Assessment of the Impact of *Enterococcus faecium* on Physiochemistry and Nutrition of Fermented Milk

Submitted

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2024

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A thesis submitted in partial fulfillment of the requirements for the

Degree of

Master of Philosophy

In

Microbiology

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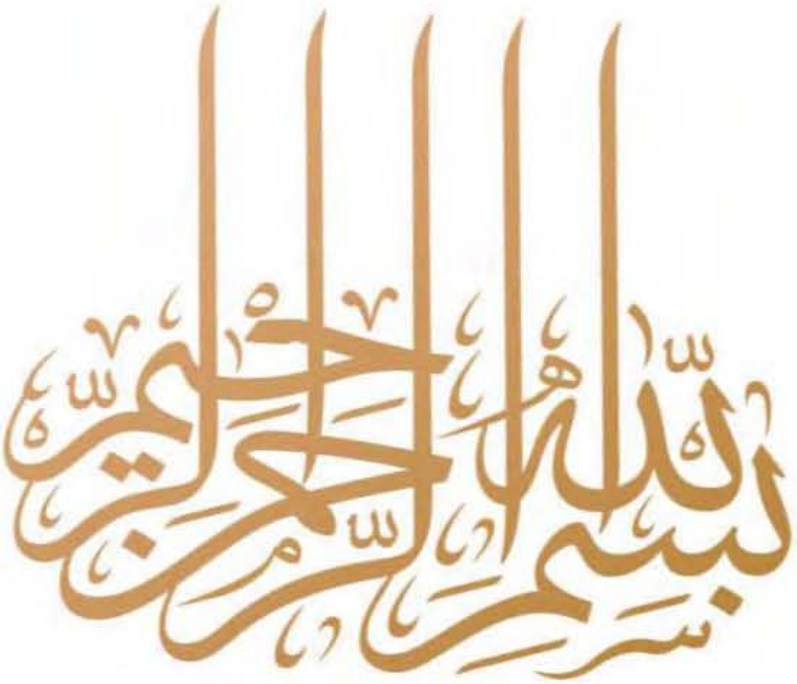
بسم الله الرحمن الرحيم

DEDICATION

With boundless love and gratitude, I dedicate this thesis to my parents and my dear brothers.

Declaration

I hereby declare that the research work presented in this thesis, "Assessment of the Impact of *Enterococcus faecium* on Physiochemistry and Nutrition of Fermented Milk," is entirely original. It represents my original research, and all the data, findings, and conclusions presented herein are solely based on my investigations and analysis.

Nimra Ghalib

Certificate

This thesis submitted by **Nimra Ghalib** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan; as satisfying the thesis requirements for the Degree of Master of Philosophy in Microbiology.

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List of Acronyms

%	Percentage
°C	Degree Centigrade
µl	Micro Liter
CFU	Colony Forming Unit
Cm	Centimeter
DNA	Deoxyribonucleic Acid
DSS	Defined Strain Strater
e.g.	For example
EMB	Eosin Methylene Blue Agar
EPS	Exopolysaccharides
Etc	Etcetra
Fig.	Figure
FTIR	Fourier Transform Infrared Spectroscopy
G+ve	Gram Positive

GIT	Gastrointestinal Tract
GDH	Glutamate Dehydrogenase
G-ve	Gram Negative
H2O2	Hydrogen Peroxide
HACCP	Hazard Analysis Critical Point
hrs	Hours
i.e.	That is
LAB	Lactic Acid Bacteria
LDH	Lactate Dehydrogenase
ml	Milli liter
MRSA	De Man Rogosa and Sharpe Agar
MSS	Mixed Strain Starter
NaCl	Sodium Chloride
NH3	Ammonia
NSLB	Non-starter Lactic Acid Bacteria

NSC	Natural Starter Culture
OGA	Oxytetracycline-Glucose Agar
ph	Potential of Hydrogen
Rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
Spp.	Species
TS	Total Solid
TSA	Tryptic Soya Agar
TTC	Triphenyl Tetrazolium Chloride
VFFA	Volatile Free Fatty Acids
WHO	World Health Organization

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, the Most Merciful.

I begin this thesis acknowledgment with heartfelt gratitude and praise to Allah Almighty, the source of all knowledge and wisdom, for bestowing upon me the strength and guidance to complete this academic journey. With His blessings, I have been able to achieve this milestone in my life.

I would like to express my sincere appreciation to my supervisor, ***Dr. Muhammad Imran****, for his unwavering support and valuable guidance throughout the course of this research. His mentorship and expertise have been invaluable in shaping my ideas and enhancing the quality of this thesis. I am truly grateful for his patience, encouragement, and belief in my abilities.*

Furthermore, I extend my deepest gratitude to my seniors, ***Muhammad Nadeem Khan and Syeda Momna Batool Bukhari****, whose invaluable insights and experiences have been instrumental in shaping my research direction. Their constant encouragement and willingness to share their knowledge have inspired me to strive for excellence in my work.*

*I am humbly grateful to my lab fellows****, Nimra Irshad, Seemab Musharaf Faiz, Alviya Zainab Kazmi and Afzal Ishaq****, for their camaraderie and unwavering support during both the challenging and rewarding times of this journey. Their motivating presence and collaborative spirit have made the research environment enjoyable and stimulating.*

I express my deepest gratitude to my loving parents, whose unwavering support and encouragement have been the driving force behind my achievements. Their sacrifices, belief in my abilities, and constant prayers have been a source of inspiration throughout my academic pursuits. I am forever indebted to them for providing me with the opportunities to pursue my dreams.

I would like to express a special acknowledgment to my brother, ***Saddam Hussain****, whose unwavering support, guidance, and belief in my potential have been instrumental*

in my academic journey. His advice and encouragement have motivated me during challenging times, and his presence has been a constant source of strength.

I am also thankful to my brothers, ***Muhammad Bilal and Muhammad Hammad****, for their support and understanding during the ups and downs of this academic endeavor. Their friendship and companionship have brought joy and positivity to my life.*

Lastly, I would like to acknowledge the support and resources provided by my educational institution, ***Microbiology department Quaid-i-Azam University, Islamabad,*** *without which this research would not have been possible.*

In conclusion, this thesis is a testament to the collective efforts and support of many individuals, each of whom has played a significant role in shaping my academic and personal growth. I am deeply grateful to all those who have contributed to this journey, and I pray that Allah rewards each one of them abundantly. May this work serve as a small contribution to the advancement of knowledge and the betterment of society.

All praise be to Allah, the Lord of all worlds.

Nimra Ghalib

Abstract

Enterococcus faecium is a lactic acid bacterium (LAB) that produces Lactic acid as a metabolic byproduct during the fermentation of milk. *Enterococcus faecium* improves the organoleptic qualities of the finished product and adds in distinctive flavor and odor of fermented milk products. A common fermented dairy product recognized for its probiotic and nutritional benefits is yogurt. This study inquires the impact of *Enterococcus faecium* on physiochemistry, and nutritional characteristics of fermented milk during fermentation. Four distinct combinations of LAB consortia, including *Enterococcus spp*., LAB consortia without *Enterococcus spp*., milk was fermented with *Enterococcus faecium* and *Enterococcus lactis* strains, and milk fermented solely with *Enterococcus faecium* strains, were used to make fermented milk samples. A comparison of fermented milk physiological and nutritional characteristics was made, including its pH, titratable acidity, solid content, syneresis, ash content, protein, and fat content. The liquid content was favorably impacted by the presence of *Enterococcus faecium* strains; it steadily grew until 48 hours, after that point it started to decline. According to an examination of ash levels, fermented milk that had been fermented with *Enterococcus faecium and Enterococcus lactis* strains contained the most ash (1.2 %). In terms of protein content, fermented milk with *Enterococcus lactis* and *Enterococcus faecium* strains had the lowest levels (3.06 %), whereas fermented milk with *Enterococcus faecium* had the greatest levels (4.7 %). When compared to milk fermented with *Enterococcus faecium, Enterococcus lactis*, or the combination of both strains, milk fermented with LAB consortia missing *Enterococcus faecium* exhibited a greater level of fat extraction, according to fat content analyses. Overall, the physiochemical and nutritional characteristics of fermented milk were dramatically impacted by the presence of *Enterococcus faecium*.

Chapter 01
INTRODUCTION

Introduction

Food fermentation is a widely accepted and safe method of preservation. This traditional process has been utilized by humans for thousands of years, even prior to the development of advanced technologies (Widyastuti, Febrisiantosa et al. 2014). The history of fermentation can be traced back to the Aryans' arrival in the subcontinent around 1500 BC, where they introduced the practice of fermenting milk (Mudgal and Prajapati 2017). Additionally, the Egyptians utilized fermentation for beer production in 1300 BC, and by 600 BC, wine, soy sauce, and cheese were also produced using this method. While researchers continue to study the origins of fermentation, and suggest that it predates recorded history and may have been used by early humans (Bourdichon, Casaregola et al. 2012).

Fermented milk products contain beneficial microorganisms like lactic acid bacterial and fungal genera, making them safe for consumption and are conventionally used as starter cultures in food fermentation. LAB bacteria are G+ve cocci/rods, are aero tolerant, non-aerobic, and utilize carbohydrates to produce energy and lactic acid (Widyastuti, Febrisiantosa et al. 2014). They follow two metabolic pathways: homofermentative, that produces two molecules of lactate, and heterofermentative, which produces lactic acid, ethanol, and carbon dioxide (Wang, Wu et al. 2021). The dairy and food industries have used lactic acid bacteria extensively to enhance the technical, nutritional, organoleptic, and shelf-life aspects of fermented foods and drinks (García-Díez, Saraiva et al. 2021). Lactic acid produced by bacteria through lactose fermentation affects milk pH, casein coagulation, environmental acidity, and dairy product rheological properties. This control of pathogenic and spoilage bacteria, positive effect on ripened cheeses' flavor quality, and environmental acidity contribute to overall dairy products (Widyastuti, Febrisiantosa et al. 2014).

Enterococcus is a type of bacteria that can be found in a broad spectrum of surrounding environment including gastrointestinal tract of animals and humans, vegetables, and food (Giraffa 2003). It belongs to the LAB group and has more than 50 species and subspecies within the Enterococcaceae family. Enterococci are gram-positive, non-spore forming, and facultative-anaerobic cocci that can occur as a single bacteria, in pairs, short chains, or groups (Švec, Franz et al. 2014). Depending on the strain, they can be categorized as starters or adjuncts, probiotics, spoilage, and pathogenic

organisms (Yuan, Zhang et al. 2023). Enterococcus species do not have been generally acknowledged as safe (GRAS) status and are allocated to risk group 2 due to the potential risks associated with their virulence and antibiotic resistance (Žugić Petrović, Ilić et al. 2020). Despite their usefulness in producing fermented food, enterococci can also cause several human diseases, including bacteremia, urinary tract infections, and endocarditis, and *Enterococcus faecalis and Enterococcus faecium*, have been identified as responsible for these infections (Vu and Carvalho 2011).

Enterococci are valuable in cheese production due to their proteolytic activity and ability to grow in harsh environments. They produce essential peptides and amino acids, as well as lipolytic and esterolytic enzymes that break down triglycerides into free fatty acids and intermediates. These enzymes are useful in dairy and meat product fermentation, particularly during ripening. Additionally, enterococci are involved in citrate metabolism, producing aromatic compounds in cheese ripening (Yerlikaya, Akpinar et al. 2020).

Enterococci produce a diverse group of antimicrobial peptides, known as enterocins, which are peptide in nature and typically cationic, amphiphilic, and membrane permeabilizing molecules (Qiao, Du et al. 2020). Enterococci, synthesizes enterocins, are effective against various pathogens, making them suitable for antibiotic alternatives or food preservatives. Natural dairy enterococcal isolates produce enterocins with a broad spectrum of activity, making them suitable for protective cultures (Kasimin, Shamsuddin et al. 2022). One strain of Enterococcus, *E. faecium* SF 68, which is produced in Switzerland, it was thoroughly researched and suggested to be effective in preventing antibiotic-associated diarrhea and treating diarrhea in children (Ghazisaeedi, Meens et al. 2022). Enterococci, known as probiotics, improve human digestive health and treat diarrhea caused by antibiotics, viral infections, chemotherapy, and foodborne pathogens (Suvorov and Foods 2019). Enterococci can inhibit pathogenic bacteria growth, have anti-mutagenic and anti-carcinogenic properties, increase intestinal mucosal barrier, stimulate immune system, prevent ulcers, and aid cholesterol assimilation in food and the human intestine (Franz, Huch et al. 2011).

Despite the positive characteristics of Enterococci, there are also several risk factors associated with them. Potential virulence factors can transfer through mobile genetic elements, increasing the risk of pathogenicity to immunocompromised individuals

(Madsen, Skov et al. 2017). This dual nature of enterococci has raised concerns in the food industry, especially in traditionally fermented food products where they may be present in large quantity (Upadhyaya, Ravikumar et al. 2009). Enterococci are becoming increasingly resistant to antibiotics, including vancomycin-resistant enterococci (VRE), posing a significant threat to food safety. These bacteria have both natural and acquired resistance, posing a significant concern for their potential in food due to the horizontal transfer of virulence and resistance genes (Ahmed and Baptiste 2018). Enterococci are broadly used as a starter or secondary cultures in fermented products due to their unique technological, probiotic, and anti-pathogenic properties, attracting scientific interest in genomic potential (Moreno, Sarantinopoulos et al. 2006).

Fermented milk products contain lactose, proteins, and lipids. These are broken down into simple sugars and organic acids, contributing to their unique flavor and aroma. Even though yogurt, the first fermented milk product, was first created only to preserve the milk nutrients, it quickly became clear that it has potential to create a wide variety of goods. Fermented Milk products have varying flavor, texture, consistency, and recently revealed health advantages and features by fermenting them with various bacteria (Hesseltine, Wang et al. 1967).

Fermented Milk products are prepared using a mixture of LAB strains, including Streptococcus, Enterococcus, Lactobacillus, Pediococcus, and Leuconostoc, during fermentation. These strains convert lactose, break down proteins, and break down milk fat, affecting the sensory properties of fermented milk (Sathe, Mandal et al. 2016). Additionally, peptidase activity of LAB and fungal strains produces volatile Sulphur compounds that are important source of free amino acids. Textural properties of fermented milk are related with the production of EPS (Shangpliang, Sharma et al. 2017).

Enterococcus faecium is essential to milk fermentation because of its unique technological features. Being a naturally occurring lactic acid bacterium (LAB) isolate, *E. faecium* has a unique collection of enzymes that are essential for several elements of the cheese-making process. Its acidification action promotes the fermentation of lactose into lactic acid, which modifies the pH of milk and the coagulation of casein, forming the flavor and texture of cheeses (Upadhyaya, Ravikumar et al. 2009). Moreover, the

casein is broken down by its proteolytic enzymes, which enhance the organoleptic properties and texture of cheese. Gelatinase facilitates the hydrolysis of proteins, which may result in the production of bioactive peptides. Furthermore, *E. faecium* demonstrates lipolytic activity, which is necessary for the hydrolysis of lipids in the course of dairy fermentation (Sathe, Mandal et al. 2016). Additionally, *E. faecium* aids in the synthesis of aromatic chemicals that are essential to the development of cheese flavor, including acetoin, diacetyl, and acetaldehyde. Its importance in milk fermentation processes is further highlighted by its capacity to generate these compounds throughout the cheese-ripening process (Suvorov and Foods 2019).

Enterococcus Faecium has capacity to convert lactose into lactic acid, which helps acidify the milk and inhibits the growth of microorganisms that cause spoiling, makes them essential for milk fermentation. Milk products with a distinctive texture and nutritional profile, such as yogurt and cheese, are altered in flavor and texture by this acidity. Additionally, Enterococcus creates flavoring substances that help give fermented milk products their flavor and scent. This study's objective is to determine how *Enterococcus faecium* affects the physiochemistry of fermented milk, which includes several elements including acidity, liquid content, syneresis, etc. The aim of study is to determine the nutritional value of fermented milk by examining how enterococcus contributes to the development of certain nutritional properties, in addition to the physiochemistry of fermented milk.

Objectives

- To produce fermented milk by Lactic Acid Bacteria (LAB) consortium, both with and without the inclusion of *Enterococcus faecium.*
- To evaluate the impact of *Enterococcus faecium* on physicochemical and nutritional attributes of fermented milk.

Chapter 02
LITERATURE REVIEW

Literature Review

One of the earliest methods of preparing food is fermentation. Fermented foods produced by controlled microbial growth and enzymatic conversion of dietary components. In the past, fermentation was used to preserve a wide range of goods, such as meat and fish, dair vegetables, soybeans, other legumes, grains, and fruits. Many variables are involved in the fermentation process, such as the bacteria, nutritional components, and environmental conditions, there are many different types of fermented meals (Dimidi, Cox et al. 2019). The most frequently used fermented milk products are yogurt, butter, cream, and cheese. Yogurt production accounts for approximately 34 billion dollars in the worldwide dairy industry each year (Sieuwerts, De Bok et al. 2008).

2.1 History of Fermentation:

Fermentation of food produces antimicrobial metabolites (including organic acids, ethanol, and bacteriocins) that reduces the risk of pathogenic microorganisms' infection, and it has long been employed as a method of food preservation. Without fermentation, some foods, like olives, are inedible because fermentation removes bitter phenolic compounds and enhances the organoleptic properties (such as taste and texture) (Dimidi, Cox et al. 2019). The process of fermentation seems to have initially developed among the societies on the Indian subcontinent that precede the well-known Indus valley civilization. Egyptians developed dough fermentation, which was used to produce leavened bread, between 4000 and 3500 BCE. However, van Leeuwenhoek and associates' discovery of microbes in 1665 signaled the start of fermentation's scientific defense. Sir John Lister revealed the role of the lone "bacteria" lactis (Lactococcus lactis) in fermented milk around the year 1877 (Ray and Didier 2014). Louis Pasture stated fermentation as "la vie sans l'air," which comes from the Latin term "fevere"(Ray and Didier 2014). He found that food might also experience yeast fermentation. In those days, fermentation was used to preserve food's nutritious worthwhile also storing it (Yadav, Ahmadi et al. 2021).

2.2 Microbial Groups Responsible for Milk Fermentation:

2.2.1 LAB in milk fermentation:

Lactic acid was initially identified by Scheela in 1780 in sour milk, and Lavoisier gave it the name "acid lactique", which is where the word "lactic acid bacteria" comes from

today (LAB) (Benning, Petermeier et al. 2003). When lactic acid bacteria are used to ferment milk, high-quality products with significant organoleptic qualities and a range of bioactive components are produced. Due to their effective utilization of mostly lactose and casein in milk, these starter cultures are utilized in dairy products production (Ziadi, Bergot et al. 2010).

Milk is a recognized natural habitat for Lactic Acid Bacteria. The strains employed for fermentation of milk yield high-quality goods with discernible organoleptic characteristics, bioactive substances, and culinary features. For industrial and commercial fermentation, LAB possess nutritional and health qualities that are highly valuable. The taxa most frequently employed as starting cultures include *Streptococcus, Pediococcus, Leuconostoc, Lactococcus,* and *Lactobacillus* (Widyastuti, Febrisiantosa et al. 2014).

As compared to trade goods, traditional fermented milk products have a combination of wild Lactic Acid Bacteria that have more diverse qualities. According to biotechnology, the wild strains are probably capable of producing probiotics called bacteriocins (Wu, Hao et al. 2020).

2.2.1.1 Growth Characteristics of LAB:

LAB are classified as Gram-+ve cocci, rods, single cells, pairs, or chains of cocci or rods. They don't sporulate, can withstand acid, and don't breathe. LAB are referred to as fastidious microorganisms since they need an external supply of peptides and amino acids. With a few notable exceptions, such as *Lactobacillus ghanensis* and *Lactobacillus agilis* LAB are mostly non-motile. They are not only nonaerobic but also aerotolerant. Temperatures between 37°C and 42°C are ideal for growth. LAB ferment sugar to produce lactic acid and energy (Adamberg, Kask et al. 2003). The metabolic pathways of LAB include homofermentative in which two molecules of lactate are generated e.g., Streptococcus, Lactococcus, Pediococcus and Enterococcus. While lactic acid, ethanol and carbon dioxide are formed in heterofermentation, e.g., Leuconostoc and Lactobacilli. Aside from their capacity to generate lactic acid, LAB play a role in enhancing the taste, consistency, and nutritional content of the food. (Gänzle 2015).

2.2.1.2 Diversity of LAB:

Lactic Acid Bacteria natural habitats are nutrient-rich settings because of their restricted metabolic skills and have excessive need of nitrogen and carbon sources. Therefore, LAB are frequently found in foods such as milk, meat, vegetables, drinks, dirt, and sewage. They are found in the intestinal, respiratory, and genital tract microbiota of higher animals such as humans. LAB are responsible for the rapid acidification of fermented milk through the production of organic acids, such as lactic acid. The atmosphere is made more acidic by LAB during this fermentation, which is thought to be a crucial property of dairy products, notably in the creation of cheese (Wu, Hao et al. 2020).

2.2.1.3 Taxonomy of LAB:

Lactic acid bacteria are categorized into distinct genera primarily based on criteria such as their morphological characteristics, glucose fermentation methods, ability to endure acidic or alkaline conditions, lactic acid production mechanisms, proficiency in thriving in elevated salt settings, and adaptability to diverse temperature ranges. Lactobacilli may live at a neutral pH and are able to survive at pH 4.0 in diets that include fermentable carbohydrates (Axelsson and Dekker- 2004). The bacterial biological group that produces lactic acid was separated by Orla-Jensen. Orla Jensen's study in 1919 illustrates the division of LAB into four genera: Streptococcus, Lactobacillus, Tetracoccus, and Betacoccus. He further subdivided Lactobacilli into the Thermobacterium, β-bacterium, and Streptobacterium subgenera. Lactobacilli that are heterofermentative are known as β-bacterium (Savadogo, Ouattara et al. 2006).

2.2.1.4 Classification of LAB at Genus Level:

To differentiate among the different cocci, growth patterns were assessed at different temperatures. For instance, Enterococci display growth at both 10°C and 45°C, while Streptococci only thrive at 45°C. Lactococci and Vagococci exhibit growth at 10°C but not at 45°C. Currently, salt tolerance (6.5 percent NaCl) is utilized in order to discriminate between Enterococci, Lactococci and Vagococci, as well as Streptococci, however in some cases, Streptococci may resist this salt concentration. Tetragenococcus genus can withstand severe alkali and acid conditions and has an 18%

salt tolerance. But the species Enterococci persisted in both high and low pH environments (Axelsson and Dekker- 2004).

2.2.1.4.1 Genus Streptococcus:

Streptococcus as a genus was originally described by Rosen Bach in 1884. Members of these genera generate lactic acid bacteria with L isomers and are catalase negative, G+ve, homofermentative, cocci and can form chains or pairs (Hardie and Whiley 1995). They can be found all over nature, including in plants, raw milk, dairy products, in human and animal mouths, and intestines. They are thermoduric and are mostly utilized to pasteurize milk, which yields vitamins B6 and B12 (Wood and Holzapfel 1992).

2.2.1.4.2 Genus Lactococcus/Lactococci:

The genera Streptococcus and Lactobacillus species were reclassified in 1985 using 16S rRNA sequencing and chemotaxonomic research, the members of this genus were given the name Lactococci. They are non-spore-forming, non-motile, catalase- and oxidase-negative, Gram-positive organisms. They are mostly homofermentative and produce L lactate (Schleifer, Kraus et al. 1985). This group consists of seven different species, including the four subspecies of *Lactococcus lactis*, namely the *L. lactic subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae, and L. lactis subsp*. *Tructae.* Lactococci are mostly found in dairy environment and in raw milk. *L. lactis subsp. cremoris* are frequently used as a starter cultures in different types of cheese and in other dairy products as a single or multiple strain starters (Teuber 1995).

2.2.1.4.3 Genus Enterococcus:

An important genus of lactic acid bacteria called Enterococcus may be found in different environments, such as fermented milk products, plants, in the intestines of both people and animals. These facultative anaerobes may ferment carbohydrates to produce lactic acid and are G+ve, catalase-negative, and oxidase-negative (Švec, Franz et al. 2014). They contain the Lancefield Group D antigen in their cell walls, group D streptococci were formerly classified as such; however, over 41 species now go by the name Enterococcus. The *Enterococcus faecalis and Enterococcus faecium* groups are a subgroup of the Enterococcus species (Zhong, Zhang et al. 2017).

2.2.1.4.4 Genus Leuconostoc:

Members of this genus are facultative anaerobes that are G+ve, catalase and oxidase negative, and non-spore producing. They might exist in pairs or in chains. Their optimal growth temperature ranges from 4 to 10°C, and growth occurs at 30°C but not at 45°C. In this genus, there are three subspecies and 22 recognized species (Holzapfel, Wood et al. 2014). This genus may be distinguished and isolated from other genera due to its resistance to the antibiotic Vancomycin. They are generally regarded as safe. Typically, they appear as nonstarter lactic acid bacteria in raw milk or traditionally made cheeses. They are hetero-fermenters, and they also generate D-lactate, CO2, ethanol, and acetate (Garvie 1960).

2.2.1.4.5 Genus Pediococcus:

Members of this genus are facultative anaerobes that are spherical, Gram-positive, do not produce spores, and are catalase and oxidase negative. The fifteen species that make up the genus have varying patterns of glucose consumption that result in D and L-lactate. Members of this species can endure severe temperatures, pH levels, and salt concentrations. They are separated from fermented milks, plants, bear, etc. The most prevalent species in fermented milks are *P. acidilactici and P. Pentocaceus* (Holzapfel, Wood et al. 2014).

2.2.1.5 Grouping of LAB based on Growth Temperature:

Depending on their optimum growth, the LAB in the dairy sector may be divided into two groups. Thermophilic bacteria thrive best at temperatures between 30°C and 45°C, whereas mesophilic bacteria prefer temperatures between 20°C and 30°C degrees (Mokoena 2017).

2.2.2 Fungal Microbial Colonies (Yeast and Mold):

Yeast are unicellular, eukaryotic, aerobic creatures that live in different kind of environments, such as soil, water, plants, fruits, animal skin, and digestive tracts. They can use lactose and galactose as well as metabolize lactic, acetic, and formic acids, among other acids. They are present in fermented foods with high acidity because they can tolerate harsh environmental conditions, such as high salts, low pH, and low temperatures (Maicas 2020). Fungal populations were detected in dairy products as a result of their introduction from polluted surroundings, equipment, handling practices,

and the processing of the goods. Kefir, koumiss, and other European dairy products contain them in conjunction with LAB. They help to taste, fragrance, and texture the fermented milk product by metabolizing acids, lipids, and proteins, primarily *Kluyveromyces marxianus* and *Debaryomyces hansenii* species of yeast. Dairy products are more commonly contaminated with, *C. Lambica, C. kefir*, *S. cerevisiae*, *S. exiguous, Torula kefir* and *S. Delbrueckii*. Due to its rapid growth and gas production, yeast causes spoiling and unpleasant odors in fermented dairy products. A yeast-like fungus called *Geotrichum candidum* is present in dairy products (Boulton and Quain 2008).

2.3 Genus Enterococcus:

Lactic acid bacteria (LAB), which include the enterococci, are crucial for understanding environmental, dietary, and clinical microbiology. Enterococci are used as probiotics in food and feed as well as in food fermentation processes, improving the sensory quality of foods and other applications. The precise identification of the strains and their unique features are crucial for their application. For the safety assessment, the absence of certain antibiotic resistances is important. Thus, it is crucial to consider enterococci's taxonomy, ecology, and antibiotic resistance in order to choose and characterise the enterococcal strains that are employed in commercial settings as well as to comprehend why these organisms are so common (Franz, Huch et al. 2011). They can be starters, adjuncts, probiotics, spoilage, or harmful organisms depending on the strain. Enterococci in terms of evolutionary relationship, are related to Firmicutes' low G+C content branch, like many other LAB-genera. They contribute to the development of cheeses' organoleptic qualities in a positive way (particularly those that have their origins in Mediterranean nations) (Ben Braiek and Smaoui 2019).

A variety of enterococci also create a heterogeneous collection of bacteriocins, or antimicrobial peptides known as enterocins, which are produced through ribosomal synthesis. Typically, enterocins are Class II bacteriocins. Peptide-based enterocins are often cationic, amphiphilic, and membrane-permeabilizing compounds. Bacteriocins operate as useful protective cultures by inhibiting harmful microorganisms (Silva, Silva et al. 2018).

Among other undesirable effects, food deterioration and the generation of biogenic amines may be present. Some enterococcal strains, however, are responsible for common opportunistic infections that spread illness, especially in nosocomial settings.

Due to enterococci's prolonged drug resistance and greater prevalence of infections in young, elderly, and immunocompromised people, they are being regarded as emerging pathogens. *E. faecium or E. faecalis* strains are responsible for most of illnesses. However, several strains of *E. hirae, E. mundtii, and E. gallinarum* have also been linked to endophthalmitis and native valve endocarditis in humans (Perin, Belviso et al. 2017).

Additionally, a large number of enterococcal isolates from various origins have potential virulence factors, which is transmissible via mobile genetic elements. The food industry is troubled by the contrasting characteristics of enterococci. They constitute the initial group among the ESKAPE bacteria (*Enterococci spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.)* recognized by the WHO for their potential to endanger public health, given their emerging association with hospital-acquired infections and antibiotic resistance (Tigabu and Getaneh 2021).

2.3.1 History of Enterococcus:

Thiercelin first identified enterococci in 1899, and he and Jouhaud proposed the name "Enterococcus" for these Gram-positive diplococci of intestinal origin in 1903. However, because of their capacity to form chains, Andrewes and Horder reclassified Thiercelin's enterococci as *Streptococcus faecalis* in 1906. In 1933, Lancefield created a technique for serologically recognizing the group D antigen in enterococci of fecal origin. This division of the Streptococcus genus into pyogenic, viridans, lactis, and "enterococcus" groups was consistent with Sherman's grouping scheme from 1937. *Streptococcus faecalis, Streptococcus faecium, Streptococcus bovis, and Streptococcus equinus*, sometimes known as "enterococcal" or group D strains, were included in the "enterococcus" category (Švec, Franz et al. 2014).

Streptococcus faecalis and Streptococcus. faecium were moved from Streptococcus to Enterococcus in 1984, reviving the genus Enterococcus in the process. Potentially harmful bacteria obtained from endocarditis patients were referred to as "*Streptococcus faecalis*" by Andrewes and Horder in 1906. Later, oral and fecal streptococci were used in place of the names "viridans" and "enterococci," respectively (Facklam and Collins 1989) .

2.3.2 Growth Characteristics of Enterococci:

Unlike other G+ve, catalase-negative cocci, Enterococcus species have unique growth properties. They grow well in a broad variety of temperatures, flourishing between 10°C and 45°C. They exhibit remarkable heat tolerance by withstanding at 62.8°C for 30 minutes. These bacteria show resistance to high salt concentrations, surviving up to 6.5 percent NaCl and 40% bile. The optimum growth for Enterococcus species range in the pH 4.0 to 9.6 (Byappanahalli, Nevers et al. 2012).

In 1984 by Schleifer and Kilpper-Balz discovered that *Streptococcus faecium and Streptococcus faecalis* are unique from other streptococci by employing 16S rRNA sequencing and DNA-DNA hybridization. They suggested moving these species to the Enterococcus genus (Facklam and Collins 1989). 69 enterococcal species have been added to the genus as a result of further research (Kireeva and Dmitriev 2023).

Selective or elective mediums are key in this process of distinguishing distinct Enterococcus species, and their growth characteristics. For instance, triphenyl tetrazolium-chloride (TTC) is strongly reduced by *Enterococcus faecalis*, causing the growth of intensely red colonies. *Enterococcus. faecium*, in contrast, either does not diminish TTC or reduces it very weakly, resulting in the development of light pink colonies. There have been several different media mentioned and used for regular testing across various sources such as Aesculin-bile-azide medium (ABA), Kanamycin aesculin azide agar (KAA), Citrate azide tween carbonate agar (CATC) etc. The CATC and ABA media treated with vancomycin have proved particularly helpful in identifying vancomycin-resistant enterococci (VRE) (Zhang, Mills et al. 2009).

Molecular-based approaches have become crucial for accurate and quick identification, particularly in sources with complex microbial flora. The ability to distinguish between several Enterococcus species has been successfully achieved using 16S and 23S rRNA targeted probes. Additional methods have been employed to differentiate between strains of enterococci within the same species. These techniques encompass protein fingerprinting, randomly amplified polymorphic DNA (RAPD-PCR), pulsed-field gel electrophoresis (PFGE), contour-clamped homogeneous electric field electrophoresis (CHEF), and analysis involving restriction enzymes (Shalaby, Eshra et al. 2016).

2.3.3 Taxonomy of Enterococci:

The traditional taxonomy of enterococci lacks definitive phenotypic characteristics to differentiate them from other G+ve, catalase-negative, coccus-shaped bacteria. However, 16S rRNA sequence contrasts and dendrogram construction have allowed for the division of Enterococcus, Lactococcus and Streptococcus into different subgroups. Over time, numerous species have been described within the genus Enterococcus, with 69 currently validly published. These newly identified species demonstrate considerable alterations in physiological and biochemical attributes compared to conventional enterococci (Kireeva and Dmitriev 2023).

Enterococcal species are often allocated to distinct species groups based on 16S rRNA sequence data. The most significant and frequent are *E. faecium and E. faecalis*, while others include *E. avium, casseliflavus, E. durans, E. dispar, E. hirae, E. gallinarum, , and E. raffinosus*. Non-faecium non-faecalis enterococci are highly considered as causes of bloodstream and endovascular infections in humans (Devriese, Baele et al. 2006).

2.3.4 Ecological Aspects in the Gastro-intestinal Tract and in Food:

The native microflora of humans is significantly influenced by enterococci and group D streptococci. *E. faecium and E. faecalis* are the most common species in the human gut. *E. faecium* is frequently found in producing animals including chickens, cattle, and pigs, along with other species like *E. faecalis, E. gallinarum, E. cecorum,* and *E. avium. E. mundtii* and *E. casseliflavus*, on the other hand, are frequently connected to plants and are distinguished by their coloured colonies. This suggests that the ecological niches inhabited by species Enterococcus are remarkably diverse (Cattoir 2022).

Enterococci and other group D streptococci are common in the animal digestive systems; thus they can be found in a variety of foods, especially those that come from animals. *E. faecium and E. faecalis* have frequently been used as a sign of fecal contamination in food as they last in the environment and are resistant to pH shifts and high salt concentrations (Fisher and Phillips 2009). Enterococci are now understood to be both typical components of the food microflora and signs of inadequate cleanliness.

According to recent investigations, *E. faecalis* rather than *E. faecium* is more commonly found in food and excrement of animal origin (Klein 2003).

2.3.5 Enterococci as Contaminants or Indigenous Starter:

The presence of enterococci in milk has historically been linked to fecal contamination, however they are able to proliferate in a variety of conditions. Research has demonstrated that fecal contamination is not necessarily a direct cause of *E. faecalis* contamination in food items (Boehm and Sassoubre 2014). Enterococci are naturally found in both raw and pasteurized milk due to their adaptability to many environments, including chilling and pasteurization. Several species of Enterococci , primarily *E. faecalis and E. faecium*, can be detected in dairy products (Fisher and Phillips 2009). As food safety indicators, the European Union has determined maximum values for coliforms and *E. coli*, but no precise acceptable thresholds for enterococci have been established (Bhardwaj, Malik et al. 2008). In the commercial food processing industry, enterococci are only of limited use as hygiene indicators. Eenterococci play a function as starting cultures in the manufacturing of various milk products and are a natural component of the microflora of raw milk and whey (Boehm and Sassoubre 2014).

2.3.6 Enterococci as Probiotic:

Enterococci are essential for the development of probiotic and fermented meals. By reducing milk fat and generating taste components, they have increased proteolytic activity and aid in cheese ripening. Due to these positive outcomes, dairy products now contain starting cultures of enterococcal strains (Faccia, Natrella et al. 2022). The bacteriocins known as enterocins, which are likewise produced by enterococci and have antibacterial capabilities against foodborne pathogens, can also be employed as bio preservatives. They have inhibitory effect against a variety of bacteria, such as Listeria and Clostridium species, and are therefore useful in preventing food from deteriorating and being contaminated (Garmasheva and Oleschenko 2023).

The probiotic properties of enterococci go beyond their function in food production. They help to maintain healthy gut flora, lessen gastrointestinal diseases, ease lactose intolerance, reduce blood cholesterol levels, have anticancer properties, boost immunological function, and enhance nutritional value. Various Enterococcus species, including *E. faecalis, E. faecium, and E. durans*, are crucial components of the human

digestive system (Faccia, Natrella et al. 2022). A probiotic enterococcal strain known as *E. faecium* SF 68 has been shown to be useful in treating diarrhea in children and avoiding diarrhea brought on by antibiotic treatment. Additionally, it has been demonstrated to boost animal immune responses and have immuno-stimulating effects against Giardia intestinalis (Ghazisaeedi, Meens et al. 2022). Although enterococci have proven probiotic advantages, their use in the food business is controversial because to worries about potential health dangers.

Overall, enterococci play a key role in the food business due to their participation in fermented foods, synthesis of enterocins as bio preservatives, and probiotic qualities; nevertheless, careful evaluation and testing are necessary before their practical application.

2.3.7 Undesirable Activities of Enterococci:

Even though enterococci have certain favorable traits, there are also some risks factors associated with enterococci. Enterococci can cause problems in the setting of traditionally fermented food items, especially if they are initially present in large numbers. The synthesis of biogenic amines by enterococci in fermented dairy products is one of their undesirable behaviors, which have been connected to cases of food poisoning. Tyramine is the most important amine generated by enterococci in dairy products, and its immense development can result in considerable quantities of biogenic amine production in milk and milk products (Perin, Belviso et al. 2017).

In the making of cheese, enterococci's degradation of casein affects the cheese taste. While certain peptides help create tastes that are palatable, others can produce bitter-tasting peptides that give cheese an unpleasant flavor (Faccia, Natrella et al. 2022). The synthesis of fatty acids by enterococci's lipolytic activity in cheese also aids in taste development. However, these fatty acids' susceptibility to oxidation can result in the development of substances known as oxidative rancidity that result in taste problems (Habibi, Shahab-Lavasani et al. 2022).

Additionally, a few nosocomial illnesses, including endocarditis, bacteremia, urinary tract infections, and newborn infections, have been linked to enterococci. The prevalence of enterococcal infections has been rising, with *E. faecalis and E. faecium* strains accounting for the majority of infections enterococci have been identified in

humans as opportunistic infections (Bhardwaj, Malik et al. 2008). The incidence of antibiotic-resistant enterococci, notably vancomycin-resistant enterococci (VRE), has increased, and several virulence factors in enterococci have been found (Ben Braiek and Smaoui 2019).

Even though enterococci have benefits like helping cheese taste better and having probiotic features, it's important to be aware of the concerns too, such as the possibility of biogenic amine production and their status as opportunistic pathogens with rising antibiotic resistance.

2.3.8 Virulence of Enterococci:

The ability of enterococci to induce infections by colonizing host tissues, eluding host defensive mechanisms, and causing pathological alterations is due to their variety of virulence features including the extracellular surface protein, adhesin-like antigens that are present in *E. faecalis and E. faecium*, and the aggregation substance, which promotes adhesion to host tissues. The cytolytic toxins, gelatinase, hyaluronidase, and sex pheromones produced by enterococci also increase their pathogenicity. cytolysins can be lyse Wide-ranging cell types, whereas gelatinase operates on collagenous tissues to facilitate invasion. In addition to promoting the acquisition of plasmid DNA, sex pheromones also cause inflammation and the creation of superoxide, which attracts neutrophils through chemoattraction (Li, Yang et al. 2022).

The rise of vancomycin-resistant enterococci (VRE) in hospitals has caused serious infections that are challenging to treat with standard medicines, making vancomycin resistance a particular concern. The vanA type is the most significant gene cluster causing glycopeptide resistance in enterococci, and it is found in *E. faecium*. Enterococci's pathogenic potential are brought up by the horizontal gene transfer of virulence genes and antibiotic resistance components, particularly when utilized as starting cultures in food manufacturing (Conwell, Dooley et al. 2022).

Even though enterococci are common in the food chain and certain strains have virulence and antibiotic resistance characteristics, they are not often regarded as foodborne pathogens. Due to enterococci's strong nature, extensive distribution, and environmental stability, the food sector has difficulties in controlling and ensuring the

safety of goods containing them. Maintaining food safety requirements requires careful handling and monitoring of enterococci in food items (Franz, Huch et al. 2011).

2.3.9 Safety Assessment of Enterococci:

Enterococcal viability for use in probiotics and food production have grown as a result of their increasing prevalence. Research shows that enterococci isolated from dietary sources also exhibit virulence features. Therefore, it is crucial to assess these strains' safety before thinking about using them in food or probiotic products. Recent research has been done to evaluate the safety of enterococci from diverse sources. Safe enterococci strains for food and probiotics should ideally not have any virulence characteristics or the capacity to acquire genes for antibiotic resistance. Furthermore, it is best to stay away from strains that cause biogenic amines in meals. Opsonophagocytic killing, an in vitro test that gauges the host's defense against enterococci, is thought to be a crucial indicator of strain safety. These tests allow for the selection of enterococci that are appropriate for use in the food sector (Bhardwaj, Malik et al. 2008).

2.4 Technological Characteristics of *Enterococci faecium*:

Enterococcus faecium strains are natural LAB isolates that possess a unique set of catabolic enzymes for citrate metabolism, lipolysis, and proteolysis that are specifically adapted to the cheese-making process. *Enterococcus faecium* is a key player in the world of traditional fermented foods, particularly artisanal cheeses, because of its technological characteristics. These include a range of processes like acidification, lipolysis, and proteolysis as well as effective citrate utilization and the synthesis of aromatic volatile chemicals, which are responsible for the unique sensory qualities of various cheese kinds (Ben Braiek et al., 2019). Some *Enterococcus faecium* strains have been shown to have favorable metabolic qualities, which has led to recommendations that these strains be added to current starter cultures to produce a variety of internationally known cheeses, including Bitto, water-buffalo Mozzarella, feta, Venaco, Cebreiro, cheddar, Koopeh, Tulum, and Lighvan (De, Vuyst et al. 2006).

2.4.1 Acidification Activity:

The main feature of lactic acid bacteria (LAB) is their ability to ferment lactose into lactic acid, which has several effects on milk pH and casein coagulation. Other effects include: (a) increased environmental acidity, which inhibits the growth of pathogenic

and spoilage bacteria; (b) decreased milk pH and casein coagulation; (c) beneficial effects of casein coagulation on dairy product rheological properties; and (d) acidification, which determines the final flavor quality of ripened cheeses (G Giraffa et al. 2003). A member of the LAB group, *Enterococcus faecium*, typically has a low to medium capacity to acidify milk. with milk's pH dropping to less than 5.0 following a 24-hour incubation period at 30–37 °C. However, because of other advantageous technological features, its potential as an auxiliary culture when paired with high-capacity acidifiers is notable (Abeijon et al. 2006).

2.4.2 Proteolytic Activity:

Enterococci's proteolytic activity is intimately associated with their contribution to the manufacture of cheese. Through the breakdown of casein, they play a crucial role in determining the texture and organoleptic qualities of cheese. Because enterococci may grow in a variety of environments, such as those with high salt content, low pH, and fluctuating temperatures, they are more common in cheeses that take a long time to mature. Their ability to produce proteolytic enzymes allows them to flourish by supplying necessary amino acids and peptides (Fuka et al. 2017).

The presence of gelatinase, an external zinc metalloprotease that can hydrolyze a variety of proteins including gelatin, elastin, collagen, and hemoglobin, is a unique feature of the proteolytic system in enterococci. *Enterococcus faecium* strains containing gelatinase have the potential to affect human health by aiding in the breakdown of casein and the production of bioactive peptides (Medeiros et al. 2017). There are strain-dependent differences, certain strains degrade casein significantly, whereas other strains only slightly activate the protease. *Enterococcus faecalis* has been found to contain gelatinase more often than *Enterococcus faecium*, which has led to observations suggesting that *E. faecalis* may have greater proteolytic activity. *E. faecium* strains has better caseinolytic activity which emphasizes their importance in cheese production (Vidojević et al. 2014).

2.4.3 Lipolytic Activity:

Like many other bacterial species, *Enterococcus faecium* is lipolytic and esterolytic, generating lipases and esterases among other enzymes. These enzymes are essential for hydrolyzing triglycerides, which allows them to be broken down into glycerol, free fatty acids, and intermediates such as mono- and diglycerides. *Enterococcus faecium's*

lipolytic and esterolytic systems are highly helpful for food fermentation, especially when it comes to ripening dairy and meat products (Tzanetaki, E., et al. 2001).

The provided data exhibits variability due to the strain-dependent nature of lipolytic activity in enterococci. According to Morandi et al., examined strains of *E. faecalis* and *E. durans* showed lower lipolytic activity than strains of *E. faecium* from dairy products in northwest Italy. Conversely, a subset of enterococci isolated from artisanal raw-milk cheese from Istrenia was found to possess lipolytic ability, with *Enterococcus faecalis* exhibiting more lipolytic activity than *E. faecium* and *E. durans.* Although Enterococcus species and strains within the same species can differ, strains that exhibit strong lipolytic properties are worth looking into further as they could be useful for commercial auxiliary cultures in the fermentation of food products (Morandi, S., et al. 2006).

2.4.4 Production of Aromatic Compounds:

LAB, most of which are enterococci, produce the majority of aromatic compounds during the citrate metabolism that occurs during cheese ripening. The unique and strong flavor of finished raw-milk cheese, as opposed to cheeses made with pasteurized milk, is caused by citrate, which can be broken down over a variety of metabolic pathways during production and ripening. These aromatic compounds are primarily acetate, acetaldehyde, acetoin, and diacetyl. The most important component of dairy products' buttery, "buttermilk" flavor and scent is diacetyl, a volatile substance that is produced as a byproduct of the process that turns citrate into pyruvate (Kilcawley, K. N. 2017).

Certain aromatic compounds develop differently in cheese depending on a variety of factors that influence citrate metabolism, such as the kind of LAB, cell density, culture conditions, environment pH, and lactate concentration. Enterococci are better diacetyl-acetoin producers than other LAB. Enterococci produce a range of volatile compounds, especially during the ripening phase, which contribute to the fragrance development of the cheese (Pretorius, N., et al. 2019). Citric acid cannot be metabolized by all LABs. There were differences between Enterococcus species and strains in terms of how they synthesized diacetyl and acetoin. The strains *E. faecalis* N8W4 and N0W5 produced the least amount of diacetyl, followed by *E. faecium* C1W5. Some authors did, however, assert that some *Enterococcus durans* strains produced more diacetyl than other Enterococcus strains (Ribeiro, S. C., et al. 2014).

2.5 Therapeutic Potential of *Enterococcus faecium*:

Enterococci have emerged as promising probiotic agents with applications spanning pharmaceuticals, human health, veterinary care, and the food industry. Despite safety concerns, they have proven to be beneficial in reducing symptoms in children with atopic dermatitis and in lowering the frequency of upper respiratory tract infections and acute gastroenteritis in healthy adults. It's interesting to note that formulations containing *B. animalis subsp. lactis* and *E. faecium* significantly reduced gastrointestinal and respiratory ailments while also increasing salivary IgA levels in children undergoing treatment (Di Pierro, F., et al. 2018). Nonetheless, there are still concerns regarding *Enterococcus faecium's* dual nature as possible probiotics and opportunistic infections because of its virulence traits and genes for antibiotic resistance. Enterococci strains are used extensively in the food and probiotic industries; however, they have not yet received the generally recognized as safe (GRAS) certification. Despite the observation of medication resistance in Enterococci strains identified in cheeses, no diseases have been linked to probiotic Enterococci such as *E. faecium* and *E. faecalis* (Russo, L., et al. 2020).

2.5.1. Antibiotic-associated Diarrhea (AAD) and Acute Diarrhea:

As a probiotic, *Enterococcus faecium* shows potential in treating acute diarrhea as well as antibiotic-associated diarrhea (AAD). *E. faecium*, when taken orally, can reduce the occurrence of diarrhea as It has capacity to rectify the upset microbial equilibrium in the gut resulting from antibiotic use, hence it reduces the likelihood and intensity of AAD (Wunderlich, P. F., et al. 1989). Additionally, by modifying gut flora and enhancing intestinal barrier integrity, *E. faecium* has shown promise in reducing the symptoms of acute diarrhea. *E. faecium* has ability to generate antimicrobial compounds, involves in competitive suppression of harmful bacteria, and strengthening of the mucosal immune response. These properties depend on variables like strain properties, dosage, and unique patient features. The effectiveness and safety of different *E. faecium* strains in treating certain ailments may differ (D'souza, A. L., et al. 2002). Meta-analysis on *E. faecium* shows the effectiveness of probiotic therapy in avoiding diarrhea. The "Biothree" probiotic blend, which includes strains of *Bacillus mesentericus, Clostridium butyricum*, and *E. faecium*, showed promise in lessening the severity and length of diarrhea in kids (Zeyner, A., et al. 2006).

2.5.2. Irritable Bowel Syndrome (IBS):

E. faecium has potential as a medicinal agent for controlling irritable bowel syndrome (IBS). Dysbiosis, or an imbalance in the gut microbiota, is thought to play a role in IBS, a chronic gastrointestinal condition marked by changed bowel patterns and discomfort in the abdomen. Probiotic therapy changed the gut microbiota and as it reduces the symptom (Fan, Y. J., et al. 2006). As a probiotic, *E. faecium* works by balancing the microbiota, modifying the immune system in the gut, and generating healthy metabolites like short-chain fatty acids (SCFAs). *E. faecium* improve the function of the intestinal barrier by lowering permeability and obstructing the transfer of toxic chemicals. Gut motility and visceral sensitivity are important components in the pathogenesis of IBS. *E. faecium* may affect these parameters via affecting neurotransmitter levels along the gut-brain axis (Gade, J., et al. 1989). Individual differences in responsiveness to treatment emphasize the significance of tailored strategies for treating IBS with supplementation of *E. faecium.* Freeze-dried *E. faecium* containing probiotic "Paraghurt" is an effective treatment for IBS. The effectiveness of auto-probiotic Enterococcus strains in treating IBS is that these strains reduce the symptoms of IBS and bring the dysbiotic microbiomes back to levels that were similar to those of healthy individuals. Additionally, *E. faecium* along with its metabolic butyrate control cytokines and maintains intestinal epithelial integrity, that demonstrate strong anti-inflammatory effects, acting as a preventative treatment for inflammatory bowel disorders (IBDs). This highlights the therapeutic potential of enterococcus-based therapies for the treatment of gastrointestinal disorders such as IBS (Enck, P., et al. 2008).

2.5.3. Cholesterol Reduction/Assimilation:

E. faecium have therapeutic benefits in the treatment of hypercholesterolemia and associated cardiovascular disorders. *E. faecium* helps to regulate the host's homeostasis and cholesterol metabolism through these enzymatic activities. Several strains of *E. faecium* can decrease cholesterol through different ways. It lowers intestinal absorption of cholesterol by converting it enzymatically into a non-absorbable byproduct called coprostanol (Agerbaek, M., et al. 1995). *E. faecium* generates two cholesterol-reducing enzymes i.e Bile salt hydrolase (BSH) and cholesterol oxidase (ChoX), that are responsible for lowering cholesterol levels, also referred to as cholesterol assimilation. Bile salt hydrolysis is catalyzed by BSH, which results in the deconjugation of bile

acids and subsequently impairs the synthesis and absorption of cholesterol micelles. Furthermore, ChoX mediates the conversion of cholesterol to cholest-4-en-3-one, which serves as a precursor to the synthesis of coprostanol (Hlivak, P., et al. 2005). *E. faecium* demonstrated comprehensive cholesterol-lowering effects, particularly on LDL cholesterol, without affecting HDL cholesterol or triglyceride levels. Isolated strains of *E. faecium* from traditional Italian cheeses exhibited cholesterol-lowering effects in vitro, suggesting their potential as probiotic candidates (Albano, C., et al. 2018). Additionally, strains of *E. faecium* isolated from rhizospheric soils and *E. lactis* from goat milk, both possessing the bile salt hydrolase (bsh) gene, exhibited cholesterol-lowering properties in vitro, suggesting their potential as probiotic agents (Larsen, L., et al. 2000).

2.5.4 Antioxidant, Anti-inflammatory and Anti-cancerous Properties of *E. faecium*:

The potential therapeutic applications of *E. faecium* and related strains are highlighted by their many positive effects, which include inflammation, oxidative stress, and cancer. Their ability to ward against oxidative damage, which is linked to a host of chronic illnesses, is facilitated by their antioxidant qualities. By regulating immune responses and lowering the synthesis of pro-inflammatory cytokines, *E. faecium* exhibits anti-inflammatory properties that mitigate inflammation linked to diseases like inflammatory bowel disease. Certain strains of *E. faecium* have been shown to exhibit anticancer capabilities by inhibiting the proliferation of cancer cells and inducing apoptosis, suggesting that the bacterium may possibly hold promise in the treatment of cancer. Emphasize the therapeutic potential of related strains of *E. faecium* in treating a variety of disorders, opening the door for more studies and clinical uses (Divyashri, G., et al. 2015).

2.5.4.1 Antioxidant Properties of *E. faecium*:

E. faecium is known to exhibit antioxidant function as it can scavenge reactive oxygen species (ROS) and prevent oxidative stress from damaging biomolecules including lipids, proteins, and DNA. Lactic acid is the primary metabolic product produced by *E. faecium*, with butyric acid coming in second. Significant antioxidant activity against ascorbate auto-oxidation, 1,1-diphenyl-2-picryl-hydrazyl, oxygen radical absorbance, and reducing power was demonstrated by *E. faecium* Hydroperoxides can be reduced

by *E. faecium* to hydroxy-octadecadienoic acid. These antioxidant qualities support the defense of cells against oxidative damage and may lessen the chance of developing chronic illnesses like cancer and cardiovascular disorders that are linked to oxidative stress (Braiek, O., et al. 2019).

2.5.4.2 Anti-inflammatory Properties of *E. faecium*:

In LPS-stimulated macrophage cell lines, E. faecium has anti-inflammatory activity, suggesting a possible therapeutic function in ailments such as inflammatory bowel disorders (IBDs). Inflammatory diseases like dermatitis, arthritis, and inflammatory bowel illness are all lessened by *E. faecium*. By regulating immune responses and lowering the generation of pro-inflammatory cytokines and mediators, *E. faecium* acts as an anti-inflammatory agent. *E. faecium* has a strong anti-inflammatory impact as it upregulates IL-10 levels and adversely influence TNF-α production in LPS-stimulated macrophage cell lines, *E. faecium* stimulates the release of anti-inflammatory cytokines and prevents the activation of inflammatory pathways. Furthermore, *E. faecium* improves the intestinal barrier's integrity, which lessens the translocation of pathogens and inflammatory chemicals across the gut mucosa (Baroja, M., et al. 2007).

2.5.4.3 Anticancer Properties of *E. faecium*:

E. faecium exhibits anticancer characteristics by preventing the growth of cancer cells, causing apoptosis, or programmed cell death, and altering the tumor microenvironment. Lactic acid and butyric acid are the two main acids produced by *E. faecium*. The lactic and butyric acid generated could contribute to the intestinal acid-probiotic actions and inhibit tumor cells' neoplastic traits. It cytotoxically affects cancerous cells and inhibits tumor growth. Moreover, *E. faecium's* antioxidant and immunomodulatory properties may improve the effectiveness and lessen the adverse effects of traditional cancer treatments, such as radiation and chemotherapy (Nami, Y. et al. 2015).

2.6 *Enterococci faecium* as a Starter Cultures:

To start fermentation operations, microorganisms are selected based on their physiological and metabolic characteristics. Although the food industry increasingly uses commercial starting cultures for fermentation, most products still use natural starter cultures to produce natural taste, quality, flavor, and fragrance. Microbial variety is a key component of commercial starter cultures, which are designed based on microbial physiological and metabolic features. A more varied starting culture produces

a product with a richer flavor because different bacterial groups work together to produce flavoring chemicals that improve the product's overall flavor and quality (Parente, Cogan et al. 2017).

The primary agents of fermentation are lactic acid bacteria (LAB), which transform carbohydrates into alcoholic metabolites, organic acids, and CO2. These substances are helpful for food preservation, taste, and flavor, and they also improve the product's overall flavor and quality. LAB also help in the manufacture of wine by raising the pH of the beverage through the creation of lactic acid from malic acid and produces flavor and texture enhancing chemicals (Coelho, Malcata et al. 2022).

Commercial starting cultures are used by companies in two different ways: either in concentrated form or by growing and multiplying the organisms in the industry. The type of product produced, economic worth, and the quantity of goods produced from a single starting culture are taken into consideration while making the decision. For this, cultures that have been freeze-dried yet are still extremely active are known as lyophilized cultures. The Hazard Analysis Critical Control Point (HACCP) principles must be adhered to in order to guarantee the security and caliber of any desired product (Hansen 2002).

2.6.1 Technological Role and Potential of *Enterococcus faecium* in Artisanal Cheese:

Enterococci are members of the non-starter lactic acid bacteria (NSLAB) found in many artisanal cheeses. They have also occasionally been added to experimental starter cultures. Their contribution to the formation of the typical sensory features of a number of artisanal cheese varieties, including Domiati (Egypt) and Izmir Tulum (Turkey), has been documented as part of the NSLAB (Giraffa, G. 2002). While several artisanal cheese variants have shown their importance as NSLAB in flavor development, their potential as cheese starters is far less obvious. A starter's primary functions include stimulating acidification and participating in the first stages of proteolysis, two fundamental events in maturation that are critical to the development of the distinct flavor, texture, and mouthfeel of cheese (Garg, S.K. et al.1991). According to multiple investigations, the enterococci have a weak capacity for acidification; only a tiny proportion of isolates can create enough acid after 16–24 hours at 37 °C to lower the pH to 5.0–5.2. While some research indicates that *E. faecalis* acidifies skim milk more

quickly than *E. faecium*, other studies reveal a significant inter-strain heterogeneity (Gelsomino, R., et al. 2002).

Cheese flavor and texture are influenced by casein breakdown, but it also contributes to the build-up of bioactive peptides in the product matrix that may have positive health effects. Of all the enterococcal isolates, caseinolytic strains have been recovered from artisanal cheeses to varying degrees (17–95%). Often considered a virulence factor, the gelE gene contributes to caseinolysis when enterococci proliferate in dairy substrates. According to the increased proteolytic activity reported for this species, *E. faecalis* has a higher frequency of this gene than other enterococcal species. When recombinant enterococcal bacteria express gelE, they produce hydrolysates of milk proteins that have a strong inhibitory effect on the angiotensin-converting enzyme (ACE). Proteolysis in enterococci appears to be strain-specific in addition to species-specific (Kagkli, M., et al. 2007). *Enterococci faecium* first break down casein by the action of the extracellular Clp proteolytic complex. The resultant oligopeptides are subsequently transported into the cell by a number of transport systems (Opp, Dpp, and DptT). Once inside the cell, they undergo additional degradation by aminopeptidases (Pep A, Pep B, Pep C, Pep F, Pep O, Pep Q, Pep S, Pep T, and Pep V). Eventually, these aminopeptidases will provide nitrogen for the growth of enterococci and act as building blocks for flavor compounds (McAuley, M., et al. 2015).

Moreover, enterococcal decarboxylase systems have the ability to transform free amino acids into biogenic amines. Since acidic surroundings trigger the activation of amino acid decarboxylation, they may aid in the enterococcal cell's ability to maintain pH homeostasis. In situations when there is a shortage of nutrients, it might also assist primary metabolism. One of the primary producers of biogenic amines in cheese has frequently been identified as Enterococci, several species of this genus have been shown to synthesize histidine, tyramine, 2-phenylethylamine, cadaverine, and putrescine, along with lysine, agmatine, and ornithine decarboxylase and agmatine deiminase for biogenic amine synthesis. Nevertheless, since these enzymes produce significant taste components and their precursors (i.e., short peptides and free amino acids), aminopeptidase activity is a desirable characteristic for cheese starting cultures. Moreover, their actions might help avoid flavor flaws (Ortigosa, M., et al. 2008).

Since aminopeptidases are located inside cells, enterococcal cells. Autolysis plays a crucial role in the maturity of cheese by encouraging contact between these enzyme systems and their substrates, which in turn speeds up peptidolysis and contributes to flavor development. It has also been suggested that starter autolysis helps regulate cheese's bitterness deficiencies. The LAB strain has a significant influence on autolytic activity. Enterococci from artisanal cheeses have been found to have strong autolytic activity, which has been connected to greater aminopeptidase activity in cheese slurries (Nieto, P., et al. 2011).

Because of the limited proteolytic capabilities and relatively low acidification capability, enterococci are not frequently considered essential components of the major starting cultures used in cheesemaking. On the other hand, they might be promising as protective or supplemental cultures. In order to boost the strength and aid in creating a well-balanced cheese flavor, adjunct cultures are purposefully introduced. Metabolic activities such lipolysis, esterase activity, proteolysis, amino acid degradation, and citrate metabolism are of interest while screening for cheese adjunct cultures because they are frequently chosen from among cheese NSLAB. Additionally, it has been proposed that the ability to produce exopolysaccharides (EPS) could be a noteworthy characteristic, particularly for low-fat cheese kinds, since EPS could enhance the texture and water-holding capacity of the cheese (Enck, P., et al. 2008).

Since the cheese microbiota releases free fatty acids (FFA) through the hydrolysis of milk lipids preserved in the matrix, lipid metabolism plays a significant role in the flavor and texture of many cheese kinds. These FFA are then converted during metabolism into volatile substances that contribute to cheese flavor, such as methylketones and thioesters. While lipases function on milk fat that has been emulsified, esterases target the dissolved lipid fractions (Nieto, P., et al. 2011). Cheese LAB, especially enterococci, are generally thought to be mildly lipolytic, nevertheless, some enterococcal strains isolated from specific cheeses have been reported to have significant lipolytic activity, which may suggest that this feature varies depending on the species and strain. Although their esterolytic activity has been reported to be higher than that of the other LAB species, the enterococci's activity appears to be mostly restricted to short-chain fatty acids (Russo, L., et al. 2020).

The metabolism of citrate by enterococci affects the flavor of cheese by releasing a number of volatile C-4 compounds, such as butanediol, acetoin, and diacetyl. Moreover, the creation of the characteristic "eyes" or cavities found in cheese varieties like Gouda and Danbo is facilitated by the CO2 emitted during the citrate process. Enterococci from cheese have been reported to produce diacetyl and, to a lesser amount, acetoin through manufacturing. In addition, genes encoding for acetaldehyde, diacetyl, and acetoin-related enzymes have been identified through the annotation of enterococcal genomes (Larsen, L., et al. 2000).

Many enterococci strains linked to cheese yield a diverse range of bacteriocins, or enterocins, with broad-spectrum activity against a number of G+ve foodborne pathogens, such as Staphylococcus aureus, Bacillus cereus, clostridial endospores, vegetative cells, and Listeria monocytogenes. One possible explanation for the success of enterococci in colonization and multiplying to significant quantities in cheese is the generation of enterocin. The exceptional capacity of the bacteria in this genus to exchange genetic material is assumed to be the cause of enterocin variety in enterococci (Baroja, M., et al. 2007). Enterolysin A is one prominent exception; it targets the cell wall. Bacteriocin-producing bacteria in cheese may help control the microbiota during ripening, encourage starter and NSLAB cell permeability and autolysis (which releases intracellular enzymes), and suppress pathogenic and deteriorating microorganisms (Yerlikaya, O.,et al. 2019).

Nonetheless, certain enterococci can function as human nosocomial, opportunistic infections; because of their inclination to exchange genetic material, they might serve as repositories of genes that confer virulence and resistance to antibiotics. As a result, there is no Qualified Presumption of Safety (QPS) designation for the genus. Before applying any enterococcal strain to food, a detailed analysis of its safety-related phenotypic and genetic determinants is required for each strain. In light of these worries, it has occasionally been suggested to use pure or semi-purified enterocins as antibacterial food additives rather than producing them in situ (Margalho, L., et al. 2020).

Chapter 03
MATERIAL AND METHODS

Material and Methods

The study was carried out at the Laboratory for Microbial Food Safety and Nutrition (LMFSN), located in the Department of Microbiology within the Faculty of Biological Sciences at Quaid-e-Azam University Islamabad, Pakistan.

3.1 Media used:

For microbiological analysis five medias were used including Tryptic Soy Agar (TSA), Oxytetracycline Glucose Agar (OGA), Eosin Methylene Blue (EMB), Nutrient Agar, De Man Rogosa and Sharpe Agar (MRSA), and MacConkey Agar. Normal saline was used for serial dilution.

3.1.1 Tryptic Soy Agar (TSA):

The medium described is a general-purpose growth medium utilized to facilitate the growth of bacteria. It was prepared by combining Trypticase soy broth with agar as a solidifying agent. An anti-fungal agent was also incorporated into the medium to prevent any potential fungal contamination. Following inoculation with the sample, the plates were incubated at a temperature of 37°C for a duration of 24 hours.

3.1.2 Oxytetracycline Glucose Agar (OGA):

To detect yeast and fungal growth, a medium known as Oxytetracycline Glucose Agar (OGA) was utilized. This medium was prepared using glucose and yeast extract as key ingredients, and agar was included as a solidifying agent. The proportions of the ingredients were determined in accordance with a specified protocol. Glucose served as the primary carbon source for growth, while yeast extract provided additional nutrients necessary for microbial growth. To ensure accurate yeast/fungal counts, Oxytetracycline was incorporated into the medium to prevent bacterial growth. Following inoculation with the sample, the media plates were incubated at a temperature of 25°C for a duration of 48 hours.

3.1.3 Eosin Methylene Blue (EMB):

EMB agar is a type of medium that is both selective and differential in nature. The differential property of EMB agar arises from the formation of a complex between eosin and methylene blue under acidic pH conditions. Eosin and methylene blue serve as

selective/inhibitory agents for this medium. Methylene blue prevents the growth of gram-positive bacteria (while eosin has a lesser effect), whereas eosin causes a color change to a dark purple when the pH around the colony becomes acidic. Agar is incorporated into the medium as a solidifying agent. Following inoculation with the sample, the plates were incubated at a temperature of 37°C for a duration of 24 hours**.**

3.1.4 De Man Rogosa and Sharpe Agar (MRSA):

It is a selective medium for growth of Lactobacilli. The yeast/meat extracts and peptone present in the medium serve as sources of carbon, nitrogen, and vitamins that are essential for the general growth of bacteria. Ammonium citrate and sodium acetate give the selectivity against streptococci and molds. Ammonium citrate promotes lactobacilli development at low pH values while inhibits the growth of other types of bacteria. Magnesium sulphate and manganese sulphate supply critical ions required for lactobacilli to multiply. Agar is included in the medium as a solidifying agent, and following inoculation with the sample, the plates were incubated at a temperature of 37°C under anaerobic conditions for a period of 48 hours.

3.1.5 Nutrient Agar:

Microorganisms are grown in nutrient agar, a multipurpose nutrition medium, to support the development of numerous non-fastidious organisms. Nutritional agar is popular because a variety of bacteria and fungi may grow on it and because it contains many of the nutrients needed for bacterial growth. It is frequently used to separate and purify cultures. NaCl, agar, yeast extract, and peptone make up the majority of the mixture. The plates were incubated for 48 hours at 37C.

3.1.6 MacConkey Agar:

This media supports the growth of gram-negative microorganisms, making it a valuable tool for assessing the quality of milk and fermented milk samples. To prepare this medium, MacConkey agar was utilized, and an anti-fungal agent was added to prevent fungal contamination. After inoculation of the media plates with the sample, they were incubated overnight at a temperature of 37°C.

3.2 Probiotic growth and culture:

The laboratory for microbial food safety and nutrition at Quaid-e-Azam University provided 22 probiotic strains that were isolated from locally produced fermented milk products (Dahi) and stored in the probiotic culture collection. The strains were maintained at a temperature of -80°C, and then rehydrated in MRS broth before being grown on TSA and MRS agar plates under anaerobic conditions at a temperature of 37°C. Gram staining, the catalase test (3% H_2O_2), and the oxidase test (1% Kovac's reagent) were used to confirm the identity of the isolates and characterize their phenotypic features. These strains were identified as lactic acid bacteria and were phenotypically and genotypically characterized, with their genomes submitted to the NCBI database. The table provides the names of the strains that were collected from the -80°C refrigerator**.**

Table 3.1: List of Lactic Acid Bacteria used along with their NCBI accession number.

Sr.	Strain	NCBI Accession no
1	*L. lactis* QAULLNA8	JAJAPA000000000
2	*E. faecium* QAUEFNA13	JAJAOH000000000
3	*E. faecium* QAUEFNA17	JAJAOI000000000
4	*E. faecium* QAUEFNN2	JAJAOJ000000000
5	*E. faecium* QAUELNN4	JAJAOK000000000
6	*E. lactis* QAUELNN14	JAJAOF000000000
7	*E. faecium* QAUEFNS1	JAJAOL000000000
8	*Lb. rhamnosus* QAULRN2	JAJAOZ000000000
9	*Lb. paracasei* QAULPN3	JAJAOW000000000
10	*Lb. delbrueckii* QAULDN14	JAJAON000000000
11	*Lb. reutori* QAULRN15	JAJAOX000000000
12	*Lb. reutori* QAULRN18	JAJAOY000000000
13	*Lb. fermentum* QAULFN21	JAJAOP000000000
14	*Lb. acidophilus* QAULAN51	JAJAOM000000000
15	*Lb. fermentum* QAULFN53	JAJAOQ000000000
16	*Lb. fermentum* QAULFN54	JAJAOR000000000
17	*Lb. fermentum* QAULFN55	JAJAOS000000000

18	*Lb. fermentum* QAULFN56	JAJAOT000000000
19	*Lb. delbrueckii* QAULFN61	JAJAOO000000000
20	*Lb. fermentum* QAULFN62	JAJAOU000000000
21	*S. thermophilus* QAUSTN63	JAJAOG000000000
22	*Lb. fermentum* QAULFN64	JAJAOV000000000

3.3 Probiotic Consortia Preparation:

Individual strains of LAB were combined to form a probiotic consortium. Each strain was inoculated into 5 ml TSB broth and incubated at 37°C for 8 hours under anaerobic conditions. The resulting cultures were combined to create a mixed broth culture to ensure mutual growth of the strains. The consortium was then incubated for 24 hours at 37°C under anaerobic conditions. To refresh the LAB consortium, 9 ml of TSB was dispensed into three test tubes, and 100 µl of the consortium was added to each tube. The tubes were incubated at 37°C under anaerobic conditions for 2 hours.

Preparation of Fermented Milk Utilizing a LAB Consortium

3.4 Study Design:

The quality of cow milk from a dairy farm was assessed by examining it. The natural microflora of the milk was examined through microbiological analysis prior to any subsequent processing steps. For the milk fermentation process, cultures were activated in tryptone soya broth (TSB) and MRS broth for a duration of 2 hours. Subsequently, they were introduced into pasteurized milk and allowed to ferment for 7 hours. This fermentation process was conducted in five distinct batches: one served as the control, utilizing a commercial starter culture, while the other four batches were inoculated with respective consortia containing selective strains of LAB.

Various parameters including pH, titratable acidity, total solids, and syneresis were monitored at different intervals to delineate the biochemical alterations taking place during the fermentation process. Additionally, experimental samples were analyzed for proteolysis and ash content using methods outlined by the A.O.A.C. (1990). The determination of pH followed method number 981.12, titratable acidity was measured according to method number 967.16, and the calculation of total solid content employed method number 925.23 as per the A.O.A.C. (1990) guidelines. The separated solid content yielded whey, from which the percentage of syneresis was determined.

Chemical changes occurring during the fermented milk fermentation were investigated using Frontier Transformed Infrared Spectroscopy (FTIR). The FTIR analysis was conducted using Perkin Elmer Spectrum 65 FTIR spectroscopy equipped with ATR. Spectra were collected within the range of 650-4000 cm^-1. An overlay of spectra was generated to highlight new peaks, allowing for a comparison of changes in the experimental samples against the control sample.

3.5 Raw milk analysis:

3.5.1 Methylene Blue Dye Reduction Test (MBRT):

The MBRT test, also known as the methylene blue reduction test, is a rapid technique used for evaluation of the microbiological quality of milk. This test relies on the principle that the color of milk, induced by the addition of methylene blue dye, will diminish over time. The discoloration is caused by the reduction of oxygen in milk and the production of reducing agents during bacterial metabolism. A sterile MBRT test tube is filled with 10 ml of milk sample and 1 ml of a 0.005% MBRT dye solution is added. The tubes are then sealed with sterilized rubber stoppers and placed in a water bath at 37°C. The color is checked every 30 minutes, and the sooner the decolorization occurs, the lower the bacteriological quality of the milk is.

3.5.2 Determination of Specific Gravity of Milk:

A lactometer is a device that is used to measure the specific gravity of milk. It is a hydrometer that works on the principle of Law of Floatation. To measure the milk density put milk in a flask. Adjust the temperature of milk between 15°C-20°C by using Dairy floating thermometer. Once the temperature is adjusted place the lactometer inside the milk. The lactometer should not touch the walls of the flask. Note the reading of the lactometer after 90minutes. The specific gravity of milk is than calculated by using given formula:

$$\boldsymbol{Sp.Gr.} = \frac{\boldsymbol{corrected\ lactometer\ reading}}{\mathbf{1000}} + \mathbf{1}$$

3.5.3 Milk Microbiology:

Microbiological analysis was conducted on both raw and pasteurized milk to examine the presence of natural microbial flora. A total of six media, namely Eosin methylene blue agar (EMB), Tryptic Soy Agar (TSA), De Man, Rogosa and Sharpe agar (MRSA),

MacConkey agar, Nutrient Agar and Oxytetracycline Glucose Agar (OGA), were utilized. The milk samples were serially diluted, and three dilutions i.e.,10-3, 10-4, and 10-5 were spread-plated in duplicate onto the media. The media plates were incubated at 37^0C to facilitate bacterial growth, and colony counts were taken after 24 hours of incubation. For the growth of yeast/fungal colonies, the plates were incubated at 28^0C, and colony counts were taken after 48 hours of incubation**.**

3.6 Milk Pasteurization:

The raw milk underwent pasteurization by heating it in a sterilized beaker, which was placed in a Memmert Water Bath (WNB 7-45), at 65°C for 30 minutes. After pasteurization, the milk was processed to produce fermented milk.

3.7 Preparation of Fermented Milk:

After subjecting the raw milk to pasteurization, an inoculum obtained from LAB was introduced to initiate the process of making fermented milk. The LAB consortium was formed by creating four different combinations, with each consortium first being revived in 5ml of TSB broth by adding 100µl of the consortium in three falcon tubes. The falcon tubes were then left to incubate for 48 hours, followed by centrifugation at 8000rpm for 15 minutes to extract a cell-free supernatant. The supernatant was then discarded, and the remaining pellet was washed with normal saline three times, followed by centrifugation at 8000rpm for 15 minutes after each wash to obtain a washed pellet. The washed pellet was then added to the pasteurized milk in triplicate and left to incubate at 42°C in an anaerobic incubator. Commercial starter culture was used to inoculate pasteurized milk as a control.

3.7.1 Consortia A:

In the first combination of probiotic consortium all twenty-two strains of LAB were used. The consortia was refreshed in 5ml of TSB in a test tube and incubated at 37°C in an anaerobic incubator for 2 hours. The individual strains of LAB used in consortium are QAUEFNA13, QAUEFNA17, QAUEFNN2 QAUEFNN4, QAUELNN14, QAUEFNS1, QAULLNA8, QAULRN2, QAULPN3, QAULDN14, QAULRN15, QAULRN18, QAULFN21, QAULAN51, QAULFN53, QAULFN54, QAULFN55, QAULDN61, QAULFN62, QAULFN64 and QAUSTN63**.**

3.7.2 Consortia B:

In the second combination of probiotic consortium fifteen strains of Genus Lactococcus, Genus Lactobacillus and Genus Streptococcus were used to inoculate milk excluding strains of Enterococcus spp. The consortia was refreshed in 5ml of TSB in a test tube and incubated at 37°C in an anaerobic incubator for 2 hours. The lactobacillus strains used in consortia are QAULRN2, QAULPN3, QAULDN14, QAULRN15, QAULRN18, QAULFN21, QAULAN51, QAULFN53, QAULFN54, QAULFN55, QAULDN61, QAULFN62 and QAULFN64. The Lactococcus strain used is QAULLNA8 and QAULLNA8 strain of Lactococcus is used.

3.7.3 Consortia C:

In the third combination of probiotic consortium only six strains of Enterococcus spp. were used. The consortia was refreshed in 5ml of TSB in a test tube and incubated at 37°C in an anaerobic incubator for 2 hours. Among these strains five were of Enterococcus Faecium including QAUEFNA13, QAUEFNA17, QAUEFNN2 QAUEFNN4 and QAUEFNS1. And one strain of Enterococcus lactis QAUELNN14 is used.

3.7.4 Consortia D:

In fourth combination of probiotic consortium only five strains of *Enterococcus faceium were* used. The consortia was refreshed in 5ml of TSB in a test tube and incubated at 37°C in an anaerobic incubator for 2 hours. Among these strains five were QAUEFNA13, QAUEFNA17, QAUEFNN2 QAUEFNN4 and QAUEFNS1**.**

3.8 Physiochemical Analysis of Fermented Milk:

3.8.1 Determination of Fermented Milk pH:

The quality, texture, and taste of fermented milk rely heavily on its pH since the enzymatic reactions that occur within the product are sensitive to pH. To determine the pH, method number 981.12 from the A.O.A.C (1990) was employed. The procedure involved taking 7ml of the fermented milk sample and homogenizing it through vertexing. The pH value was measured using a pH meter probe (Sartorius Professional Meter PP-15) inserted into the sample, and then rinsed with distilled water. All readings were taken in triplicate at room temperature.

3.8.2 Determination of Titratable Acidity:

Milk and milk products are naturally acidic because they contain citrates, phosphates, casein, and albumin. To measure this acidity, a strong base like Sodium Hydroxide (NaOH) can be used for titration. The titratable acidity of fermented milk was determined using method number 967.16 of the A.O.A.C (1990). Titratable acidity is quantified in relation to the equivalent weight of lactic acid. To carry out this procedure, 7 ml of the fermented milk sample was extracted and subjected to vortexing and then 2 to 3 drops of phenolphthalein were added as an indicator. NaOH was added gradually to the sample until a light pink color appeared. Titratable acidity was then calculated.

$$total\ acidity = \frac{Eq.wt\ of\ lactic\ acid \times Normality\ of\ NaOH \times ml\ of\ 0.1N\ NaOH\ used}{weight\ of\ sample} \times 100$$

3.8.3 Determination of Total Solid Content:

According to (A.O.A.C, 1990) method no. 925.23, the total soluble solid content of the fermented milk sample was calculated. The sediments in the fermented milk sample were separated by centrifuging a 2-4 ml sample of the inoculated milk at 14000 rpm for 10 minutes. To entirely eliminate all water content, the solid phase was next dried in a dry air oven at 100°C and weighed. The percentage of total soluble solids was calculated using the following formula.

$$\%\boldsymbol{age\ solid} = \frac{\boldsymbol{weight\ of\ residue}}{\boldsymbol{weight\ of\ fermented\ milk}} \times \mathbf{100}$$

3.8.4 Determination of Syneresis:

To determine the syneresis content in fermented milk, a 2ml sample of the fermented milk was centrifuged at 12000 rpm for 10 minutes, and the resulting liquid phase was weighed. The syneresis content was then calculated using the following formula:

$$\%\boldsymbol{age\ of\ liquid} = \frac{\boldsymbol{weight\ of\ liquid}}{\boldsymbol{total\ weight}} \times \mathbf{100}$$

3.9 Nutritional Profiling of Fermented Milk:

Fermented milk samples were subjected to nutritional profiling to determine their nutrient composition, such as proteins, fat and minerals that are essential for human growth and health. The aim was to estimate the percentage of these components in the samples.

3.9.1 Determination of Ash Content:

According to the procedure outlined by, the dried ash technique was used in a muffle furnace (carbolite Model No. CWF 1200) at 500 C for 6 hours (overnight) (Hernandez and Park, 2014). After weighing each sample (10 mL), 2-3 drops of acetic acid (10%) were applied. The mixture was heated to between 102 and 105°C before being burned at 500 degrees. The ash content was then determined using a specified formula and tested gravimetrically (AOAC, 2000).

$$\boldsymbol{Ash}\ \% = \frac{\boldsymbol{Weight\ of\ crucible\ and\ Ash - wight\ of\ crucible}}{\boldsymbol{weight\ of\ sample}} \times \mathbf{100}$$

3.9.2 Determination of Protein Content:

The protein was analyzed by the Kjeldahl System. For this purpose, 1g of sample is taken in a flask. Add 10g of Na2SO4, 1g of CuSO4 and 20ml of H2SO4 into the sample. Preheat the digester for 1 minute at full temperature. After preheating place, the sample containing tubes on to the digester and set the temperature on 400C until tubes become transparent and solution becomes bluish green. After digestion replace the tube container from heater and place it for 45 min for cooling. After cooling tubes make it 100ml of volume by adding Distilled water. For neutralization and distillation prepare the receiving flask by adding 25ml of 4% boric acid and a few drops of methyl blue indicator and place it at the receiving point of distillation unit. Place 100ml of digested sample containing tube into the distillation unit, set 4 min time on distillation unit, pour the NaOH until the color of solution become completely dark and press the start button to start the distillation process. After distillation titrate the receiving solution with 0.1N HCL, until the end point, which is blue color. Note the reading of HCL. Calculate the % protein using given formula:

$$\%\ \boldsymbol{protein} = \frac{\boldsymbol{R \times 0.014 \times 6.25 \times N}}{\boldsymbol{Sample\ weight}} \times \mathbf{100}$$

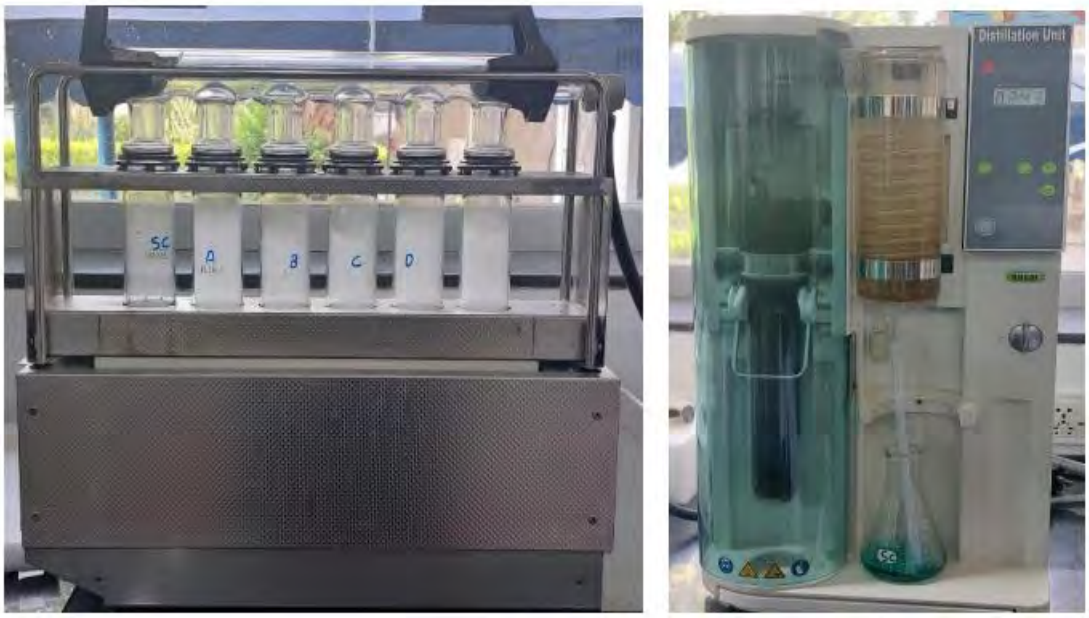

Figure 3.9.2 Kjeldahl System comprising Protein Digestion Apparatus (left) & Distillation Unit (right) at the Institute of Nutrition and Health, Islamabad.

3.9.3 Determination of Fat Content:

The fat content of fermented milk was ascertained following the Rose Gottlieb Method No. 905.02 (AOAC, 2000). To extract fat, 10 grams of the fermented milk sample were combined with 1.25 ml of NH_3 and 10 ml of ethyl alcohol within a separating funnel. Through vigorous shaking, protein precipitation occurred. After this, 25 ml of diethyl ether and 25 ml of petroleum ether were introduced, and the solution was shaken for approximately a minute. Following settling, the clear ethereal layer was poured into an empty China dish. This extraction procedure was repeated twice using 15 ml of each solvent. The weight of the empty China dish was documented prior to ethereal layer decantation. The isolated ethereal layer was then subjected to a hot air oven at 102°C for 30 minutes to eliminate the solvent entirely. The residual fat in the China dish was placed in a desiccator to eliminate moisture, and the dish's weight was re-measured. The fat percentage was computed using the subsequent formula.

$$\boldsymbol{fat\%} = \frac{\boldsymbol{weight\ of\ fat}}{\boldsymbol{weight\ of\ Dahi}} \times \mathbf{100}$$

3.9.4 FTIR Analysis of Extracted Fermented Milk Fat:

Fourier Transformed Infrared spectroscopy (FTIR) was employed to investigate the alterations in the chemical composition of fermented milk fat specimens. The FTIR analysis was carried out utilizing a Perkin Elmer spectrum 65 FTIR spectrophotometer equipped with ATR. This methodology is capable of detecting a wide array of chemical modifications occurring within the sample. Placing the fat samples on the FTIR plate, a spectrum ranging from 650 to 4000 cm^-1 was recorded for each individual sample. Subsequently, an overlay was generated to discern any novel peaks or deviations in the sample when compared to the control.

Chapter 04
RESULTS

Results

4.1 Raw Milk Analysis:

4.1.1 Methylene Blue Dye Reduction Test (MDRT):

Based on the methylene blue dye reduction test raw milk sample was analyzed and the results suggest that raw milk exhibit good microbiological quality as no color change was observed in the 6 hours of incubation period. Lack of color change in the sample shows that there was no significant bacterial activity observed that could reduce the methylene blue dye within 6 hours incubation period. The absence of significant microbial activity implies minimal contamination and low bacterial count. The results show that the milk sample met the acceptable microbiological quality standards for consumption (fig 4.1).

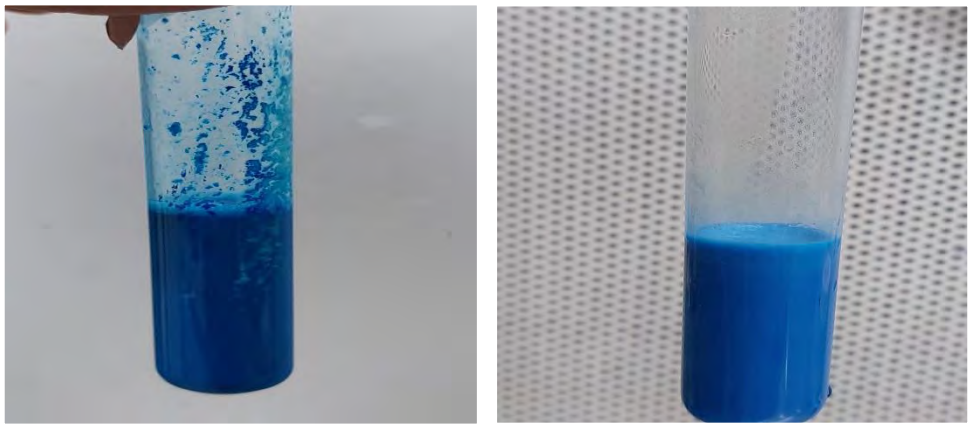

Figure 4.1 Pictorial representation of MDRT, before incubation (left), after 6hours of incubation (right).

4.1.2 Determination of Specific Gravity of Milk:

The lactometer reading of raw milk was 27 and normal milk lactometer reading range between 26-32. By using this lactometer reading the milk density was calculated i.e. 1.0284g/ml that falls within the standard density range for normal milk.

4.1.3 Raw Milk Microbiology:

Different types of media were used to check the microbial load in the raw milk used for fermentation. MacConkey agar was used for the detection of gram-negative bacteria, total aerobic count was observed by using TSA. TSA is a general-purpose media used for a variety of colonies having different morphology, color, and size. Further confirmation was provided by performing gram staining of colonies obtained on TSA. For fungal count OGA media was used.

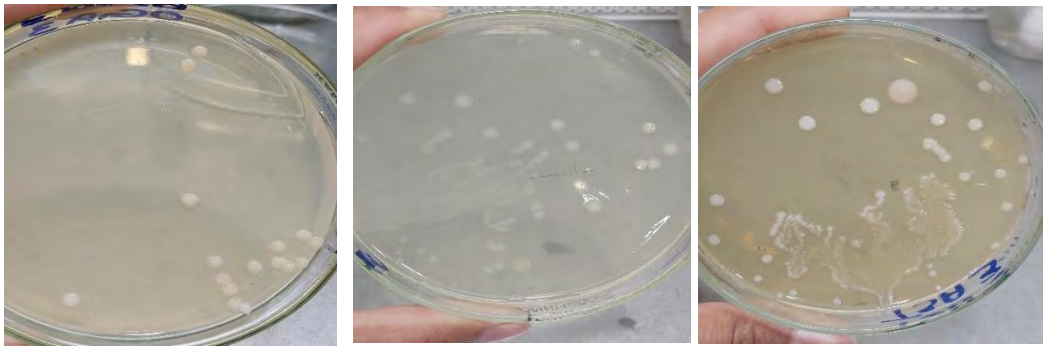

Figure 4.2 Raw Milk microbiology on OGA, Nutrient Agar and TSA.

4.2 Pasteurized Milk Microbiology:

For fermentation milk was pasteurized at 65C for 30 minutes. To ensure the quality of pasteurized milk microbiology was done. MacConkey was used for Enterobacteriaceae count and no colony was observed. TSA was used for total aerobic count and adequate colonies were observed on TSA plates. OGA was used for total fungal count. No colony was observed on Nutrient Agar.

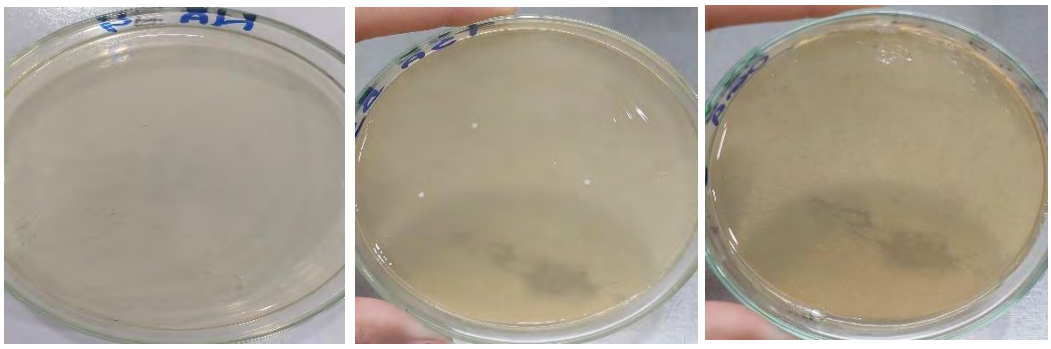

Figure 4.3 Pasteurized Milk microbiology on OGA, Nutrient Agar and TSA.

4.3 Microbial Colonies present in Raw and Pasteurized Milk:

Raw and Pasteurized milk microbial colonies were calculated on different media including OGA, TSA, MRSA etc., using following formula:

$$\boldsymbol{cfu/ml = \frac{(no.of\ colonies) \times (Dilution\ factor)}{Volume\ plated}}$$

Four dilutions were made and 50µl of volume were plated from dilution factor 10^{-4}. Pasteurized milk shows 00E+00 colonies on each media plate. While raw milk has shown 4.8E+06 cfu/ml on OGA plate that was used for total fungal count, 3.50E+07 cfu/ml on Nutrient Agar plate, 2.40E+07 cfu/ml on TSA plate and 5.00E+06 cfu/ml on MRSA plate.

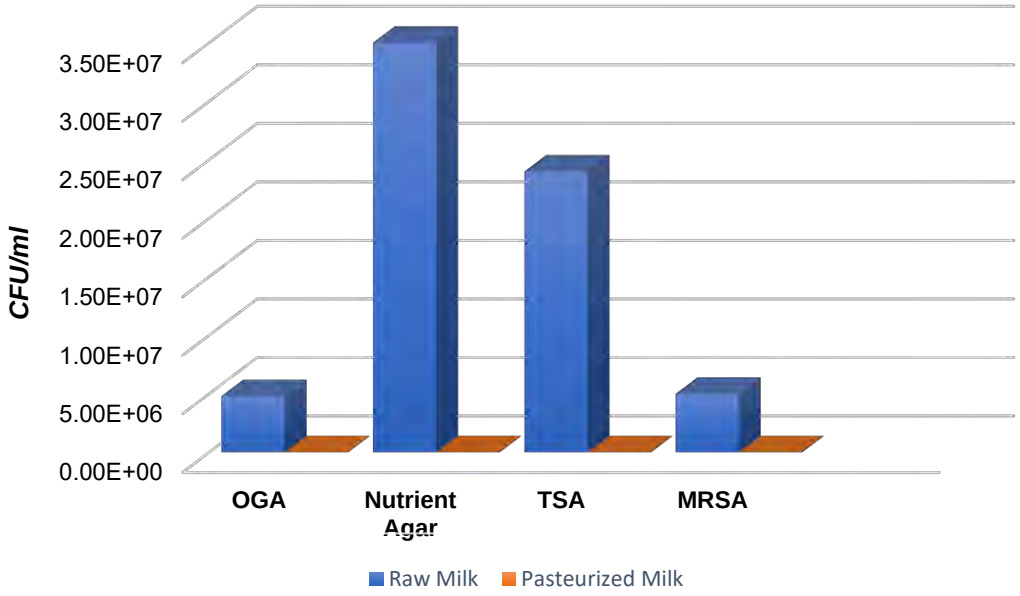

Figure 4.4 CFU/ml of microbial colonies present in Raw and Pasteurized milk.

4.4 Fermented Product of different Combinations of LAB Consortium:

Pasteurized cow milk was inoculated with a starter culture that acts as a control and 4 different combinations of LAB consortia were used to ferment milk. The milk inoculated with consortia was incubated in aerobic conditions for 6 hours at 42°C. After 6 hours proper curd was formed with whey on the top of curd indicating that fermentation has occurred, and fermented milk is formed.

Figure 4.5 Fermented Milk (left), closeup view of curd and whey separation in fermented milk (right).

4.5 Physiochemical Analysis of Fermented Milk:

4.5.1 Effect of Different LAB consortia on the pH of Fermented Milk:

Once fermented milk was set, fermented milk pH was continuously monitored after every 24 hours. In all four combinations of fermented milk (fermented with consortia A, consortia B, consortia C and consortia D) and in control (fermented with commercial starter culture) a continuous increase in pH was observed till 48 hours. After 48 hours there is a decrease in the pH. The pH of fermented milk C (fermented with consortia C that contain strains of *E. faecium* and *E. lactis*) has lowest pH i.e 5.35±0.014 before storage as compared to other experimental fermented milk (fig 4.6).

pH of raw milk was 5.7. Milk was than inoculated with consortia A, consortia B, consortia C and consortia D. It was then allowed to set for 6 hours at 42°C following inoculation. Once milk had fermented, it was chilled, and a pH meter was used to determine its pH.. The pH was found to be 5.615±0.021 in control, 5.62±0.014 in fermented milk A, 5.55±0.007 in fermented milk B, 5.35±0.014 in fermented milk C and 5.38±0.014 in experimental fermented milk D.

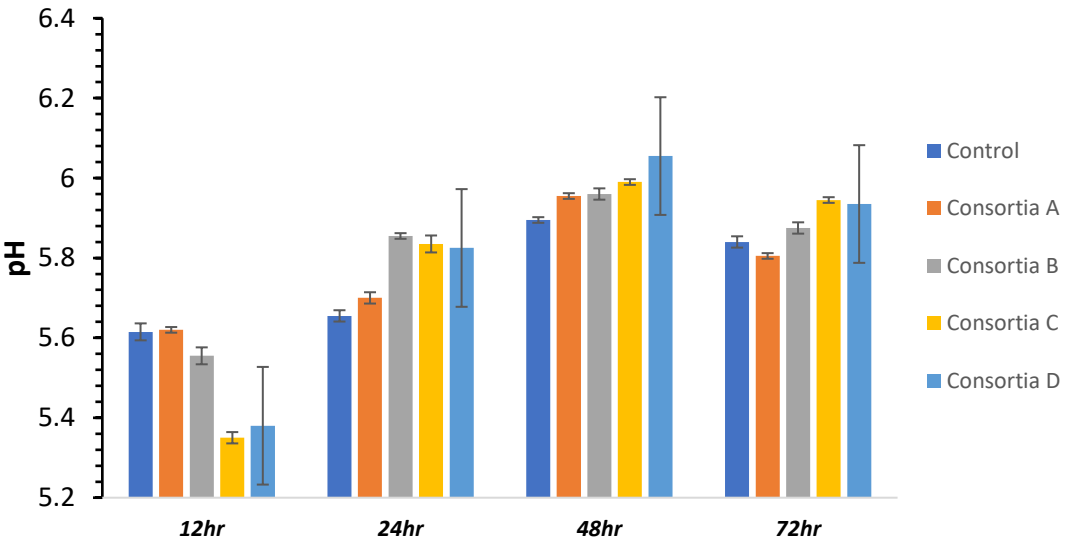

Figure 4.6 Graph showing change in pH with time.

4.5.2 Titratable Acidity:

Titratable acidity was determined by an indicator (phenolphthalein) that gives faint pink color at the end. The acidity of control fermented milk (fermented with commercial starter culture) and experimental fermented milk A (fermented with consortia A) remain same after 12hrs and 24hrs while there is an increase in the acidity of fermented milk

B, fermented milk C and fermented milk D after 24 hrs.The acidity of the fermented milk increases abruptly after 48hrs and no further increase or decrease in acidity is seen after the 48hr in any of the experimental fermented milk.

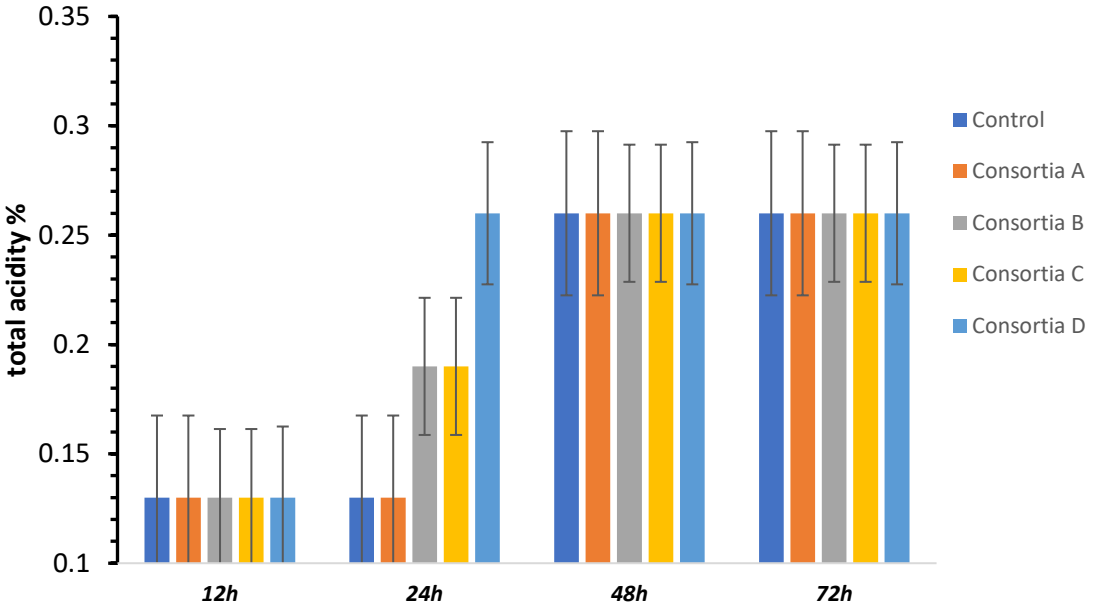

Figure 4.7 Graph showing change in titratable acidity with time.

4.5.3 Solid Content:

Increased solid content indicates thicker consistency of fermented milk. By centrifuging the fermented milk sample and then drying it in a dry oven, the solid content of the fermented milk was determined (fig 4.9). Solid content of fermented milk was continuously monitored after regular intervals. The solid content of control was very high as compared to experimental fermented milk after 12hrs of fermentation i.e. 19.5 % (19.5±1.414 ml). The solid content of control and fermented milk D decreases after 12hrs and then there is an increase in solid content after 24hrs. While the solid content of fermented milk A, fermented milk B and fermented milk C increases after 12hrs and after 48hrs there is as slight decrease in the solid content as compared to the reading taken on 24hrs (fig 4.8).

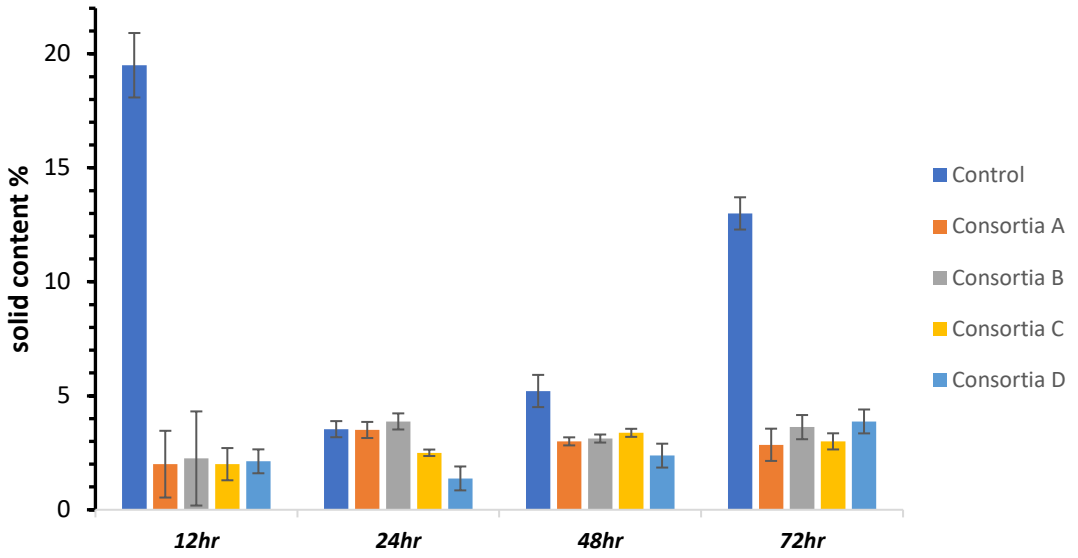

Figure 4.8 Graph showing change in solid content of fermented milk with time.

Figure 4.9 Pictorial representation of solid content of fermented milk; before moisture removal (Left), after complete moisture removal (right)

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4.5.4 Syneresis:

Syneresis is defined as the liquid content of fermented milk. Syneresis was calculated after regular intervals just like solid content. The highest and lowest syneresis was shown by control fermented milk. After 12hrs the syneresis of control was highest i.e., 98% (97±1.414ml) with the passage of time the syneresis of control decreases gradually after i.e., 90% (90±1.414ml) at 72hrs. The syneresis of fermented milk A and fermented milk B decreases after 12hrs and it increases after 24hrs. The syneresis of fermented

milk C and fermented milk D increases after 12hrs. fermented milk D achieves maximum syneresis at 48hrs i.e., 98% (98.5±0.707ml), after 48hrs the syneresis of fermented milk D decreases. While fermented milk C achieves maximum syneresis at 24hrs i.e., 98% (97.5±0.707ml) and after 24hrs its syneresis decreases (fig 4.10).

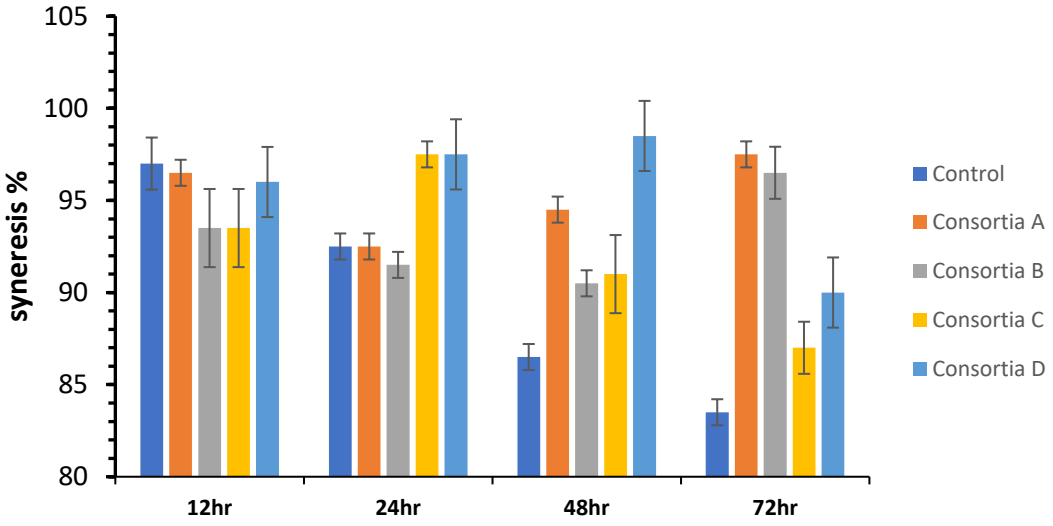

Figure 4.10 Graph showing change in syneresis of fermented milk with time.

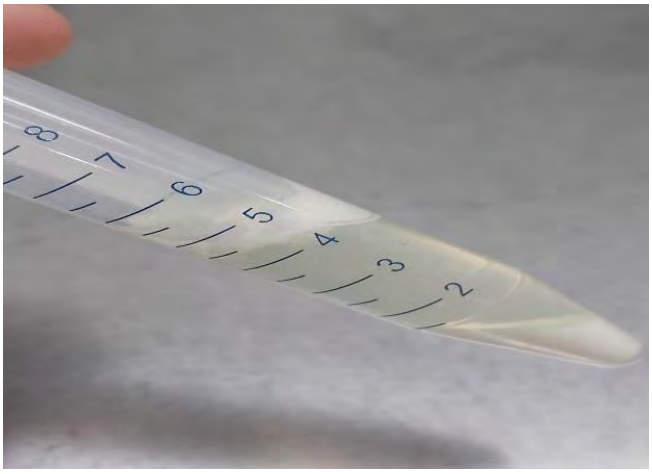

Figure 4.11 Pictorial representation of separation of solid and liquid content of fermented milk.

4.6 Nutritional profiling of Fermented Milk:

4.6.1 Determination of Ash Content:

Ash of samples contain different minerals Potassium (K), Sodium (Na), Zinc (Zn), Calcium (Ca), Iron (Fe), and Magnesium (Mg) etc. High value of ash content indicates high amount of minerals in the fermented milk sample. fermented milk C has the highest ash content i.e., 1.2% (1.35±0.212g). The ash content of control is 0.502% (0.547±0.063g). The lowest ash content is of fermented milk A i.e., 0.414% (0.40±0.010g).

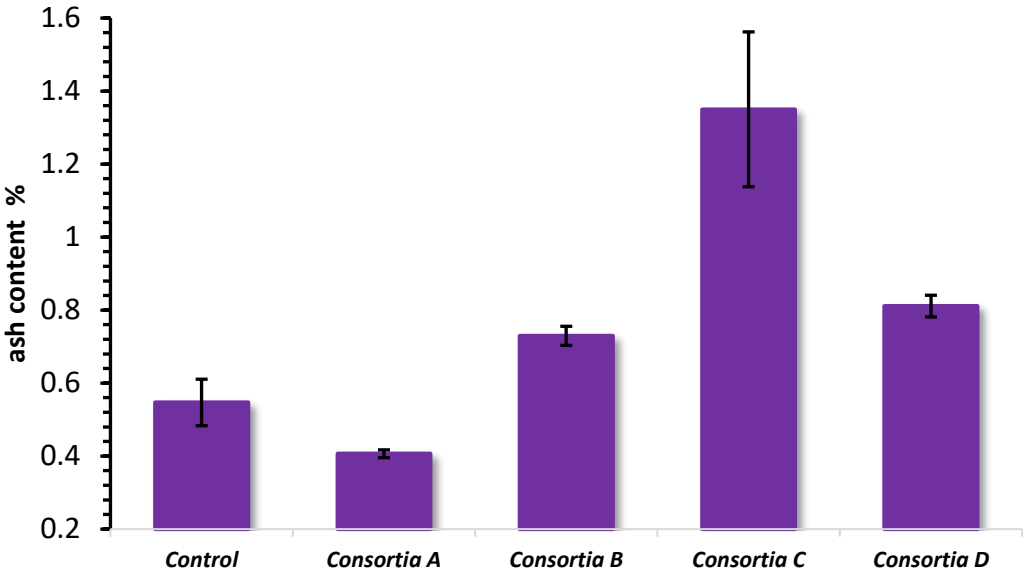

Figure 4.12 Graph showing ash content of control and experimental fermented milk.

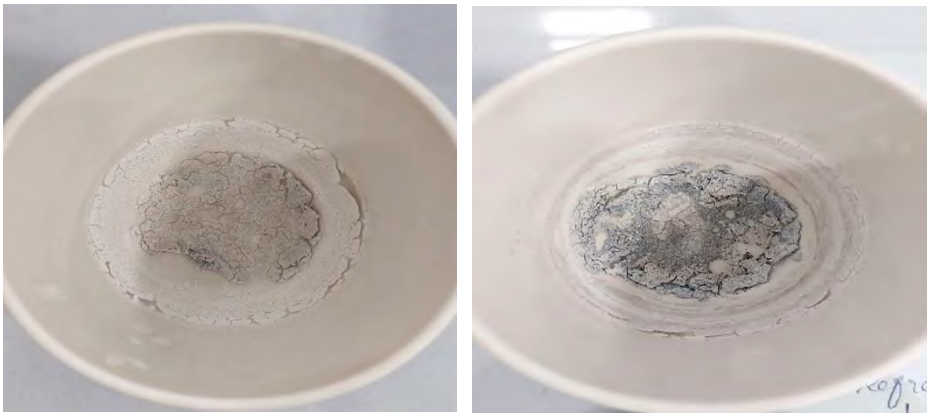

Figure 4.13 Pictorial representation of ash content, incomplete ash (left), complete ash (right).

4.6.2 Determination of Protein Content:

The protein content of fermented milk ranges between 3%-5%. The highest percentage of protein was present in control (fermented with commercial starter culture) and fermented milk D (fermented with consortia D) i.e., 4.7%. The lowest amount of protein was present in fermented milk C i.e, 3.06% (fig 4.14).

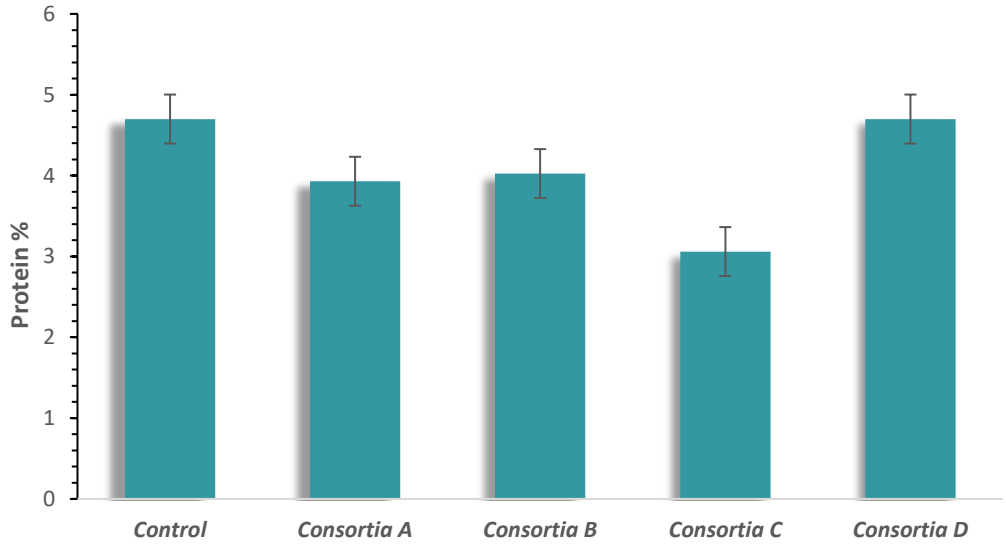

Figure 4.14 Graph showing protein content of control and experimental fermented milk.

4.6.3 Fat Extraction:

The highest fat percentage was recorded in fermented milk B (fermented with consortia B) i.e., 6.54% (6.56±0.028). While the lowest fat percentage was recorded in control (fermented with commercial starter culture) i.e., 3.55% (3.52±0.042g). The overall fat content of experimental fermented milk including control ranges between 3.5%-6.5% (fig 4.16).

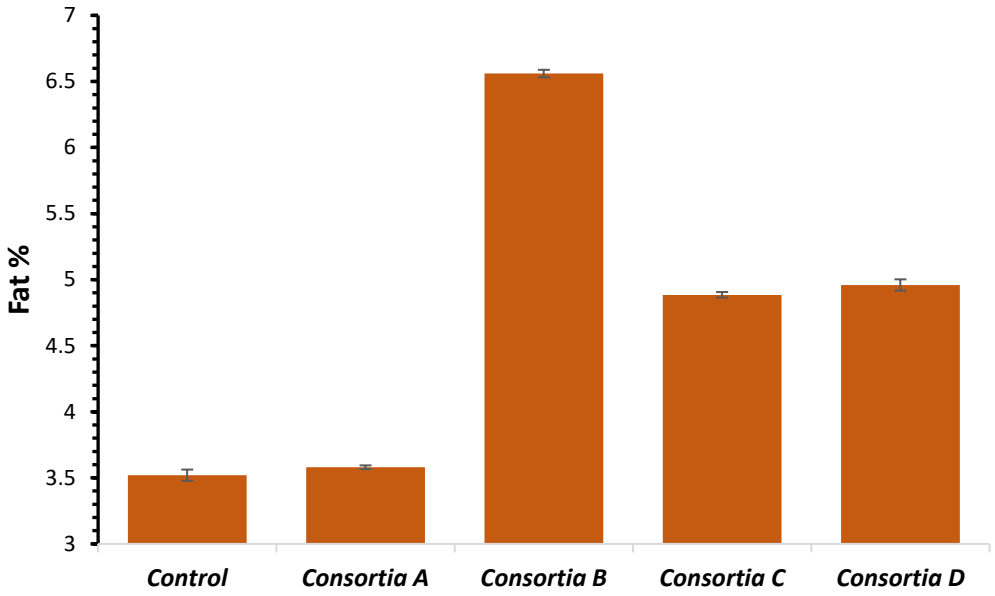

Figure 4.16 Graph showing fat content of control and experimental fermented milk.

4.6.4 Fat Composition by FTIR:

ATR-FTIR of fat extracted from contol, fermented milk was performed. The first wave number obtained in fat extracted from fermented milk C is 3418.91cm-1 indicates the presence of alcohols and phenols. The second main strecthes were obtained at wavelength 2920.91cm-1 and 2851.91cm-1 and these streches indicates the presence of alkanes. While 3rd main stretch were obtained at 1742.30cm-1 indicating esters and saturated aliphatics. While fat extracted from fermented milk D indicates first main stretch at 3416.91cm-1 indicating phenols and alcohols, 2nd at 2922.91cm-1 & 2853.91cm-1 indicating alkanes and 3rd main stretch at 1744.91cm-1 indicating esters and saturated aliphatics. Minor peak at wavelength 1377.42 cm-1 in fermented milk C fat sample and 1458.34cm-1 in fermented milk D fat samples indicates aromatic compounds. No significant difference was obsereved between Control , fermented milk C and fermented milk D fat samples.

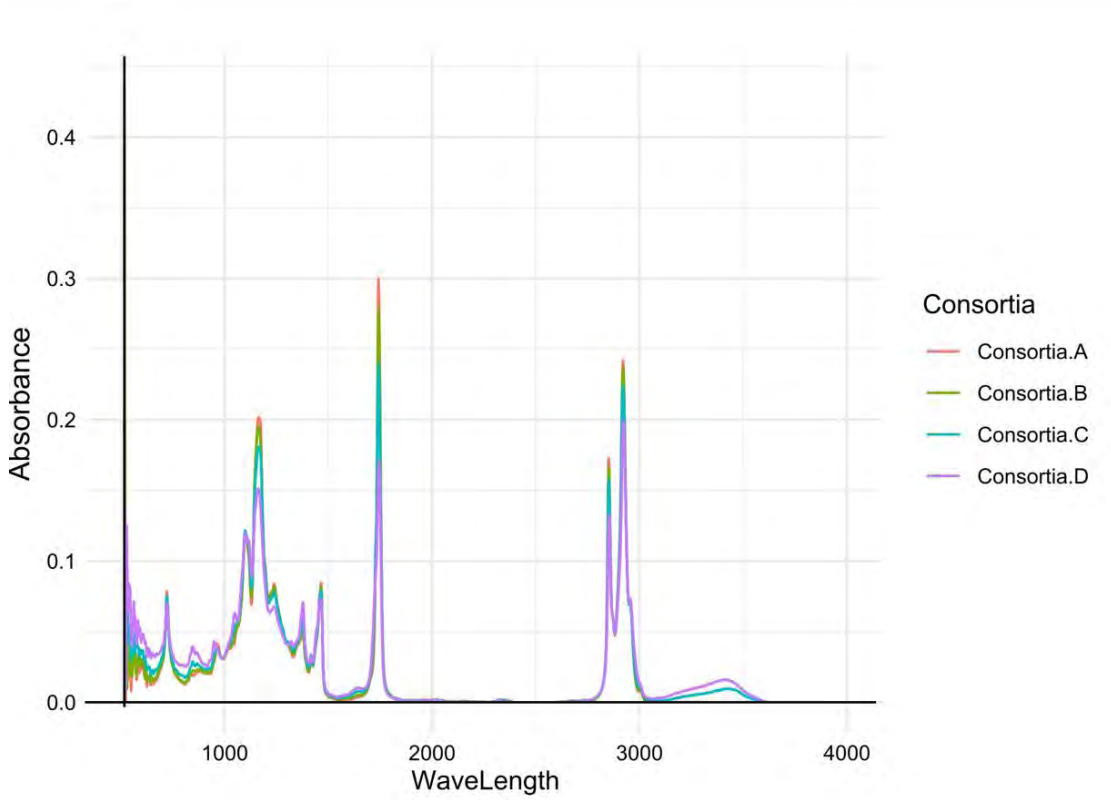

Figure 4.17 Overlay of FTIR Spectra for Extracted Fat Samples.

Chapter 05

DISCUSSION

Discussion

During the fermentation process, particular lactic acid bacteria (LAB) strains have an impact on fermented milk, a common fermented milk product. Fermented milk physiochemical makeup and nutritional value are impacted by *Enterococcus faecium's* critical contribution to fermentation. These strains alter the dynamics of pH, texture, and consistency in addition to the breakdown of proteins and lipids, which affects the mouthfeel and gel formation. They also help to produce lactic acid, which has an immediate impact on the titratable acidity of fermented milk. Through processes including protein breakdown, release of peptides, amino acids, and fatty acids, as well as the creation of vitamins and bioactive substances, *Enterococcus faecium* also affects the nutritional qualities of fermented milk. Some strains are regarded as probiotics and may provide health advantages when included in a balanced diet.

The impact of *Enterococcus faecium* on fermented milk products has been the subject of several studies utilising *Enterococcus faecium* in various combinations with LAB. These investigations have demonstrated that the utilization of Enterococcus contributes positively to the fermentation process, without imparting any adverse impact on the physical, chemical, rheological, or sensory attributes of the products. Instead, its incorporation has led to notable levels of bacterial viability, a crucial element in the creation of probiotic foods (Akpinar, Saygili et al. 2020). Our work is distinctive in that it compared various combinations of LAB consortia with and without enterococcus faecium while using just enterococcus strains for milk fermentation. According to recent research, the pH of milk fermented with LAB consortia including *Enterococcus faecium* and lacking *Enterococcus faecium*, as well as fermented milk that was fermented only with *Enterococcus faecium* strains, displays a lower pH on the first day of storage before rising up to 48 hours later and then falling.This was in line with the discovery made by Akpinar, et al. (2020), which demonstrated that the pH of a product fermented with *Enterococcus faecium* strains increases after the first day of storage and decreases on the second day. The conclusion of work done by Lankaputhra et al. (1995) demonstrated that the pH of a product fermented with *Enterococcus faecium* strains is lower on the first day of storage and increases afterward.

Acidity of the fermented milk feremented with *Enterococcus faecium* strains only incease after 12 hrs showing that *Enterococcus faecium* directly effects that acidity of

the product due to increase in the lactic acid content of the product. Same results were expressed by Saygili et al. (2020) which states that the titratable acidity of probiotic fermented milk increased with storage. All samples had an increase in lactic acid concentration after storage. Titratable acidity is known as the essential factor in evaluating the fermented dairy products structural qualities. Numerous studies found that the starting cultures in probiotic fermented milk enzymatic activity caused the acidity and lactic acid levels to rise during storage.

Syneresis occurs when fermented milk liquid phase separates from the gel, causing whey loss. The liquid content during fermentation is influenced by specific LAB strains, metabolic activities, and interactions within the consortium. The study found that milk fermented with *Enterococcus faecium* strains solely shows decrease in syneresis with increased storage time and fermented milk fermented with LAB consortia containing *Enterococcus faecium* increase in syneresis after, suggesting that different strains may contribute differently to the fermented milk matrix's liquid-holding capacity. This corresponds with the research conducted by Shenana et al. (2015) in which an inverse relationship between storage period and susceptibility to syneresis is observed. Syneresis takes place as a result of the rate of acid development, reorganization of casein particles within the gel network, and the dissolution of colloidal calcium particles. Some strains of LAB used in fermented milk manufacturing produce EPS, which affects syneresis and reduces it (Lee and Lucey 2004).

The ash value is a measure of the amount of trace minerals like zinc and iron as well as major minerals like calcium, phosphorus, potassium, and magnesium that are vital for human health. As was previously mentioned, the presence and combination of particular LAB strains can affect the ash content of the final fermented milk product. The significant differences in ash content between milk fermented with *Enterococcus faecium and Enterococcus lactis* strains and milk fermented with a LAB consortium containing *Enterococcus faecium* supported by Amjad (2010). The metabolic activities of these LAB strains during fermentation may account for the greater ash content (1.2%) in milk fermented with *Enterococcus lactis and Enterococcus faecium* strains. Due to the capacity of *Enterococcus lactis and Enterococcus faecium* to use numerous minerals and micronutrients found in the milk matrix, fermented milk has a greater mineral content, including ash (Mahmood, Abbas et al. 2008).

The amount of protein in fermented milk is a useful measure of the nutritional value of the product, the efficiency of the fermentation process, and the activity of LAB strains. These factors can be used to determine the end product's quality and functioning (Saldo, Sendra et al. 2022). In our study, we found that milk that had been fermented with *Enterococcus faecium* had less protein. This is due to the fact that distinct metabolic inclinations and enzymatic activity exhibited by *Enterococcus faecium* strains impact protein synthesis throughout fermentation. This conclusion is consistent with research by Dapkivicius et al. (2021), which indicates that the presence of particular genes, like gelE, which are frequently linked to virulence factors, may be the cause of the decreased protein concentration in fermented milk. One important protein in milk, casein, is broken down via a process called caseinolysis, which is aided by the gelE gene (Perin, L. M., et al. 2017). Enterococcal decarboxylase systems have the ability to transform free amino acids generated during caseinolysis into biogenic amines, which further modifies the fermented milk's protein composition and flavor profile. The amount of protein produced during fermentation may be further reduced by *Enterococcus faecium* strains whose metabolic pathways redirect metabolic precursors away from protein synthesis and toward other cellular functions (EI-Din, B., et al. 2002).

In a study done by Centeno and his team in 1999, they found that the samples that have *Enterococcus faecium and Enterococcus faecalis*, contain high levels of volatile free fatty acids (VFFA). They noticed this especially in cheeses made with these specific bacteria. This suggests that having more of these bacteria can make cheese break down fats more(Centeno, Menendez et al. 1999). Another research (Hesari et al. 2012) also talked about how having more of these bacteria can affect cheese fats. Our study agrees with Menendez et al. (1999) that enterococcus strains, increase fats levels in food and can also break down fats more. Enterococcus spp are responsible for breaking down proteins and fats, and even changing certain substances. They can even use citrate, which helps cheese develop its taste and flavor. Enterococci have been suggested as part of specified starting culture combinations for several European cheeses because of these intriguing metabolic characteristics(Morandi, Brasca et al. 2006)

The distinctive fragrance of fermented milk products can exhibit considerable variation due to the influence of enzymatic activities carried out by naturally occurring and/or introduced microorganisms (Routray, Mishra et al. 2011). Among the constituents

contributing to the aromatic profiles of fermented milk, alcohols and aldehydes, followed by ketones and acids, appear to be the most prevalent. Ketones, which arise from the oxidation of acyl lipids, are abundantly present in various dairy products, and they possess distinct odors with low thresholds for detection (Tian, Shi et al. 2019). Notably, prior research has indicated that *Enterococcus faecium* is particularly adept at generating ketones, accounting for approximately 53.19 percent of ketone production (Guarrasi, Sannino et al. 2017). However, our findings are contrary to those of Gao et al. (2022). Acids, recognized aromatic compounds, exert significant influence over the flavor of fermented milk. These acids can originate from processes like lipolysis and glycolysis, with lactose metabolism being a primary contributor. Furthermore, it's worth noting that the formation of other aromatic molecules, such as ketones and alcohols, can also stem from acid-mediated reactions (Mousavi, Heshmati et al. 2019).The complex interplay of these aromatic components underscores the intricate nature of fermented milk sensory characteristics and offers a nuanced perspective on the factors shaping its olfactory and gustatory properties.

CONCLUSION

Conclusion

Significant insights have been gained from the extensive study of *E. faecium's* effects on the physiochemical and nutritional characteristics of fermented milk during fermentation. The study shows that throughout fermented milk fermentation process, *E. faecium* is extremely important in determining the physiochemical and nutritional properties of the product. According to the results, adding *E. faecium* to milk fermentation causes noticeable changes in the fermented milk properties. This suggests that early in the process, *E. faecium* may be responsible for a quicker acidification process. Additionally, the pace at which acidity developed during fermentation appeared to be modulated by *E. faecium's* presence in the LAB consortium. The fat content of fermented milk fermented only with *E. faecium* strains was noticeably greater, underscoring the effect of *E. faecium* on fat metabolism during fermentation. It highlights the numerous ways that bacterial interactions affect the production of proteins during fermentation.

FUTURE PROSPECTS

Future Prospects

- Present study reveals the probiotic potential of fermented milk, which might result in new fermented milk products that promote gut health as well as clinical studies on immune system, digestive, and general health.
- The study of the effects of *Enterococcus faecium* on the physiochemical and nutritional characteristics of fermented milk may lead to the development of specialised formulations that satisfy certain dietary needs and customer preferences.
- A study recommends investigating the presence of *Enterococcus faecium* in fermented dairy products in order to possibly create nutrient- and probiotic-rich dairy products.
- Future studies may examine the detailed safety profile of *Enterococcus faecium* by using it in different fermented products.
- Fermented Milk manufacturing with *Enterococcus faecium* has the potential to increase quality and nutritional value while also raising output for the dairy sector.

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