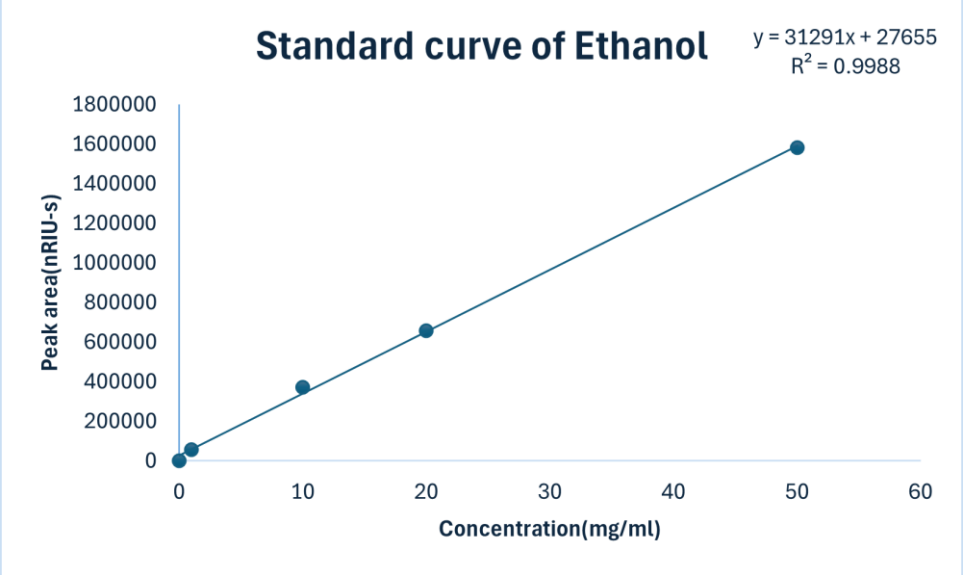
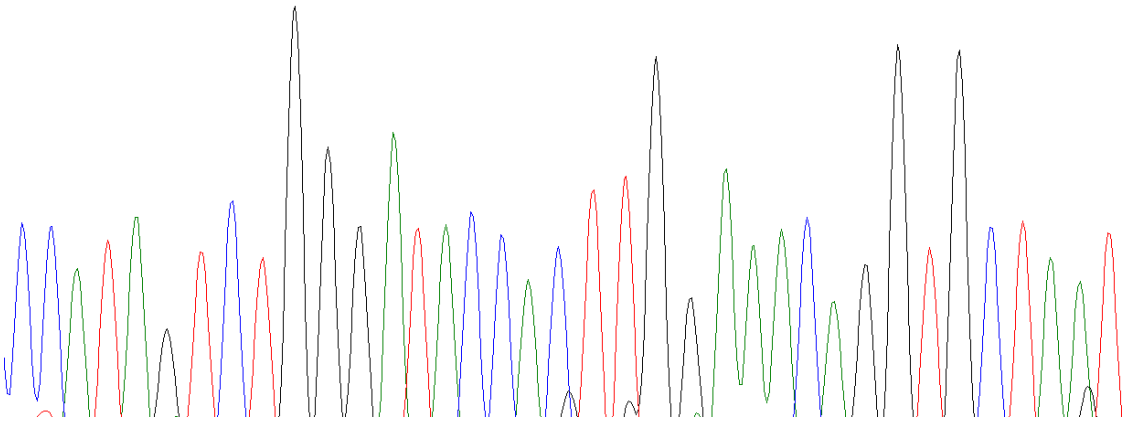
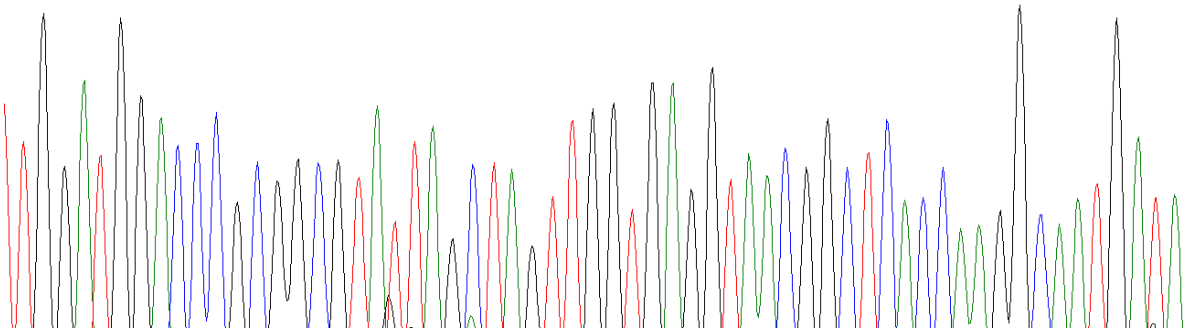



Fig 5: Standard curve of ethanol production assay

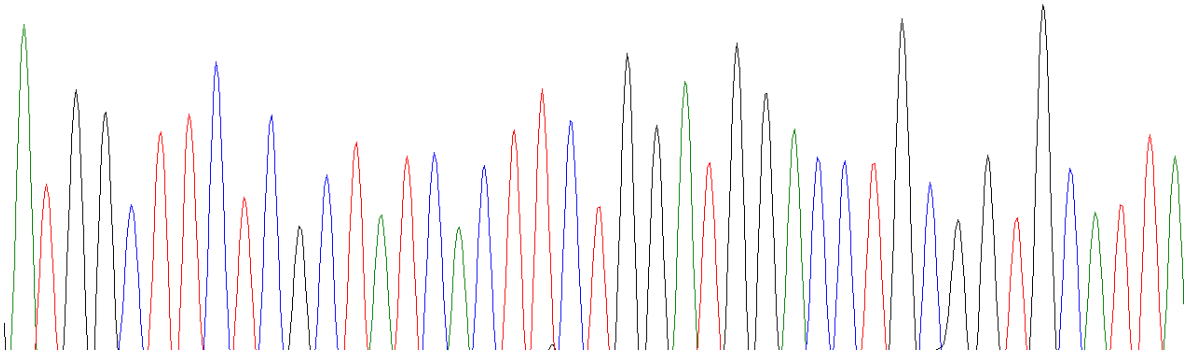
## APPENDIX IV: CHROMATOGRAMS & SEQUENCES OF ISOLATES



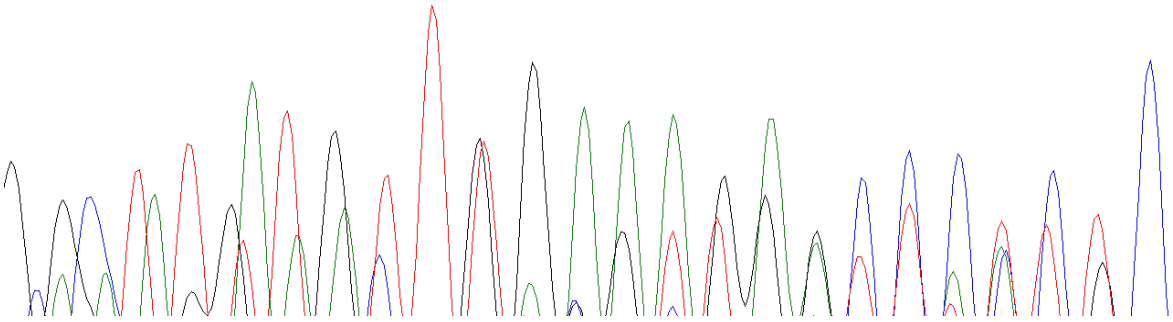
Chromatogram of *Lactobacillus helveticus*



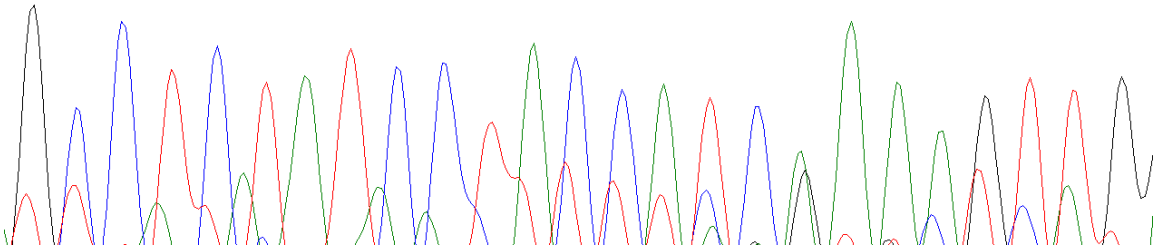
Chromatogram of *Lacticasiebacillus rhamnosus*



Chromatogram of *Limosilactobacillus fermentum*



Chromatogram of *Kazachstania martinaie*



Chromatogram of *Pichia chibdodasensis*

>Seq1: *Lactobacillus helveticus*

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>Seq2*: Lacticasiebacillus rhamnosus*

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>Seq3: *Limosilactobacillus fermentum*

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CACCCCGCGGTAA

>Seq 4: *Kazachstania martinaie*

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>Seq5: *Pichia chibdodasensis*

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