STUDY ON ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF LACTIC ACID BACTERIA DERIVED FROM FERMENTED DAIRY PRODUCTS

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CERTIFICATE

*This is to certify that the thesis entitled "**STUDY ON ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF LACTIC ACID BACTERIA DERIVED FROM FERMENTED DAIRY PRODUCTS**" submitted to the Department of Dairy Science, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **DAIRY SCIENCE**, embodies the result of a piece of bona fide research work carried out by **ABDUR RAHMAN**, Registration No. **15-06838** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma at any other institution.*

I certify that any help or source of information received during this investigation has been duly acknowledged.

Dated: December, 2022
Place: Dhaka, Bangladesh

Prof. Dr. Md. Asaduzzaman
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Dedicated to
my beloved parents

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
ATP	Adenosine Tri Phosphate
BLASTN	Basic Local Alignment Search Tool Nucleotide
bp	Base pair
DNA	De-oxy Ribo Nucleic Acid
ESP	Exopolysaccharide
et al.	*et alibi* (and others)
etc.	*et cetra* (and so on)
GIT	Gastrointestinal Tract
GRAS	Generally Regarded as Safe
IBD	Inflammatory Bowel Disease
LAB	Lactic Acid Bacteria
lb	Pound
mg	Milligram
ml	Milliliter
MRS	de Man, Rogosa, and Sharpe
NCBI	National Center for Biotechnology Information
NSLAB	Non-Starter Lactic Acid Bacteria
PCR	Polymerase Chain Reaction
RNA	Ribo Nucleic Acid

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ABSTRACT

Lactic acid bacteria (LAB) are important in dairy food fermentation which have beneficial health properties. The research was conducted to isolate, characterize and identify the LAB derived from fermented dairy products like *dahi*, *borhani*, *matha*, *lassi*, cheese and to assess their antibiotic susceptibility patterns. The bacteria were isolated based on their colony morphology on de Man, Rogosa, and Sharpe (MRS) agar and selected based on gram-staining properties, biochemical properties and molecular tests. Salt tolerance, sugar fermentation and growth at different temperatures and salt concentrations were observed for all isolates. PCR followed by 16S rDNA sequencing was done to identify the bacteria. All isolates survived at low (≤5%) NaCl concentration, but could not tolerate high (≥6%) NaCl concentration. All isolates can ferment glucose, sucrose, and lactose. All the tested isolates were sensitive to antibiotics like gentamicin, ciprofloxacin, tetracycline and ceftriaxone. Finally, the identified bacterial species were *Lactobacillus rhamnosus* from *dahi*, *lassi* and *matha* samples and *Lactobacillus fermentum* was identified from the cheese sample which are known as probiotic species. Ultimately, the research revealed two antibiotic-sensitive LAB species suitable for incorporation into fermented dairy product formulations.

Keywords: Lactic acid bacteria, fermented dairy products, isolation of bacteria, antimicrobial resistant

CHAPTER I
INTRODUCTION

Lactic acid bacteria (LAB) are important microorganisms for food fermentation. LAB are the dominant bacteria of all fermented dairy products (Mathara *et al*., 2004; Yu *et al*., 2015). As most LAB have health-beneficial properties, products made from fermented dairy are an excellent source of probiotics, as well as prebiotics and bioactive substances (Kumar *et al*., 2022; Park, 2009). The consumption of fermented dairy products has been shown to have many positive effects on health (Garcia-Burgos *et al*., 2020). Fermented dairy products depend on microbial metabolism for their tastes, textures, and appearances (Bintsis, 2018). LAB has been consumed through fermented dairy products (Yu *et al*., 2015). Since lactic acid is their main metabolic end product, LAB are most important for the preparation of fermented dairy products (Wang *et al*., 2022; Bintsis, 2018). LAB are the subject of extensive research due to their potential to produce antimicrobial substances that promote probiotic properties such as antitumor activity, lactose intolerance relief, plasma cholesterol reduction, gut microflora stabilization and immune system stimulation (Ayivi *et al*., 2020; Taye *et al*., 2021). Consumers readily embrace LAB as a natural method of preserving food and promoting health (Quinto *et al*., 2014). LAB are grouped into several genera under the Lactobacillaceae family. They are always an integral part of fermented dairy products and are widely utilized in the fermented food industry (Wang *et al*., 2022). Although LAB includes more than 60 genera, the most frequent genera in food fermentation generally include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, (Mokoena, 2017; Li *et al*., 2020). *Lactobacilli* such as *Lactobacillus acidophilus, L. casei, L. paracasei, L. rhamnosus, L. delbrueckii* subsp. *bulgaricus, L. brevis, L. johnsonii, L. plantarum* and *L. fermentum* are commonly used as probiotic products (Nawaz *et al*., 2011, Panesar, 2011). So, the isolation of LAB can be useful for the improvement of the quality of dairy products.

LAB are bacteria with a rod or coccoid-shaped morphology. They are characterized as gram-positive, lacking the ability to form spores, non-motile, not being acid-fast and catalase-negative (Pot *et al*., 1994). These organisms exhibit optimal growth under anaerobic conditions, but they are capable of growing microaerophilic and

aerobic conditions as well. These organisms exhibit optimal growth in a mildly acidic setting with a pH range of 5.5 to 6.0, whereas their growth is typically impeded in a neutral or slightly alkaline environment with a pH range of 7.0 to 7.5. The exclusive metabolic activity of these microorganisms involves the fermentation of carbohydrates, resulting in the production of lactic acid as the principal end product (Axelsson, 2004). There exist various categories of LAB, distinguished by their morphological characteristics, their glucose fermentation capabilities, their adaptability to varied temperatures and salt concentrations and their capacity for lactic acid production (Axelsson, 2004). LAB are responsible for the flavor and texture formation of the products. So, it is important to know about the LAB strains that are used for the preparation of fermented dairy products to make the products desirable and safe for human health. LAB exopolysaccharides are employed as emulsifiers, stabilizers, thickeners, gelling agents, moisture retainers, rheology and hardness modifiers, syneresis modifiers and to enhance texture, mouthfeel and sensory qualities in the food industry (Korcz and Varga, 2021). Many studies have shown that LAB may produce antioxidant metabolites (Wang *et al*., 2017). Antioxidant compounds produced by LAB in fermented foods are quite safe and may have several positive impacts on human health (Ayivi *et al*., 2020). The other health advantages of LAB include anti-allergic, anti-carcinogenic, prevention of gastrointestinal infection, constipation relief, immune system activation and cholesterol-reducing properties (Panesar, 2011).

Antibiotics are drugs generally used to treat microbial diseases and 50% of the antibiotics will be resistant within a few years (Alanis, 2005). At present, antibiotic resistance is life-threatening and one of the major issues of food safety and health. The overuse of antibiotics has been found to contribute to the emergence of antibiotic resistance in bacterial populations (Singer *et al*., 2003). This antibiotic resistance can cause significant danger and suffering for many people with common bacterial infections, those once easily treated with antibiotics. LAB can be resistance to antibiotics and the primary hazard posed by these bacteria is their ability to spread resistance genes to other harmful bacteria (Mathur and Singh, 2005). The emergence of antibiotic resistance in microorganisms is a significant area of concern among researchers. Therefore, antibiotic sensitivity pattern of LAB is necessary to better understanding of their safe use in the fermented dairy products.

Fermented dairy products are very popular in Bangladesh (Hossain and Kabir, 2016), but the current status of LAB is still unknown. There is a lack of information about the starter cultures used in fermented dairy products. But dairy industry and value-added dairy products are becoming popular in Bangladesh. Identification of the potential LAB is necessary to nourish the dairy industry as well as human health. Due to the importance of LAB in many food applications, the food industry is constantly looking for strains with superior traits and qualities to improve product and sensory quality. Recently, there has been a rise of interest in identifying LAB, as scientists are trying to determine their impact as starter cultures and their probiotic activity. The information about the variety of LAB in different fermented dairy products in Bangladesh from a microbiological standpoint is still limited. Therefore, the main objectives of the study are

i. To isolate, characterize and identify potential LAB from fermented dairy products
ii. To evaluate the antibiotic susceptibility of LAB isolated from fermented dairy products

CHAPTER II
REVIEW OF LITERATURE

Fermented dairy products are significant components of the human diet due to their desirable attributes, such as a mild acidic flavor and a pleasantly firm consistency (Widyastuti *et al*., 2014). Various fermented milk products are gaining popularity globally. Milk fermentation, which uses LAB, may happen naturally or intentionally. These microbes may grow in new environments and ferment milk into a variety of healthful fermented milk products, including yogurt, cheese, *kefir*, fermented milk, *lassi*, *borhani*, kumys, and others (Garcia-Burgos *et al*., 2020). The utilization of microorganisms in the fermentation process of various foods and beverages encompasses a range of bacterial species such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Bifidobacterium* (Xiang *et al*., 2019; Liu *et al*., 2014). Multiple research studies have demonstrated that the consumption of fermented milk products has a positive impact on human health. LAB have been widely studied for their diverse range of advantageous effects, including the production of bacteriocins, antiviral properties, anti-diabetic properties, anti-allergic properties, anti-obesity properties, anti-diarrheal properties and the prevention of lactose intolerance. There exist multiple methods by which LAB can be employed as a probiotic to promote and maintain human health (Ayivi *et al*., 2020; Hati *et al*., 2013; Zhang *et al*., 2019).

The primary objective of this study is to isolate and identify potential LAB present in fermented dairy products and subsequently study of their antibiotic susceptibility. To ensure the study was conducted effectively, diligent efforts were made to comprehend the prior research conducted on these aspects. This was accomplished by thoroughly reviewing the studies conducted by previous researchers. The ensuing section provides a comprehensive overview of these studies, organized under appropriate headings. This chapter provides an overview of the background of LAB, various types of fermented dairy products, and the utilization of LAB in their production. It also discusses the laboratory techniques presently employed for the isolation, identification and characterization of LAB. Furthermore, the chapter outlines the diverse health advantages associated with these fermented dairy products and probiotics.

2.1 Lactic acid bacteria (LAB)

2.1.1 Introduction to lactic acid bacteria

A genus of bacteria that ferment and coagulate milk is called "lactic acid bacteria". In 1919, Orla-Jensen designated the taxonomic classification "Lactobacteriaceae" to a group of bacteria that produce lactic acid, acetic acid, alcohol, and carbon dioxide. According to Breed *et al*. (1957), LAB are gram-positive bacteria with unique morphological, metabolic, and physiological traits. They may ferment carbohydrates and produce lactic acid without spores. In addition, these organisms can tolerate acidic conditions in anaerobic habitats and inhibit catalase. Organisms under investigation are immobile and cannot reduce nitrite. The classification of LAB includes four genera, namely *Lactobacillus*, *Streptococcus*, *Leuconstoc*, and *Pediococcus*. LAB has been widely utilized in the fermentation of diverse food items, such as cheese, yogurt, sourdough, buttermilk, brined vegetables and sauerkraut (Ammor *et al*., 2006) and various strains of LAB are frequently used as starter cultures in the manufacturing of dairy products (Ayad *et al*., 2004; Klaenhammer *et al*., 2002). The generation of organic acid, hydrogen peroxide and various enzymes has been observed in bacteria during the process of fermentation (Grobben *et al*., 1998). The occurrence of LAB in fermented food items leads to the synthesis of antimicrobial compounds, which efficiently impede the proliferation of both spoilage and pathogenic bacteria (Amezquita and Brashears, 2002). Therefore, LAB is a part of extensive research in dairy industry.

2.1.2. Role of LAB as a starter culture in fermented dairy products

"Starter culture" refers to a microbial preparation that consists of substantial populations of cells from at least one microorganism. This preparation is added to raw materials to initiate the fermentation process and improve its efficiency. The utilization of LAB in the preparation of fermented milk products and beverages was well-established and an essential component of these manufacturing processes (Hati *et al*., 2013). LAB play a crucial role in numerous processes, such as milk fermentation and food preservation. According to Ayivi *et al*. (2020), a significant proportion of products produced using LAB exhibit enhanced health advantages for consumers, thereby playing a crucial role in promoting a well-functioning digestive system.

LAB enhances fermented product flavor and aroma. They also induce acidity in food. Additionally, they exhibit proteolytic and lipolytic activities and produce aromatic compounds. According to Hati *et al.* (2013), it has been found that the LAB is accountable for the manufacturing of fermented food products and the enhancement of their taste profiles via the mechanisms of proteolysis, glycolysis, and lipolysis. Although the primary metabolic process involves the conversion of carbohydrates into lactic acid through fermentation (Bintsis, 2018).

LAB are gram-positive, non-spore-forming, catalase-negative, cytochrome-deficient, anaerobic, but aero-tolerant microorganisms. These bacteria can survive in acidic environments and engage in strict fermentation processes, with lactic acid being the primary byproduct of sugar fermentation (Konig and Frohlich, 2017). LAB is classified into two categories, homofermentative and heterofermentative, based on their distinct carbohydrate fermentation capabilities. Homofermentative LABs, including *Lactococcus* and *Streptococcus*, convert a single molecule of glucose into two molecules of lactate. On the other hand, heterofermentative LABs, such as *Leuconostoc*, *Wiessella* and certain *lactobacilli*, convert glucose into lactate, ethanol, and carbon dioxide (Mokoena, 2017). These organisms have specific habitat requirements, necessitating their presence in locations abundant in particular compounds, such as those found in animals, plants, and other multicellular organisms. According to Konig and Frohlich (2017), the majority of LAB strains exhibit the ability to flourish within a pH range 4.4 to 5.5. Furthermore, these bacteria are capable of withstanding temperatures ranging from 5 to 45 degrees Celsius. LAB exert a significant influence on the process of fermented food production, thereby contributing to its widespread popularity. Table 1 presents the species of LAB that have traditionally been employed in the production of fermented dairy products.

Table 1: Starter cultures used in different fermented dairy products

Fermented Dairy Products	Starter Cultures	References
Cultured buttermilk	*Streptococcus lactis* subsp. *diacetylactis*, *S. cremoris*	Ayivi *et al*., 2020
Dahi	*Lactobacillus fermentum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis* subsp. *lactis biovar diacetylactis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroids* subsp. *mesenteroids*	Bhattarai *et al*., 2021; Bangaragiri *et al*., 2021; Rai, 2020
Hard Cheese	*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Streptococcus thermophiles*, *L. delbrueckii subsp. bulgaricus*, *Lactobacillus helveticus*	Hayaloglu, 2016; Gobbetti *et al*., 2018
Hard cheese without eyes	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* ssp. *cremoris*	Hayaloglu, 2016
Kefir	*Lactobacillus acidophilus*, *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*	Chen *et al*., 2008; Rosa *et al*., 2017
Kumiss	*L. acidophilus*, *L. bulgaricus*, *L. casei*	Ghosh *et al*., 2019; Panesar, 2011
Lassi	*Lactococcus lactis* ssp. *cremoris*, *Lactobacillus casei*	Adiver and Hiremath, 2021
Leben	*S. lactis*, *S. thermophilus*, *L. bulgaricus*	Panesar, 2011
Shrikhand	*Lactobacillus bulgaricus*, *Streptococcus thermophiles*	Sahu, 2021
Surface mould cheese	*Lactococcus lactis* subsp. *lactis and L. lactis* subsp. *cremoris*	Panesar, 2011; Hayaloglu, 2016
Swiss cheese	*Lactobacillus delbrueckii* ssp. *lactis*, *Lb. helveticus*, *Streptococcus thermophilus*	Hayaloglu, 2016
Tofu	*Lactobacillus plantarum*	Li *et al*., 2020
Yogurt	*Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *diacetylactis*, *Leuconostoc* spp., *Lactobacillus* spp., *Streptococcus thermophiles*, *Lactobacillus delbrueckii* subsp. *bulgaricus*	Shah, 2017; Chen *et al*., 2017; Laino *et al*., 2013

2.1.3 *Lactobacillus* species

Lactobacillus is a diverse group of gram-positive bacteria that are non-spore-forming and have a negative catalase reaction. These bacteria are capable of producing lactic acid as the primary end product during the fermentation of carbohydrates (Pelinescu *et al*., 2009). LAB are rod-shaped, typically elongated and slender in shape. Some of them exhibit microaerophilic characteristics, while others adhere strictly to anaerobic conditions (Claesson *et al*., 2007). It belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales and family Lactobacillaceae, consisting of 154 species (Claesson *et al*., 2007). According to Sozzi and Smiley (1980), the majority of *lactobacillus* species that can metabolize glucose and convert it into lactate are referred to as *Lactobacillus*. This characteristic makes *Lactobacillus* a valuable organism for the industrial production of fermented food products.

Due to their long history of consumption, presence in fermented foods and safety profile, *lactobacilli* are conferred with the 'generally regarded as safe' (GRAS) status. *Lactobacilli* are known probiotics and are reported to exert several health benefits on the host. Owing to these beneficial effects and their widespread application as starters in the food industry, several *lactobacillus* species have been commercially available in the market in the form of various products or pharmaceutical preparations. Species of *L. acidophilus, L. casei, L. rhamnosus, L. plantarum, L. fermentum, L. salivarius, B. longum, B. lactis* and *B. breve* are some of the commonly available and well-characterized probiotics found in the market (Archer, 2017).

2.2 Fermented dairy products

There are many fermented dairy products such as yogurt, cheese, *kefir*, fermented milk etc. The utilization of LAB is integral to the fermentation process of milk, where in unprocessed milk transforms into fermented milk products. The proteolytic activities of LAB lead to modifications in milk protein, which subsequently induce acidification and contribute to the development of taste and texture. Yogurt and cheese are two instances of dairy products that undergo fermentation. These products are characterized by a pleasing, mildly acidic flavor and a pleasantly firm consistency (Widyastuti *et al*., 2014). Fermented dairy products can be derived from the milk of various domestic milch animals, such as cows, buffaloes, ewes, and goats. Numerous

distinctive fermented milk products are experiencing a significant surge in popularity across various regions globally.

A number of these microorganisms possess the ability to inhabit novel habitats and convert milk into a diverse range of nutritious fermented dairy products, including yogurt, cheese, *kefir*, fermented milk, *lassi*, *borhani*, kumys, and others. According to the study conducted by Garcia-Burgos *et al*. in 2020, the growing consumer demand for therapeutic goods has led to the widespread incorporation of probiotic microorganisms in dairy products. The investigation of novel fermented milk products is of great interest due to their widespread acceptance as safe and their possession of various health-promoting properties. This paper aims to explore various widely consumed fermented dairy products and the specific LAB strains employed in their production.

2.2.1 Fermented milk

Fermented milk is produced by reducing the pH of milk with suitable microorganisms, with or without coagulation. Fermented milk products or cultured dairy products that have been fermented by a group of LAB cause milk curdling or sourness and also help milk retain its flavor and nutritional value (Mokoena, 2017). Starter cultures for fermented milk serve many purposes, including improving the sensory, textural properties, and nutritional qualities of the final product by increasing the production of bio-preservative compounds that preserve the milk and ensure it is safe to consume. The production of fermented milk relies heavily on mesophilic cultures (Surono and Hosono, 2011). Different types of fermented milk are gaining popularity due to their numerous beneficial effects on human health.

2.2.2 Cheese

Cheese is made by a process of microbial fermentation in which certain LAB convert lactose into lactic acid. Various strains of LAB are widely used in the production of a wide variety of cheeses. LAB starter cultures may either be mesophilic from the genera *Lactococcus* and *Leuconostoc* or thermophilic from the genera *Streptococcus* and *Lactobacillus*. *Streptococcus thermophilus*, *Lactococcus lactis*, and *Lactobacillus helveticus* are the three species that have undergone the most thorough research (Widyastuti *et al*., 2014). Cheese made from fermented milk may be eaten months or even years after the milk itself has gone bad. Cheeses that have been

matured for 12 months or more have a more robust taste, aroma, and texture than their fresh counterparts, which may be served immediately after production (Hayaloglu, 2016). Mesophilic *lactobacilli* such as *Pediococcus* spp., *Enterococcus* spp., and *Leuconostoc* spp. are the most frequent NSLAB in white-brined cheeses (Hayaloglu, 2016). Mesophilic starters consisting of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* are employed as starters in the production of cheddar cheese, while the other strains of *lactobacilli* may be added just to aid in taste formation and hasten the cheese's ripening process (e.g., by using *L. helveticus*, *L. casei*). It has been suggested that *S. thermophilus* may be utilized for rapid acidification (Hayaloglu, 2016). Therefore, there is a use of different starter LAB and NSLAB in the production of various soft, semi-hard and hard types of cheese.

2.2.3 *Dahi*/ Yogurt

Dahi is the oldest and most widely consumed fermented milk product. *Dahi* can be eaten plain or with the addition of salt or sugar. Usually, *dahi* is prepared using a simple art or technology following the traditions. It is preferable to apply well-known starter mixtures for producing *dahi* on a big scale with known, uniform quality. *Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *diacetylactis*, *Leuconostoc* spp., *Lactobacillus* spp., and *Streptococcus thermophilus* are the most used starter microorganisms in the preparation of *dahi* (Shah, 2017). *Dahi* samples were found to have predominantly *Lactobacillus* (62%) and *Streptococcus* (38%) bacteria (Nahidul-Islam *et al*., 2018). The taste of yogurt depends on several variables, including the starter cultures used, the temperature and time of processing, the quality of the milk used, and the chemicals and additives that are employed. The fermentation's first starter cultures have a significant impact on the final product's flavor.

Yogurt often contains commercial probiotic microorganisms from the families *Lactobacillus* and *Bifidobacterium* (Nyanzi *et al*., 2021). Yogurt, Bulgarian buttermilk, and several other products that utilize gut bacteria, including *lactobacilli* and *bifidobacteria*, commonly employ thermophilic initial cultures. The production of yogurt with diverse flavors is dependent on the utilization of thermophilic starter cultures. The inclusion of some ingredients in yogurt is attributed to their alleged health benefits, although it should be noted that they also have an impact on the flavor

profile. Therefore, yogurt is very popular in many parts of the world as a dessert after heavy meals due to its efficiency in digesting foods and other beneficial health properties.

2.2.4 *Borhani*

Borhani is a yogurt-based drink that is often offered during lavish gatherings like weddings and celebrations. Pepper, mint leaves, cumin seeds, green chilies, sugar, salt, and water are all added before being blended till smooth. After that, it's chilled by being stored in a fridge (Hossain and Kabir, 2016). Sour yogurt is an essential ingredient in making *borhani*. It's a great nutritional choice since it has a wide variety of nutrients including protein, carbohydrates, fats, vitamins, and minerals (Bari *et al*., 2020). *Lactobacillus* (43%), *Streptococcus* (20%), and *Pseudomonas* (3%), in addition to the most frequent LABs, were discovered as predominant bacteria in *borhani* (Nahidul-Islam *et al*., 2018). As *borhani* is made from *dahi* blended with mint leaves, it affects digestion.

2.2.5 *Kefir*

Kefir is a dairy beverage that undergoes a process of symbiotic fermentation involving LAB and yeasts within a complex structure known as a *kefir* grain (Garrote *et al*., 2001). *Kefir* distinguishes itself from other fermented products due to the unique attributes of its starting culture, which contribute to its distinct flavor and taste profile. LAB are the predominant population within *kefir* grains, coexisting with acetic acid bacteria and yeasts, as reported by Dong *et al*. (2018). LAB are known to synthesize lactic acid, acetic acid, and antimicrobial compounds, which contribute to the preservation of *kefir* (John and Deeseenthum, 2015). Additionally, these bacteria play a role in enhancing the organoleptic properties of *kefir* through the production of volatile compounds, such as acetaldehyde, exopolysaccharides (Rimada and Abraham, 2003), and free amino acids (Guzel-Seydim *et al*., 2011). The predominant bacterial genera detected in *kefir* samples utilizing culture-dependent methods include *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc* (Witthuhn *et al*., 2004). According to Rosa *et al*. (2017), the majority of the *Lactobacillus* species observed in the study were identified as *Lactobacillus kefir*.

2.3 Probiotic properties of LAB

Probiotics refer to living microorganisms that, when administered in sufficient quantities, provide a beneficial effect on the host's health. Probiotics offer a range of health benefits that extend beyond the scope of promoting digestive health. Probiotics function as a supplementary measure to the existing microbial population within a host organism, offering a safeguard against various enteric pathogens. Probiotics possess diverse physiological functions that play a crucial role in maintaining the health of the host environment by regulating microflora. Additionally, they are beneficial in addressing issues related to overweight and obesity (Kobyliak *et al*., 2016). Probiotics are commonly ingested in the form of preparations that contain active live cultures, which consist of bacteria such as *lactobacilli*, *lactococci*, or *bifidobacteria* (Bongaerts *et al*., 2016). According to Dixit *et al*. (2016), numerous microorganisms have been extensively examined as potential probiotics due to their observed in-vitro and biological or clinical impacts. The vast majority of probiotic organisms are derived from human sources. The genera *Lactobacillus* and *Bifidobacterium* are widely recognized as the most prevalent probiotic organisms, encompassing numerous species and strains that have been identified as promising candidates for probiotic use (Kleerebezem and Vaughan, 2009).

The study aims to isolate LAB from fermented dairy products that are locally accessible. The results of this study are anticipated to promote increased consumption of fermented dairy products. In recent times, there has been a surge in scholarly attention towards the identification of LAB. When LAB are detected in fermented dairy products, they can be analyzed to assess their potential in enhancing the overall quality of the end product. It is advisable to utilize widely recognized starting combinations while engaging in large-scale production of goods. Traditional small-scale enterprises often rely on nonspecific strains of LAB that are passed down from one generation to another. However, this practice can pose a significant threat to consumer health as these strains may be contaminated. Molecular methods in food microbiology provide a complete understanding of ecological dynamics and microbial diversity.

2.4 Strength, weakness, opportunity and threat (SWOT) analysis of the research

1. **Strength**
   i. Fermented dairy products are very popular among people
   ii. Availability of raw materials and laboratory facilities
   iii. Lactic acid bacteria are normally present in fermented dairy products
   iv. Probiotic organism
   v. Previous work in other countries
2. **Weakness**
   i. High chance of contamination at the laboratory
   ii. Lack of laboratory equipment and skilled laboratory technician
   iii. Lack of sufficient funding
   iv. Lack of molecular detection facilities of unknown bacteria in fermented products at the departmental laboratory
   v. Molecular detection facilities were costly
   vi. Lack of infrastructural development
3. **Opportunities**
   i. Isolate the LAB from the fermented dairy products
   ii. Identify the specific LAB isolates
   iii. Know the physiological and biochemical properties
   iv. Know about the antibiogram of LAB isolates
   v. Impact on public health
   vi. Molecular detection of specific LAB
   vii. Using specific LAB for a specific function
   viii. Using known starter culture in the production of fermented dairy products
4. **Threats**
   i. LAB can be resistant to antibiotics and a threat to human health
   ii. Isolated LAB can be contaminated during research work and transport

CHAPTER III
MATERIALS AND METHODS

3.1 Research conducting place and study period

The present study was carried out at the MS Laboratory of Dairy Science Department at Sher-e-Bangla Agricultural University (SAU) and the Laboratory of Animal Biotechnology Division (ABD) at the National Institute of Biotechnology (NIB). The study was conducted from March 2022 to August 2023.

3.2 Sample collection

A total of 40 samples of locally available fermented dairy products (*Dahi*-20, *Borhani*-5, *Lassi*-5, *Matha*-5 and Cheese-5= Total of 40) were collected from local shop, restaurants and supershops of Dhaka city. Samples were collected in the sterile beaker from different retail shops and restaurants in Dhaka city and brought to the laboratory as soon as possible. After collection, samples were stored immediately in the aseptic condition in refrigerator at 4°C temperature to protect them from contamination and deterioration.

3.3 Materials

The following materials are used during the study.

3.3.1 Different bacteriological culture media

In the present study, various types of solid media were employed to conduct the bacteriological analysis, which included nutrient agar, de Man, Rogosa and Sharpe (MRS) agar and Muller Hinton agar. A diverse range of liquid media like nutrient broth, de Man, Rogosa and Sharpe (MRS) broth are used.

3.3.2 Chemicals and reagents

The chemicals and reagents employed in this investigation encompassed alcohol, 0.1% peptone water, phosphate buffered saline (PBS), reagents for Gram's staining (crystal violate, gram's iodine, safranin, ethyl alcohol), sugar media (dextrose, lactose, sucrose etc.), 3% hydrogen peroxide, phenol red, methyl red, normal saline, PCR reagents, deionized water as well as other standard laboratory chemicals and reagents.

3.3.3 Materials used for polymerase chain reaction (PCR) amplification

The following materials and primers are used in the process of PCR (shown in Tables 2 and 3). Mastermix for PCR is obtained from Jiangsu Cowin Biotech Co. Ltd., Beijing, China.

Table 2: Preparation of PCR mixture

Materials	Quantity
Nuclease free water	6.5µl
Forward primer	2.5µl
Reverse primer	2.5µl
2x master PCR mix	12.5µl
DNA template	1.0 µl
Final Volume	25.0 µl

The primers used for the PCR amplification is obtained from GeneCreate Co., Beijing, China.

Table 3: Primers and sequences used in PCR amplification

Primer Name	Primer Sequences (5´- 3´)	Size (bp)	Reference
27F 1492R	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGACTT	1500	(Hou *et al*., 2018; Frank *et al*., 2008; Kang *et al*., 2020)

Note**:** PCR=Polymerase chain reaction; F= Forward, R = Reverse, bp = base pair.

3.3.4 Antimicrobial discs used for the study

Antibiotic sensitivity and resistance patterns were interpreted using commercially available antimicrobial discs (OXOID Limited, Canada). The sensitivity and resistance pattern of the detected bacterial pathogens (LAB) isolated from fermented dairy products were evaluated using the following antimicrobial agents and their disc concentrations and ranges (Table 4).

Table 4: Antimicrobial agents with their disc concentration

Antimicrobial agents	Symbol	Disc concentration (µg/disc)
Gentamicin	GEN	10
Ciprofloxacin	CIP	5
Tetracycline	TE	30
Ceftriaxone	CTX	30

Note: µg = Microgram

3.4 Methods

The study consisted of three phases. The first step consisted of identifying sources, collecting samples, isolating, identifying, and characterizing microorganisms based on their colony morphology, staining properties, and biochemical features and the second step was using commercially available antibiotic discs, antibiotic sensitivity and resistance patterns were seen. The final step involved the molecular characterization of particular isolates.

3.4.1 Preparation of essential glassware

All essential glassware, including test tubes, pipettes, slides, cylinders, flasks, conical flasks, glass, and vials, were soaked overnight in a household dishwashing detergent. Before washing, contaminated glassware was disinfected with a 2% sodium hypochlorite solution. The glassware was then brushed, washed thoroughly in running tap water, rinsed in distilled water, and sterilized either by dry heat at 160°C for two hours or autoclaving at 121°C for 15 minutes at 15 lbs pressure per sq. inch. The autoclaved items were dried in a 50°C hot air oven. Through autoclaving, disposable plastic (e.g., micropipette tips) was sanitized, and all the glassware was stored at 50°C for future use.

3.4.2 Preparation of bacterial culture media

a) Preparation of nutrient broth (NB)

The nutrient broth was made by dissolving 13 grams of dehydrated nutrient broth (HiMedia, India) in 1000 ml of distilled water and autoclaving it at 121ºC under 15 lbs pressure per square inch for 15 minutes. The broth was then poured into test tubes (10 ml/tube), incubated at 37ºC overnight to ensure sterility, and refrigerated at 4ºC until used.

b) Preparation of de Man, Rogosa and Sharpe (MRS) broth

The MRS broth was made by dissolving 55.15 grams of dehydrated MRS broth (HiMedia, India) in 1000 ml of distilled water and autoclaving it at 121ºC under 15 lbs pressure per square inch for 15 minutes. The broth was then poured into test tubes (10 ml/tube), incubated at 37ºC overnight to ensure sterility, and refrigerated at 4ºC until used.

c) Preparation of nutrient agar (NA)

The NA was made by dissolving 28 grams of NA agar (HiMedia, India) in 1000 ml of distilled water and heated to boiling in a conical flask to dissolve the medium completely. The solution was sterilized by autoclaving at 121ºC for 15 minutes at 15 lbs pressure per square inch. The medium was autoclaved before being put into sterilized petridishes and allowed to harden. After the medium had solidified, the plates were inverted and incubated at 37ºC overnight to ensure sterility and stored at 4ºC in the refrigerator until required.

d) Preparation of de Man, Rogosa and Sharpe (MRS) agar

MRS agar was prepared by dissolving 67.15 grams of dehydrated *Lactobacillus* MRS agar (HiMedia, India) in 1000 ml of distilled water in a conical flask and heated until the medium was fully dissolved. Then, the solution was sterilized by autoclaving it for 15 minutes at 121ºC and 15 lbs per square inch of pressure. The liquid was then put into sterilized petridishes and allowed to be set. After the solidification of the medium, the plates were inverted and incubated at 37ºC for one night to test their sterility before being refrigerated at 4ºC until use.

e) Preparation of Muller Hinton (MH) agar

MH agar was made by dispersing 38 grams of dehydrated MH agar (HiMedia, India) in 1000 ml of distilled water. The liquid was then brought to a boiling temperature to dissolve the medium thoroughly. The solution was sterilized by autoclaving at 121ºC under 15 lbs of pressure for 15 minutes before being cooled to 45 to 50ºC. The mixture was then vigorously agitated and put into sterilized petridishes to solidify. After the solidification of the media, the plates were inverted and incubated at 37ºC for 24 hours to ensure their sterility and then stored at 4ºC in the refrigerator until further used.

f) Preparation of phosphate buffered saline (PBS)

To make PBS, 8.0 grams of sodium chloride (NaCl), 2.89 grams of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 grams of potassium chloride (KCl), and 0.2 grams of potassium hydrogen phosphate (KH_2PO_4) were dissolved in 1000 ml of distilled water. The solution was heated to complete dissolving, and the pH was adjusted using a pH meter. The solution was then autoclaved for sterilizing and maintained at 4ºC for future use.

g) Preparation of carbohydrate solutions

To prepare the medium, 1% peptone water was used, to which fermentable sugars were added. Peptone water was made by combining 1 gm of bacto peptone (Difco, USA) and 0.5 gm of sodium chloride in 100 ml of distilled water, boiling it for 5 minutes, adjusting the pH to 7.6 using a phenol red (0.02%) indicator, and filtering it through filter paper. The solutions were transferred in 5 ml amounts into test tubes containing inverted Durham's fermentation tubes and cotton plugs. The sugars that were utilized for fermentation, fructose, lactose and sucrose, were each prepared as 10 percent solutions in distilled water. After applying a little heat to dissolve the sugar, autoclave it for 15 minutes to ensure sterility.

3.4.3 Procedure for the isolation and identification of LAB

The following procedure was followed for the isolation and identification of LAB:

3.4.3.1 Procedure for the isolation of LAB

A volume of 1 mL from each thoroughly mixed sample was subjected to enrichment in 9 mL of sterile MRS broth for 24 hours at a temperature of 37°C. Before the

inoculation of the sample, the pH of the MRS broth was carefully adjusted to a value of 6.5±0.2. The samples that had been enriched were streaked onto petridishes that contained *Lactobacillus* MRS agar. This was done using a calibrated inoculating loop. The plates were then incubated aerobically at a temperature of 37 °C for 48 hours. During this time, the plates were inspected for the presence of colony growth.

3.4.3.2 Procedure for the identification of LAB

The process of identifying and characterizing LAB isolates cultivated on MRS agar primarily involved several tests, including microscopic examination through gram staining, catalase test, assessment of growth at varying temperatures (15ºC and 45ºC), examination of growth at different NaCl concentrations and analysis of carbohydrate fermentation and finally molecular identification by PCR and 16S rDNA sequencing.

a) Gram's staining of LAB

The isolates were subjected to gram staining to confirm their morphological identification as LAB. Using a sterile inoculation loop, a drop of saline solution and a bacterial colony were picked up to make a smear. The smear was then "heat fixed" and rapidly passed through a flame twice or thrice. Fixation was followed by gram's staining as follows: primary stain (crystal violet) for 2 minutes, mordent (gram's iodine) for 2 minutes, decolorizer (ethyl alcohol) for 10 seconds and counter stain (Safranin) for 1 minute. Tape water was used to gently rinse the process after each stage. Bacteria were characterized by seeing the slide under a microscope at 100X with immersion oil.

b) Catalase test of LAB

The catalase test was performed for the LAB isolates by picking up a colony into the surface of a sterile, desiccated glass slide using a loop. Subsequently, a solution containing 3% hydrogen peroxide (H_2O_2) was applied to the slide and thoroughly mixed. The formation of bubbles within 5-10 seconds indicated a positive test and the absence of bubbles indicated a negative test.

c) NaCl tolerance test of LAB

The NaCl tolerance of isolated LAB was assessed by subjecting it to MRS broth containing NaCl concentrations of 4%, 5%, and 6%. The culture was freshly

administered and thereafter incubated at a temperature of 37ºC for 24 hours. The tolerance test was determined by the growth of the bacteria. Positive results show turbidity and the negative results show no turbidity.

d) Growth of isolates at 15°C and 45°C

The isolates were subjected to growth assessment in MRS broth at a temperature of 15°C for a duration of 24-48 hours, as well as at a temperature of 45°C for 24-48 hours. In this experiment, 10 mL of MRS broth was inoculated with isolated LAB cultures at a concentration of 1%. The observation of turbidity formation in culture tubes was documented as an indication of the isolates' capacity to thrive under temperatures of 15 °C and 45 °C, with outcomes categorized as either positive or negative.

e) Sugar fermentation test of LAB

The experiment involved conducting a sugar fermentation test utilizing a 1% (w/v) sugar concentration in MRS broth. The test utilized glucose, sucrose and lactose as the experimental substances. The indicator employed in the experiment was a solution of phenol red. A volume of 10 mL of medium was put into each test tube, and a Durham's tube was placed in an inverted manner into each of the test tubes. The culture was freshly injected and thereafter incubated at a temperature of 37°C for 24 hours. The outcomes were detected through the occurrence of color alteration and the generation of gas. A red color indicates negative results and a yellow color indicates positive results.

3.4.4 Antibiotic sensitivity patterns of isolated LAB

The susceptibility and resistance of the isolated bacteria were assessed against four different types of commonly employed antibiotics using the modified Kirby-Bauer disc diffusion method, as previously described by Bauer *et al*. (1959). The overnight culture of LAB into the MRS broth was done. A volume of 100 microliters of the test organism's broth culture was carefully dispensed onto a plate containing Muller-Hinton agar (Merck, Darmstadt, Germany) using a micropipette. The culture was uniformly distributed within the media using a sterilized glass spreader. The antibiotic discs were thereafter applied in a sterile manner and arranged in a precise pattern on the MH agar plates. Subsequently, the plates were subjected to incubation for 24 hours at a temperature of 37°C. Following the incubation period, the

measurement of the diameter of the zones was conducted. Measurements were achieved without removing the lid by utilizing a ruler positioned beneath the plate. The updated interpretation tables provided by the Clinical and Laboratory Standards Institute (CLSI) (Table 5) were used to classify the results into three categories: susceptible, intermediate, or resistant.

Table 5: List of antibiotics used for LAB to assess antibiotic sensitivity and zone of ranges (mm) of inhibition

Antimicrobial agents	Resistant (R) (mm)	Intermediate (I) (mm)	Sensitive (S) (mm)
Ciprofloxacin	≤ 15	16-20	≥ 21
Ceftriaxone	≤ 13	14-20	≥ 21
Tetracycline	≤ 14	15-18	≥ 19
Gentamycin	≤ 12	13-14	≥ 15

3.4.5 Molecular detection and characterization of LAB

The main steps in molecular identification include:
DNA extraction from bacterial cells, amplification using 16S rDNA gene, Sanger's sequencing and BLASTN search into NCBI database.

3.4.5.1 Isolation protocol of bacterial genomic DNA

A single bacterial colony in 2 ml nutrient broth was inoculated at 37˚C overnight. Then 1.5 ml culture content was transfer to the eppendorf tube. Centrifuged at 13000 rpm for 10 minutes. A volume of 350 µl lysis buffer and 20 µl of proteinase-K was added (20mg/ml) and kept in the water bath for 30 minutes at 65˚C. The tube was cooled, 3µl of RNAse was added to the solution and kept on a heat block at 37˚C for 10 minutes. An equal volume (373 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution and mixed by vortex machine. The suspension was centrifuge at 1400 rpm for 10 minutes. The aqueous layer (about 200 µl) from the top was transferred to a fresh micro-centrifuge tube carefully to avoid any protein debris. An equal volume of chloroform: isoamyl alcohol (24:1) to the tube was added and centrifuged at 1400 rpm for 10 minutes. The upper layer (150 µl) was transferred to the new eppendorf tube. Add 800 µl of ice-cooled absolute ethanol to the aqueous phase to precipitate the DNA. The tube was inverted by handshaking and keep it at -

40°C for 1 hour. Centrifuged at 13000 rpm for 10 minutes. The liquid was discarded and 1000 µl of chilled 70% ethanol was added with 20 µl of 7.5M ammonium acetate, and keep it at -20˚C for 30 minutes. The content was centrifuged again at 13000 rpm for 10 minutes and the aqueous layer was eliminated, and let it for air dry. The 50µl of TE buffer (pH 7.6) was added to dissolve the DNA by heating at 37°C for 10 minutes.

3.4.5.2 PCR protocol for identification of LAB

The final identification of LAB was carried out by PCR. A set of universal primers (27F and 1492R) was used to amplify the 1400 bp region of the 16S rDNA gene (Acinas *et al*., 2004). The reaction volume of the polymerase chain reactions (PCR) was 25 µL, containing 10 ng of template DNA, 2 mM MgCl2, 1 mL of each primer (10 mM) and 1 × Taq Master Mix. The conditions of the PCR temperature cycle were as follows: the initial heating was 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 10 min using a gel documentation system (Bio-Rad). 1 Kb plus DNA ladder (New England Biolabs, UK) was used to compare the band on an agarose gel. The PCR products were confirmed on agarose gels (1%) stained with ethidium bromide on the gel documentation system (EZEE Clearview UV transilluminator).

3.4.5.3 Visualization of the PCR products by agarose gel electrophoresis

The PCR products underwent separation using the process of agarose gel electrophoresis. A gel tray was assembled in a suitable configuration by placing a comb into the tray. Subsequently, a 1.5% agarose solution was prepared. The solution was then placed in a water bath set at a temperature of 50°C to facilitate cooling. Additionally, 5µl of ethidium bromide was introduced into the solution. Subsequently, the agarose solution was carefully transferred into the designated gel tray. To achieve gel solidification, the gel was subjected to ambient room temperature conditions for 20 minutes. Subsequently, the gel was carefully introduced into an electrophoresis chamber that was appropriately filled with a solution of 1X TAE buffer. A volume of 5.5 µl of each PCR product corresponding to a specific gene was introduced into a well on the gel, while a volume of 3 µl of a ladder was employed. The electrophoresis experiment was conducted with a voltage of 90 volts and a current of 120 Amps for 35 minutes. Subsequently, the DNA fragments were

observed under UV light and captured by a gel documentation system (BDA digital, biometra GmbH, Germany).

3.4.5.4 Sequencing protocol

PCR products were purified, then single-stranded products were generated using cycle sequencing PCR with forward or reverse primers. PCR products were run on a Sanger machine using the di-deoxy chain termination method at Wuhan Tianyi Huayu Gene Technology Co., Ltd. (Sanger *et al*., 1977).

3.4.6 Storage of isolated LAB

One millilitre of sterile buffered glycerol was produced by mixing 800 µl of pure glycerol with 200 µl of phosphate-buffered saline (PBS). Following that, 0.5 ml of pure bacterial culture and 80% sterile buffered glycerol were placed into 1.5 ml cryo-vials, which were then kept at -18^{o}C.

CHAPTER IV

RESULTS AND DISCUSSION

In this research, fermented dairy products collected from different sources and lactic acid bacteria (LAB) were isolated and identified based on morphology, staining properties, biochemical properties and finally molecular identification of LAB species was done. The results of the study were discussed accordingly.

4.1 Isolation and identification of selected bacteria

The bacteria were identified primarily by their cultural and morphological characteristics, staining properties and biochemical properties.

4.1.1 Morphological characteristics of the selected bacteria

The colony morphology was observed to isolate presumptive bacteria. The bacteria were grown on MRS agar medium at a temperature of 37°C for 48 hours and growth of the bacterial colony was observed. The distinctive patterns of growth seen on the MRS agar medium are shown in Figure 1. In this study, all of the presumptive isolates exhibited growth characterized by a creamy white color, circular shape, convex elevation, and shiny appearance with a smooth edge, except isolate D12 (*Dahi* sample no 12), which displayed a colony of greyish white color (as shown in Table 6).

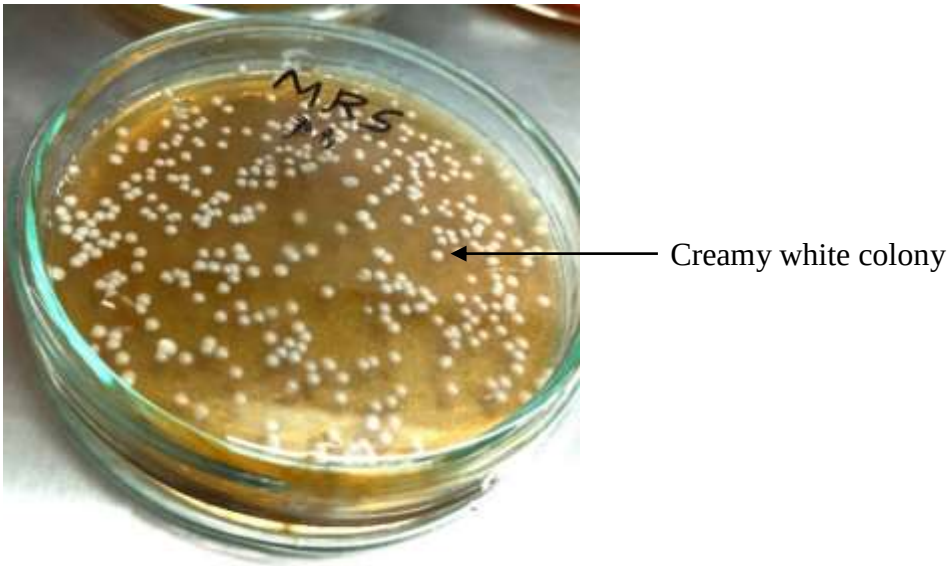

Figure 1: Growth of bacterial colony on MRS agar media

Table 6: Colony morphology of the selected bacterial cultures

SI No	Isolate No	Colony Color	Shape	Margin	Surface
1	D1	Creamy White	Circular	Entire	Smooth
2	D2	Creamy White	Circular	Entire	Smooth
3	D3	Creamy White	Circular	Entire	Smooth
4	D4	Creamy White	Circular	Entire	Smooth
5	D5	-	-	-	-
6	D6	Creamy White	Circular	Entire	Smooth
7	D7	Creamy White	Circular	Entire	Smooth
8	D8	Creamy White	Circular	Entire	Smooth
9	D9	Creamy White	Circular	Entire	Smooth
10	D10	Creamy White	Circular	Entire	Smooth
11	D11	-	-	-	-
12	D12	Grayish White	Circular	Entire	Smooth
13	D13	Creamy White	Circular	Entire	Smooth
14	D14	Creamy White	Circular	Entire	Smooth
15	D15	Creamy White	Circular	Entire	Smooth
16	D16	Creamy White	Circular	Entire	Smooth
17	D17	Creamy White	Circular	Entire	Smooth
18	D18	Creamy White	Circular	Entire	Smooth
19	D19	Creamy White	Circular	Entire	Smooth
20	D20	Creamy White	Circular	Entire	Smooth
21	L1	Creamy White	Circular	Entire	Smooth
22	L2	Creamy White	Circular	Entire	Smooth
23	L3	Creamy White	Circular	Entire	Smooth
24	L4	Creamy White	Circular	Entire	Smooth
25	L5	-	-	-	-
26	M1	Creamy White	Circular	Entire	Smooth
27	M2	Creamy White	Circular	Entire	Smooth
28	M3	Creamy White	Circular	Entire	Smooth
29	M4	Creamy White	Circular	Entire	Smooth
30	M5	Creamy White	Circular	Entire	Smooth
31	B1	Creamy White	Circular	Entire	Smooth
32	B2	Creamy White	Circular	Entire	Smooth
33	B3	Creamy White	Circular	Entire	Smooth
34	B4	Creamy White	Circular	Entire	Smooth
35	B5	Creamy White	Circular	Entire	Smooth
36	C1	Creamy White	Circular	Entire	Smooth
37	C2	Creamy White	Circular	Entire	Smooth
38	C3	Creamy White	Circular	Entire	Smooth
39	C4	Creamy White	Circular	Entire	Smooth
40	C5	Creamy White	Circular	Entire	Smooth

Note: (-) denotes no growth

Note: D (1-20): *Dahi* sample; L (1-5): *Lassi* sample; B (1-5): *Borhani* sample; M (1-5) *Matha* sample; C (1-5): Cheese sample

In this experiment, three (3) sample have no growth out of forty (40) samples on MRS agar medium and it was discarded for further identification. From each plate three colony was picked and again cultured at MRS agar. Clearly visible thirty-seven (37) bacterial colony from each representative sample plate was picked for further identification of the bacteria. MRS agar is made selective for lactic acid bacteria by lowering the pH to 5.7 and the addition of 0.14% sorbic acid (Janet *et al*., 2003). It may be indicates a potential scenario in which the product underwent a heating process after its preparation. Carr *et al*. (2002) reported creamy white to greyish white, circular, convex, shiny colonies as LAB when grown at MRS agar which supports the present study. Dhameliya *et al*. (2020) also observed white color colony in MRS agar. According to Roos *et al*. (2005), LAB are the rod-shaped, gram-positive, facultative anaerobic, non-motile, catalase-negative bacteria which are similar with the present study.

4.1.2 Gram staining properties of isolated bacteria

The selected bacterial isolates were tested for gram staining to identify bacteria whether it is gram positive or negative. A total of thirty-seven (37) isolated colony was further tested for their gram staining properties. All the 37 selected isolates were found only gram-positive by observing as purple-colored and rod-shaped under the light microscope (shown in Figure 2 and Table 7).

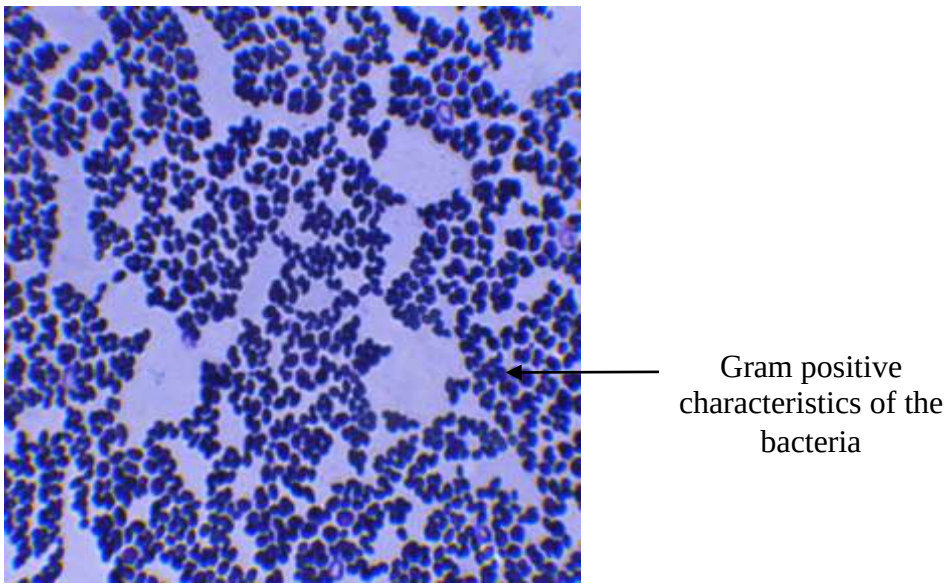

Figure 2: Gram's staining of selected isolates observed under the microscope

Table 7: Staining properties of the isolated bacterial cultures

SI No	Isolate No	Gram Staining	Shape
1	D1	+ve	Rod
2	D2	+ve	Rod
3	D3	+ve	Rod
4	D4	+ve	Rod
5	D6	+ve	Rod
6	D7	+ve	Rod
7	D8	+ve	Rod
8	D9	+ve	Rod
9	D10	+ve	Rod
10	D12	+ve	Rod
11	D13	+ve	Rod
12	D14	+ve	Rod
13	D15	+ve	Rod
14	D16	+ve	Rod
15	D17	+ve	Rod
16	D18	+ve	Rod
17	D19	+ve	Rod
18	D20	+ve	Rod
19	L1	+ve	Rod
20	L2	+ve	Rod
21	L3	+ve	Rod
22	L4	+ve	Rod
23	M1	+ve	Rod
24	M2	+ve	Rod
25	M3	+ve	Rod
26	M4	+ve	Rod
27	M5	+ve	Rod
28	B1	+ve	Rod
29	B2	+ve	Rod
30	B3	+ve	Rod
31	B4	+ve	Rod
32	B5	+ve	Rod
33	C1	+ve	Rod
34	C2	+ve	Rod
35	C3	+ve	Rod
36	C4	+ve	Rod
37	C5	+ve	Rod

Note: (+ve) denotes positive results

Note: D (1-20): *Dahi* sample; L (1-5): *Lassi* sample; B (1-5): *Borhani* sample; M (1-5) *Matha* sample; C (1-5): Cheese sample

LAB are universally recognized as gram positive because they have a thick cell wall made of peptidoglycan (Chapot-Chartier and Kulakauskas, 2014). The gram positive and rod shaped bacteria were selected for further identification of bacteria.

4.1.3 Catalase test of selected isolates

The catalase test is used to detect the ability of isolates to produce the enzyme catalase, which releases oxygen from hydrogen peroxide. A total of thirty-seven (37) selected isolates were tested for catalase test from which 2 isolates were catalase-positive and 35 isolates were catalase-negative (shown in Table 8). LAB do not produce catalase enzyme that converts hydrogen peroxide into water and oxygen (Mokoena, 2017). Fevria and Hartanto (2020) reported catalase negative bacteria in fermented dairy products. There may be presence of catalase enzymes in 2 isolates, therefore they gives positive catalase test. So, these two (2) isolates was discarded and proceed for further identification with catalase-negative isolates.

4.1.4 Carbohydrate fermentation of selected isolates

Some bacteria can be able to ferment carbohydrates and produce acid and gas. Different bacteria ferment different types of sugar. In this study, fructose, sucrose, and lactose were employed in the sugar fermentation test and the results was observed. Among 37 selected isolates, 2 isolates were not able to ferment fructose, sucrose and lactose, another 35 isolates were able to ferment the sugars (shown in Table 8). So, the final 35 isolates was selected for further study. One of the characteristics of heterofermentative LABs, such as *Leuconostoc, Wiessella*, and certain *lactobacilli* is the production of gas from glucose, which ferments hexoses into lactic acid, acetic acid, or ethanol and carbon dioxide and homofermentative LABs such as *Lactococcus* and *Streptococcus* cannot produce carbon dioxide from glucose (Das *et al*., 2019). So, the isolated bacteria can be homofermentative or heterofermentative LAB which can be confirmed through molecular tests. From the biochemical test, 35 catalase negative isolates were selected and their physiological properties was observed for further identification.

Table 8: Biochemical characteristics of the isolated bacterial cultures

SI No	Isolate No	Catalase test	Fermentable Sugars		
			Fructose	Sucrose	Lactose
1	D1	-ve	+	+	+
2	D2	-ve	+	+	+
3	D3	-ve	+	+	+
4	D4	-ve	+	+	+
5	D6	-ve	+	+	+
6	D7	-ve	+	+	+
7	D8	-ve	+	+	+
8	D9	-ve	+	+	+
9	D10	+ve	-	-	-
10	D12	-ve	+	+	+
11	D13	-ve	+	+	+
12	D14	-ve	+	+	+
13	D15	-ve	+	+	+
14	D16	-ve	+	+	+
15	D17	-ve	+	+	+
16	D18	-ve	+	+	+
17	D19	-ve	+	+	+
18	D20	-ve	+	+	+
19	L1	-ve	+	+	+
20	L2	-ve	+	+	+
21	L3	-ve	+	+	+
22	L4	-ve	+	+	+
23	M1	-ve	+	+	+
24	M2	-ve	+	+	+
25	M3	-ve	+	+	+
26	M4	-ve	+	+	+
27	M5	-ve	+	+	+
28	B1	-ve	+	+	+
29	B2	-ve	+	+	+
30	B3	-ve	+	+	+
31	B4	-ve	+	+	+
32	B5	+ve	-	-	-
33	C1	-ve	+	+	+
34	C2	-ve	+	+	+
35	C3	-ve	+	+	+
36	C4	-ve	+	+	+
37	C5	-ve	+	+	+

Note: (+ve) = Positive, (+) = able to ferment sugar; (–) = not able to ferment sugar

Note: D (1-20): *Dahi* sample; L (1-5): *Lassi* sample; B (1-5): *Borhani* sample; M (1-5) *Matha* sample; C (1-5): Cheese sample

4.1.5 Growth of selected isolates at different temperatures

Bacteria can be grown at a wide range of temperatures depending on species. The growth of bacteria at various temperatures helps to identify their species. In this study, the growth of the isolated cultures was evaluated at 15°C and 45°C temperatures. All the isolates can grow at 45^0C. Among 35 isolates, 28 isolates can grow at 15^0C, but 7 isolates could not grow at 15^0C (shown in Table 9). The research conducted by Banik *et al*. (2023) and Schillinger and Lucke (1987) has identified certain strains of bacteria as presumptive homo-fermentative strains which observed to grow optimally at a temperature of 45ºC but fail to grow at a temperature of 15ºC. On the other hand, hetero-fermentative strains were found to grow well at a temperature of 15ºC which was reported by Monika *et al.* (2017). Furthermore, strains such as *L. acidophilus* and *L. helveticus* can grow at 45°C but not at 15°C (Harrigan, 1998). So, some bacteria can be identified from their growth at various temperatures.

4.1.6 Growth of selected isolates at different NaCl concentrations

The growth of isolates at different NaCl concentrations is one of the indicators of bacteria identification. NaCl is an inhibitory substances which may inhibit the growth of different types of bacteria but the probiotic bacterial species should have the ability to survive at gastrointestinal salt concentrations (FAO, 2001). A NaCl tolerance test was conducted to assess the ability of bacteria to tolerate NaCl at different concentrations. NaCl has been identified as an inhibitory agent that effectively suppresses the growth of some bacterial strains, as demonstrated in the study conducted by Hoque *et al*. (2010). In this study, all the 35 test isolates can grow at 4% and 5 % NaCl concentrations, but could not survive at 6% NaCl concentration (shown at Table 9). Monika *et al*. (2017) also observed the growth of bacteria in 1.5%, 2.5%, 5% NaCl concentrations and observed no growth at 8% and 10% NaCl concentrations. Prabhurajeshwar and Chandrakanth (2017) observed excellent growth at 1–5% NaCl concentrations.

Table 9: Physiological characteristics of selected isolates

SI No.	Isolate No.	Physiological characteristics				
		Growth at different temperatures		Growth at different NaCl concentrations		
		15°C	45°C	4	5	6
1	D1	+	+	+	+	-
2	D2	+	+	+	+	-
3	D3	-	+	+	+	-
4	D4	+	+	+	+	-
5	D6	+	+	+	+	-
6	D7	+	+	+	+	-
7	D8	+	+	+	+	-
8	D9	+	+	+	+	-
9	D12	+	+	+	+	-
10	D13	+	+	+	+	-
11	D14	+	+	+	+	-
12	D15	+	+	+	+	-
13	D16	+	+	+	+	-
14	D17	+	+	+	+	-
15	D18	+	+	+	+	-
16	D19	-	+	+	+	-
17	D20	+	+	+	+	-
18	L1	+	+	+	+	-
19	L2	+	+	+	+	-
20	L3	+	+	+	+	-
21	L4	+	+	+	+	-
22	M1	+	+	+	+	-
23	M2	+	+	+	+	-
24	M3	+	+	+	+	-
25	M4	+	+	+	+	-
26	M5	+	+	+	+	-
27	B1	+	+	+	+	-
28	B2	+	+	+	+	-
29	B3	+	+	+	+	-
30	B4	+	+	+	+	-
31	C1	-	+	+	+	-
32	C2	-	+	+	+	-
33	C3	-	+	+	+	-
34	C4	-	+	+	+	-
35	C5	-	+	+	+	-

Note: + = able to grow; - = not able to grow

Note: D (1-20): *Dahi* sample; L (1-5): *Lassi* sample; B (1-5): *Borhani* sample; M (1-5) *Matha* sample; C (1-5): Cheese sample

After observing the physiological characteristics, all the 35 selected isolates was tested for their antimicrobial sensitivity.

4.2 Antibiotic sensitivity test of selected isolates

Antibiotic susceptibility is one of the important criteria for the safety of using bacteria in the preparation of fermented products. After characterizing the isolates physiologically, the antibiotic susceptibility of 35 selected isolates were studied by disc diffusion method at Mueller Hinton (MH) agar and antibiotic sensitivity was determined as per the Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2015). Most of the LAB isolates showed sensitivity toward gentamicin, ciprofloxacin, tetracycline and ceftriaxone (shown in Figure 3). The overall susceptibility patterns of isolated bacterial colony was shown in Table 10. Out of 35 isolates screened, only the M1 isolate showed intermediate results (shown in Table 11). No isolates were resistant to gentamicin, ciprofloxacin, tetracycline and ceftriaxone, which is a matter of great happiness.

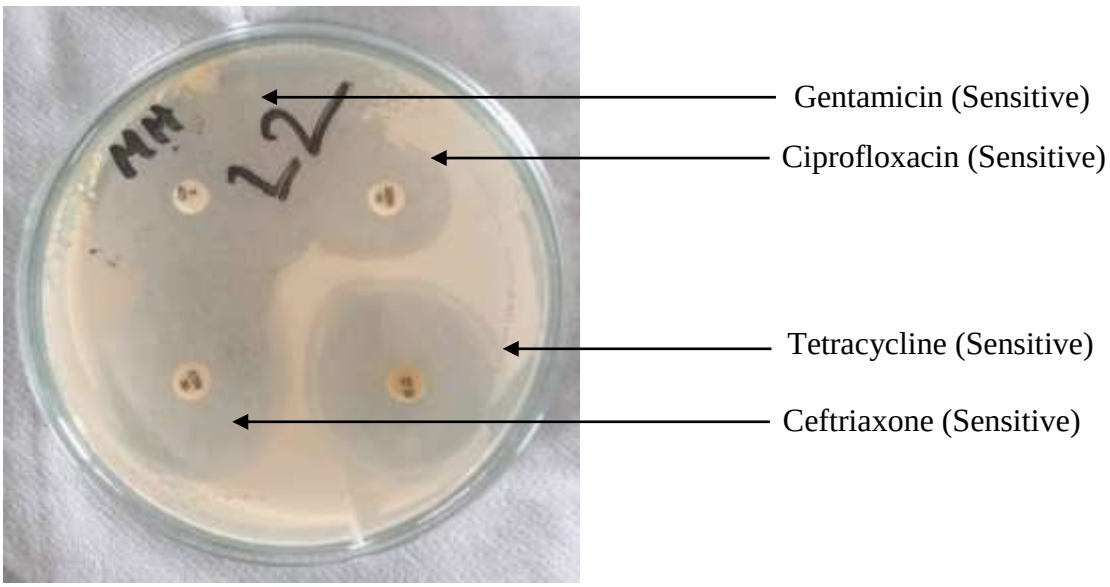

Figure 3: Antibiotic sensitivity test of selected isolates on Mueller Hinton agar

Table 10: Overall susceptibility patterns of isolated bacterial colony

Antibiotics	Total no. of isolates	Resistant (%)	Intermediate (%)	Sensitive (%)
Ciprofloxacin	35	0	2.86	97.14
Ceftriaxone		0	0	100
Gentamicin		0	0	100
Tetracycline		0	0	100

Table 11: Antibiotic sensitivity of isolated bacterial colony

SI No	Isolate No	Name of the antibiotics			
		Gentamicin	Ciprofloxacin	Tetracycline	Ceftriaxone
1	D1				
2	D2				
3	D3				
4	D4				
5	D6				
6	D7				
7	D8				
8	D9				
9	D12				
10	D13				
11	D14				
12	D15				
13	D16				
14	D17				
15	D18				
16	D19				
17	D20				
18	L1				
19	L2				
20	L3				
21	L4				
22	M1				
23	M2				
24	M3				
25	M4				
26	M5				
27	B1				
28	B2				
29	B3				
30	B4				
31	C1				
32	C2				
33	C3				
34	C4				
35	C5				

Yellow refers to intermediate Green refers to Sensitive

Note: D (1-20): *Dahi* sample; L (1-5): *Lassi* sample; B (1-5): *Borhani* sample; M (1-5) *Matha* sample; C (1-5): Cheese sample

LAB are the important bacteria in food fermentation which should sensitive to antibiotics. Prabhurajeshwar and Chandrakanth (2017) reported sensitivity of LAB to tetracycline and demonstrating sensitivity to ceftriaxone, which aligns with the present study (Hummel *et al*., 2007; Nawaz *et al*., 2011; Hawaz, 2014). The findings of the present study similar with the results of Jiang *et al*. (2016), as they demonstrated the susceptibility of bacteria to gentamicin. Chen *et al*. (2022) also found the sensitivity pattern of LAB against gentamicin, ciprofloxacin, tetracycline and ceftriaxone. Therefore, antibiotic sensitive isolates was selected for molecular identification.

4.3 Molecular identification of selected bacteria

Molecular techniques are the most reliable methods for the identification of bacteria at their species level. Among 35 selected isolates, the representative 8 isolates were tested for molecular identification. As there was a limited facility for molecular test in the departmental laboratory, a shortage of fund during conducting the research and the high cost of molecular test, only 8 isolates was sent to the NIB Animal Biotechnology Laboratory. From 35 presumptive isolates, C1, C5, D4, D9, D15, L1, L2, and M3 were tested as a representative sample of *dahi*, cheese, *lassi* and *matha*. No isolates from the *borhani* sample could not be sent because of their contamination during the study. The presumptively identified isolates were then subjected to molecular identification by polymerase chain reaction (PCR). After PCR of representative samples, gel electrophoresis of PCR product was seen (shown in Figure 4) and a clear band was selected and sequenced for further confirmation of the bacterial species.

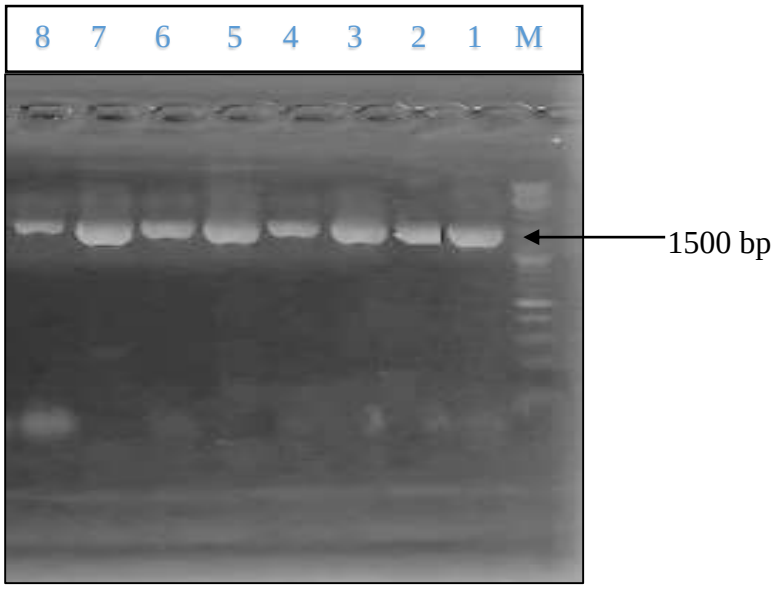

Figure 4: Gel electrophoresis of PCR product of selected isolates [Molecular identification of isolated bacteria by amplification of 1500bp DNA from 16S rDNA gene. Lane M: 50 bp DNA ladder marker, Lane 1-8: Test samples (This figure illustrates fragments specifically amplified by PCR using the primer set 27F/1492R (1500bp)]

After all the 16S rDNA sequences were obtained, it was edited using the Bioedit software package, and consensus sequences were subjected to BLASTN analysis in GenBank of NCBI to identify the organisms present (Hall, 2011). Among 8 test isolates, 7 isolates showed significant similarity of 98.5-99.5% in the GenBank. From the BLASTN search, among 8 isolates, C1 and C5 were confirmed as *Limosilactobacillus fermentum* (previously known as *Lactobacillus fermentum*) and D4, D9, L1, L2 and M3 isolates was confirmed as *Lacticaseibacillus rhamnosus* (previously known *Lactobacillus rhamnosus*), but D15 isolates do not show any significant similarities with the other genomes of the database, so D15 is unidentified. The percentage of similarity of tested strains against representative species in the NCBI BLASTN search is shown in Table 12.

Table 12: Percentage similarity of tested strains against representative species in NCBI BLASTN search

Sample no	Identified bacterial species	Similarity % (BLASTN)	Length (bp)
C1	*Limosilactobacillus fermentum*	98.91	1466
C5	*Limosilactobacillus fermentum*	99.39	1486
D4	*Lacticaseibacillus rhamnosus*	98.58	1481
D9	*Lacticaseibacillus rhamnosus*	99.46	1477
D15	Unidentified	79.65	1541
L1	*Lacticaseibacillus rhamnosus*	98.92	1482
L2	*Lacticaseibacillus rhamnosus*	98.85	1076
M3	*Lacticaseibacillus rhamnosus*	99.46	1482

Note: D4, D9 and D15: *Dahi* sample; L1 and L2: *Lassi* sample; M3 *Matha* sample; C1 and C5: Cheese sample

Finally, the LAB species were identified as *Lactobacillus fermentum* and *L. rhamnosus*. In a study conducted by Haryani *et al*. (2023), it was observed that *Lacticaseibacillus rhamnosus* accounted for the majority (34.5%) of LAB species found in fermented foods in Malaysia. This finding aligns with the results of the present study. The probiotic activity of *Lacticaseibacillus rhamnosus* and *Limosilactobacillus fermentum* species was reported by Li *et al*. (2022) and Seo *et al*. (2021). *Lactobacillus rhamnosus* and *L. fermentum* have been identified as viable probiotic strains with potential for sustainable utilization in the commercial sector of the fermented dairy industry. These strains have demonstrated various health benefits, such as the prevention and treatment of gastrointestinal infections and diarrhea, stimulation of immune responses, toxin binding capabilities, suppression of pathogens, and enhancement of food safety (Archer and Halami, 2015; Parker *et al*., 2018). The use of both strains *L. rhamnosus* and *L. fermentum* as a starter culture will be safe and advantageous for human health as they are not resistant to antibiotics and known microbial populations.

CHAPTER V
SUMMARY AND CONCLUSION

The present study was conducted to isolate, identify and molecular detection of lactic acid bacteria from five different fermented dairy products e.g. *dahi*, *lassi*, *borhani*, *matha* and cheese based on gram's staining properties and colony characteristics. A total of 37 isolates were selected based on their colony morphology from 40 different samples and catalase test and sugar fermentation test was done. Out of thirty seven (37) isolates, 2 were tested catalase positive and 35 catalase-negative isolates were selected for further test. All the selected 35 isolates was tested for their growth at different temperatures and NaCl concentrations and their growth was observed. The antibiotic sensitivity were observed for 35 isolates on MH agar to know their resistance and sensitivity patterns and found a satisfactory results of antibiotic sensitivity for 34 isolates with intermediate sensitive for 1 isolate to the antibiotics like gentamicin, tetracycline, ciprofloxacin and ceftriaxone.

Molecular identification of the selected isolates was done to know their microbial status. From the 35 isolates, 8 representative isolates were selected for molecular identification by PCR followed by 16S rDNA sequencing. From the research, the LAB species were identified as *Lactobacillus rhamnosus* and *Lactobacillus fermentum*. *Lactobacillus rhamnosus* was identified from *dahi*, *lassi* and *matha* samples and *Lactobacillus fermentum* was identified from the cheese sample. They can be utilized as potential starter cultures in the manufacturing process of fermented dairy products. The study helps the dairy industry by providing potential LAB and improving the quality of fermented dairy products.

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