Technological characterization of cholesterol-lowering probiotic *Lactobacillus* strains for the synthesis of dairy products

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I, **Saba Talib,** declare that this research work titled "**Technological characterization of cholesterol-lowering probiotic *Lactobacillus* strains for the synthesis of dairy products**" is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of Dr. Abdur Rahman. The material that has been used from other sources has been properly acknowledged / referred.

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(Supervisor)

Dr. Abdur Rahman

DEDICATION

To Allah SWT for this amazing journey and paving me, the paths of my life, and success in a way that I would have never dream of.

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In the name of Allah, the Most Gracious and Most Merciful, He only has to say "BE" to it, and it will BE (Al-Qur'an 2:117). For He who knows all the plans, He has for me, plans to prosper me, to give me hope and a future. Indeed, He is the best of all the planners. He knows what I want, what I deeply desire, and what will make me happy and only He can give it to me. Throughout this work, all the time when I fail and was alone His mercy remained upon me. I would like to thank Allah for blessing me with this opportunity, faith, strength, and discipline to endure this task with patience and fulfill all its requirements. My guidance cannot come except from Allah, in Him I trust and to Him, I repent (Al-Qur'an 11:88).

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LIST OF ABBREVIATIONS

%	Percent
^oC	Degree Centigrade
CFU	Colony Forming Unit
cfu/g	Colony Forming Unit per Gram
cfu/ml	Colony Forming Unit per Millilitre
CSH	Cell Surface Hydrophobicity
CVD	Cardiovascular Disease
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
F_0F_1-ATPase	F-Type ATPase
FAO	Food and Agriculture Organization
×g	g-force
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
h	Hour
H^+	Protons
H^+-ATPase	Proton ATPase
HCl	Hydrogen Chloride
I.e.	That is
KCl	Potassium Chloride

KH_2PO_4	Monopotassium Phosphate
L	Litre
LAB	Lactic Acid Bacteria
min	Minutes
µL	Microlitre
mL	Millilitre
mm	Millimeter
mM	Millimolar
MRS	de Man Rogosa and Sharpe medium
Na_2HPO_4	Disodium Hydrogen Phosphate
$Na_2HPO_4.2H_2O$	Disodium Phosphate Dihydrate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometer
OD	Optical Density
PBS	Phosphate-buffered saline
pH	Potential Hydrogen

SD	Standard Deviation
spp.	Species
subsp.	*Subspecies*
w/v	Weight by Volume
WHO	World Health Organization

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ABSTRACT

Food fermentation by microbes is being carried out for ages due to its many benefits like improved functionality, shelf life, flavor, and texture of food products. Probiotic microorganisms act as microbial food supplements by providing prompt health benefits after consumption of probiotic enriched foods. Hence, they are an example of food fermenting microbes that can be used to improve organoleptic characteristics and to ensure long-term product safety. *Lactobacillus* species such as *L. delbrueckii*, *L. rhamnosus,* and *L. fermentum* are the most common starter or adjunct cultures used in the fermentation of foods such as instant yogurt, and cheese, beer, wine, cider, and chocolate. The objective of the present study was to assess the technological properties through in vitro testing of five indigenous *Lactobacillus* strains, which were already evaluated for their promising cholesterol-reducing profile in both *in vivo* and *in vitro* studies, for their suitability as starter or adjunct culture in dairy products. *L. delbrueckii* (I-17) followed by *Lacticaseibacillus rhamnosus* (Y-59) had the greatest technological potential as diacetyl producers as well as being able to tolerate heat and high NaCl concentration along with a good acidification profile in both MRS broth and skim milk, their autolytic potential was better as compared to other strains and remain viable in acidified milk. Based on technological characterization indigenous *L. rhamnosus* (Y-59) was used as an adjunct culture for yogurt production. According to the sensory evaluation analysis, the indigenous *L. rhamnosus* (Y-59) supplemented yogurt proved to be better in all tested sensory attributes like aroma, taste, texture, odor, and overall acceptability as compared to starter culture (control) and commercial yogurt (reference control). Hence, these strains showed promising attributes for further applications in fermented functional products as a starter or adjunct culture to produce probiotic dairy foods.

CHAPTER 1

INTRODUCTION

Hippocrates, a Greek philosopher, and the founder of medicine introduced the idea of consuming food as medicine by stating "Let food be thy medicine, and let medicine be thy food" (Wegener, 2014). Hence, the concept of using food as medicine to provide therapeutic effects to the host has its roots in ancient history. Back in the19th-century scientists started exploring the role of different natural compounds in disease treatment (Weststrate *et al*., 2002).

At the start of the 20th century, the prime focus of the scientific community was on investigating the role of vitamins in dietary deficient diseases (Betoret *et al*., 2011). Later this led to the discovery of physiologically bioactive compounds from plants and animals which are known as phytochemicals and zoo chemicals respectively (Wrick, 1995). These potentially bioactive compounds conferred health-promoting benefits along with reducing the risk of various chronic diseases and hence in 1990 all such foods were coined under the term 'Functional Foods' (Milner, 2000).

The term 'Functional Food' was first proposed in Japan and later it gained immense importance all over the world. "The traditional foods which in addition to basic nutrition requirement confer positive health effects along with decreasing the risk of chronic diseases", are known as functional foods (Al-Sheraji *et al*., 2013). Nowadays people are concerned about nutritious food and a healthy lifestyle, whereas, in past, the prime goal of food was only to ensure survival, hunger satisfaction, and prevention of harmful effects (Granato *et al*., 2010). As a result, the need for novel functional foods has risen in recent years.

The utilization of probiotics is the most rapidly growing field in the production and research of functional foods. World health organization (WHO)/Food and Agriculture Organization (FAO), defines probiotics as; "live microorganisms which, when administered in adequate amount, confer a health benefit on the host" (FAO, 2006). In other words, the consumers gain beneficial effects from the use of probiotics which can be called microbial food supplements (Reid, 2016). Probiotic microorganisms provide prompt health benefits after the consumption of probiotic enriched foods (Losio *et al*., 2015). Food fermentation by microbes is being carried out since ages due to its many benefits like improved functionality, shelf life, flavor, and texture of food products. Probiotic cultures are an example of such microbes and can be used to improve organoleptic properties and to guarantee long term product safety (Hill *et al*., 2017).

Probiotics improve the intestinal peristalsis, immune system responses and decrease cholesterol level in our body along with their adhesion to the intestinal cells and competitive activity against pathogenic microbes (Losio *et al*., 2015). Tolerance to acid and bile salts, production of bacteriocins are some of the main properties of probiotics but most important for a probiotic strain is to be nonpathogenic and attains a status of generally regarded as safe (GRAS) (Granato *et al*., 2010).

Probiotics have qualities that are antimutagenic, anticarcinogenic, improve lactose metabolism, reduce intestinal inflammation and allergic illness symptoms, prevent diarrhea and infections during pregnancy, and control allergic diseases (Minervini *et al*., 2017). Probiotics play a vital role in disease prevention and enhance the bioregulation (Hill *et al*., 2017). Probiotics help in the synthesis of nutrients such as vitamins which prevents against hepatic diseases and *Helicobacter pylori* infections (Parvez *et al*., 2006). They mainly produce vitamin K and water-soluble vitamin B such as biotin, cobalamin (vitamin B_{12}), folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine (LeBlanc *et al*., 2013).

*Lactobacillu*s spp. and *Bifidobacterium* spp. are well-known among probiotics (Granato *et al*., 2010). *Lactobacillus* spp. are used in fermented foods such as, instant yogurt, cheese, beer, wine, cider, chocolate, and animal feeds (Losio *et al*., 2015). *Lactobacillus* spp. are characterized as Lactic Acid Bacteria (LAB) due to their lactic acid-producing ability. Among *Lactobacillus* spp. *Lactobacillus delbreukii* is known as yogurt bacteria along with *Streptococcus thermophilus* and both are used as starter culture for yogurt production.

Lactobacillus fermentum is typically found in fermented foods and dairy products. Studies indicate *L. fermentum* as pro-inflammatory, anti-pathogenic, ability to reduce esterified cholesterol, hepatic free cholesterol, phospholipids in fatty liver disease and it produces exopolysaccharide (EPS) which imparts to functional properties of the strain (Minj *et al*., 2021). Moreover, *L. fermentum* has been reported to develop a wide range of antimicrobial peptides that can be used as food preservatives or as a substitute to antibiotics. *L. fermentum* is a key microorganism in food fermentation technology in terms of flavor, texture, or health boosting advantages and has recently been employed to generate novel food items such as fortified and functional foods with positive health effects for humans (Naghmouchi *et al*., 2020).

Since the 1980s, *Lactobacillus rhamnosus* has been the most studied probiotic species (Westerik *et al*., 2018). *L. rhamnosus* plays an important part in the management of obesity and diabetes, homeostasis of intestinal microbiota, and promoting innate immune responses along with producing antibacterial compounds (Minj *et al*., 2021). According to previous studies, the ability of *L. rhamnosus* to create EPS in milk distinguishes it as a functional starter culture (Masiá *et al*., 2021). *L. rhamnosus GG (LGG)* was the first *Lactobacillus* strain to be patented in 1989 and has been used in biofilm production, owing to its capacity to live and grow at stomach acid pH and in bile-rich medium, as well as adhere to enterocytes (Capurso, 2019).

The functional probiotic product should meet all the demands and expectations of the consumer i.e., the probiotic product should be safe with good sensory properties (Patrignani *et al*., 2006). Hence, for this reason, a microbial strain developed as a dietary adjunct ought to by default should have favorable technological characteristics. (Rönkä *et al*., 2003). To meet the commercial requirements there is a need for the dairy industry to manufacture products with different textures, flavors, and nutritional values by using different starter or probiotic adjunct cultures (Bai *et al*., 2020).

Food is used as an ideal carrier system to deliver probiotics because it serves as a buffer within the gastrointestinal tract (GIT), contains active compounds that increase the effectiveness of probiotics and controls colonization (Ranadheera *et al*., 2010). To attain health benefits viable probiotic bacteria should be ingested in high cell concentrations with the minimum value of 6 log cfu/g and while passing through the gut to make up for the potential loss of microorganisms which are probiotic in nature the daily intake should be at least 10^8 cfu/g (Shah, 2007). According to Kligler and Cohrssen, (2008) dosage for children ranges from 5 to 10 billion CFU/day and 10 to 20 billion CFU/day for adults.

Among probiotic fermented food products yogurt has immense consumer acceptance for being nutrient-dense probiotic food with high nutritional value due to the presence of calcium, zinc, and vitamin B (El-Abbadi *et al*., 2014). Yogurt's global market has grown from around 77 billion dollars in 2016 to over 86 billion dollars in 2019 and is expected to exceed 100 billion dollars by next year (Osorio-Arias *et al*., 2020).

Yogurt is made by fermenting milk with bacteria that include a mix of *Streptococcus subsp. thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. Due to lactic acid fermentation, yogurt is a sort of coagulated milk product with a smooth texture and a mildly sour yet pleasant flavor. Yogurt is the most well-known food carrier for the effective transfer of beneficial microbes to the host (Sarwar *et al*., 2019). Yogurt is not only good for people with gastrointestinal problems and lactose intolerance, but it also helps to strengthen the immune system. Moreover, yogurt is also a source of several minerals mainly calcium, proteins, and micronutrients (Madora *et al*., 2016). A typical method to produce set-type yogurt is given below (Lee *et al*., 2010).

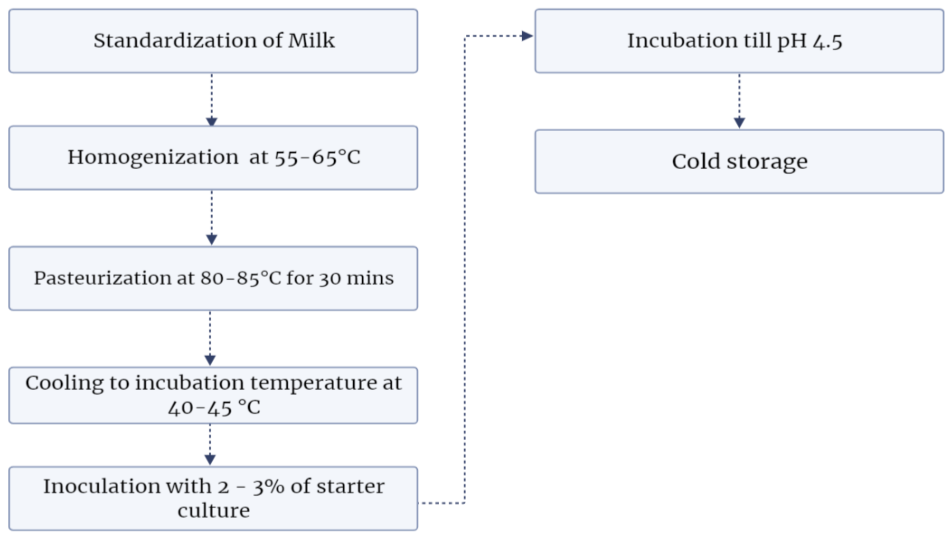


Figure 1: Main processing step involved in yogurt production.

Yogurt starter culture such as *Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus* are crucial for yogurt fermentation to produce lactic acid and to attain a good consistency. Starter cultures provide favorable metabolic substances to maintain the viability of probiotics in yogurt. For this reason, both probiotics and starter cultures are used as adjunct cultures in the production of probiotic yogurt (Kaur Sidhu *et al*., 2020). Production of novel functional yoghurts with enhanced nutritional merits and positive health effects is the need of the hour (Ahmed *et al*., 2021).

It has been proven that collaborative use of probiotics with yogurt starter cultures results in a product with improved functional qualities and increased health advantages. Hence, mineral or vitamin fortification, as well as the addition of probiotics, can improve the nutritional-physiological value of traditional yogurt and the resulting product could be suggested for use as a dietary adjunct (Bai *et al*., 2020). The primary requirements for these products are retention potential and sensory attributes (Rouhi *et al*., 2013). Therefore, food industries can project probiotic yoghurt as a functional food and have a profitable company.

Research Objectives:

1) Technological assessment of probiotic *Lactobacillus* strains for their suitability as starter or adjunct culture in dairy products.

2) Development of indigenous probiotic yogurt and its sensory evaluation.

CHAPTER 2

LITERATURE REVIEW

2.1 Nutraceuticals

Hippocrates, the Greek physician, and father of medicine, coined the term nutraceutical approximately 2000 years ago when he remarked, "Let your food be your medicine, and your medicine be your food." In 1989, Dr. Stephen DeFelice coined the word nutraceutical, which is an amalgamation of the food and pharmaceutical industries. Nutraceuticals are defined as naturally occurring foods, or parts of the food having physiological benefits or protecting human health against chronic diseases (DeFelice, 1995). Nutraceuticals have been used as ayurvedic remedies since 500 BC. Dietary fibers, polyphenols, antioxidants, spices, flavonoids, vitamins, probiotics, and polyunsaturated fatty acids are all examples of nutraceuticals. Animal, plant, and marine sources can all be used to make nutraceuticals (Ross, 2000).

Nutraceuticals are renowned for their impact on biological processes such as cell proliferation, antioxidant defense, and gene expression from a health standpoint. They can slow down the aging process and lower the risk of cancer, heart disease, hypertension, obesity, high cholesterol, diabetes, osteoporosis, arthritis, sleeplessness, cataracts, constipation, indigestion, and a variety of other lifestyle-related illnesses (Jacobs & Tapsell, 2013). Recently, the term 'nutraceuticals' has come to encompass a wide range of items, including functional foods, fortified foods, dietary supplements, separated nutrients, herbal remedies, and particular diets, all of which are offered in pharmaceutical forms and are not always related to food (Nworu *et al*., 2014; Shahidi, 2012; Venhuis *et al*., 2016).

2.2 Functional foods

Foods that alter certain activities or systems in the human body, giving health benefits beyond energy and nutrition, are known as functional foods. The phrase "functional food" originated in Japan in the 1980s to describe foods intended for certain health purposes (FOSHU) (Roberfroid, 2000). However according to the worldwide accepted definition, the phrase "functional foods" refers to foods or nutrients that cause significant physiological changes in the body that are distinct from those caused by their role as nutrition (FDA, 2004). All foods are thought to be functional at some physiological stage because they contain nutrients or other compounds that supply energy, support growth, or maintain or repair essential functions. Functional foods go above and beyond these requirements by delivering additional health attributes that may minimize risk or promote optimal health. Traditional foods, modified foods (fortified, enriched, or enhanced), medical foods, and foods for special dietary needs are all examples of functional foods (Hasler and Brown, 2009).

Mostly functional foods are a combination of one or more beneficial compounds such as prebiotic, probiotic, antioxidant polyphenols, sterols, and carotenoids (Andlauer and Fürst, 2002). Components such as vitamins, fiber, omega-3 fatty acids, minerals, bacterial cultures, and flavonoids can add functionality to any kind of food that is produced (Day *et al*., 2009). Frequent use of these foods will aid in the management of disorders such as cardiovascular disease (CVD), tumours, diabetes, and hypertension (Brown *et al*., 2018; Cassidy *et al*., 2018; Mak *et al*., 2018). The dairy sector is already a hot bed of functional foods, with tremendous commercial success. Because of dairy products calcium content, various proteins that benefit health, sphingolipids, butyric acid, conjugated linoleic acid, and probiotic cultures, dairy products may be considered functional foods (Khalaf *et al*., 2021).

2.3 Discovery of probiotics

There is a grave difference between the microflora present in the GIT of non-diseased healthy individuals as compared to the diseased individuals. This advantageous microflora of GIT was termed as probiotics. Elie Metchnikoff (1907) first discovered probiotics by observing individuals who ingested fermented milk products daily in Bulgaria, he connected this to their prolonged healthy life. Later he concluded that yogurt contains essential microorganisms which protect intestine from harmful bacteria (Parvez *et al*., 2006).

2.4 Definition of probiotics

Literally probiotics mean ‘for life’, and are microorganisms recognized for their help in promoting human and animal health (Marteau *et al*., 1995). Definition evolved from 1965 to 2012. The term ‘probiotic’ was first published as; ‘growth promoting factors produced by microorganisms and it was further observed that the consumers gained nutritional and therapeutic benefits from the lactic cultures and their fermented products (Lilly and Stillwell, 1965).

Parker in 1974 was the first one to define probiotics as “organisms and substances which contribute to intestinal microbial balance” (Parker, 1974). Then later in 1989, Fuller defined probiotics as “microbial food supplements that have a healthy impact on host, balancing their intestinal flora” (Fuller, 1989). Word probiotics originate from two Greek words that mean ‘for life’ (Fooks *et al*., 1999). Schrezenmeir and de Vrese defined probiotics as “a product or preparation containing viable microorganisms in sufficient numbers, which by implantation or colonization alter the microflora in the compartment of the host exerting health benefits on the host (Schrezenmeir *et al*., 2001). The final worldwide accepted definition proposed by the Food and Agriculture Organization of the United Nations (FAO/WHO) states; “live microorganisms which, when administered in adequate amount, confer a health benefit on the host” (FAO, 2006).

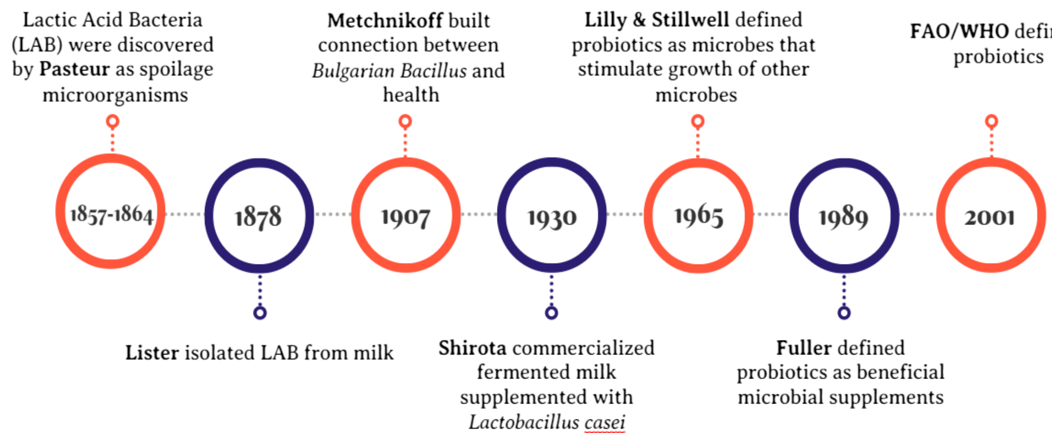


Figure 2.1: History of probiotics (O’Toole *et al*., 2017).

2.5 Probiotic species

The main genera of gram-positive bacteria; *Lactobacillus* and *Bifidobacterium* are currently characterized as probiotics and are largely being used in food products in market. LAB consists of genera including *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus*, and *Enterococcus* (Hill *et al*., 2017). The industrially consumed probiotic strains as described by Granato *et al*. (2010) are "*Lactobacillus acidophilus, L. johnsonii, L. reuteri, L. delbrueckii subsp. bulgaricus, L. casei, L. thermophilus, L. rhamnosus, Bifidobacterium longum, B. bifidum, B. animalis* and *B. infantis*."

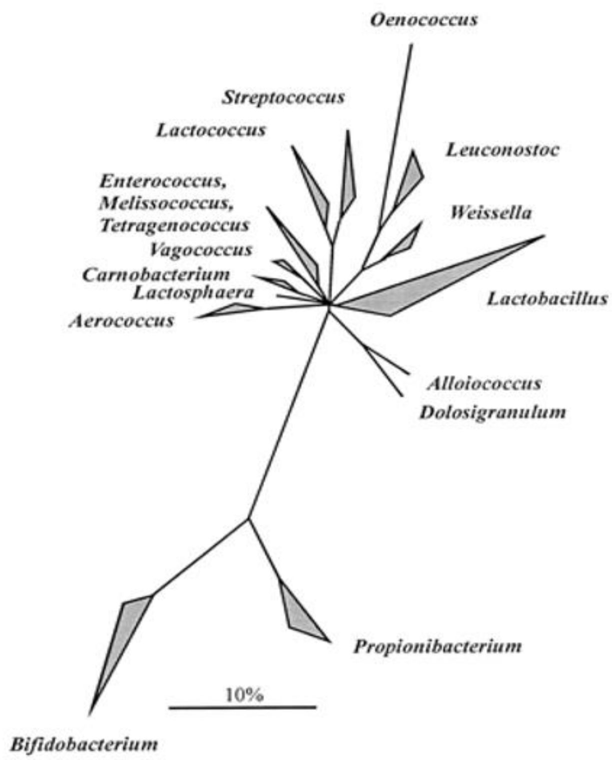


Figure 2.2: Classification of major phylogenetic groups of lactic acid bacteria (Holzapfel *et al*., 2001).

According to Senok (2009) yogurts and frozen desserts are supplemented with *Lactobacillus delbrueckii* spp., *Bulgaricus*, and *Streptococcus thermophiles*. *Enterococcus faecalis, E. faecium* and *Sporolactobacillus inulinus* are included among LAB having probiotic properties whereas "*Propionibacterium freudenreichii, Saccharomyces boulardii* (yeast) and *Saccharomyces cerevisiae*" are characterized as non-lactic microbes possessing probiotic characteristics (Holzapfel and Schillinger, 2002).

Lactobacillus plantarum and *Bacteroides species* are also known to own probiotic properties (Vanderhoof, 2000). Other LAB species which are used in probiotic preparations are: *Lactobacillus salivarius, L. cellbiosus, L. curvatus, L. fermantum, L. lactis, L. brevis, B. adolescentis, B. thermophilum, Streptococcus cremoris, S. salivarius, S. diacetylactis* and *S. intermedius* (Parvez *et al*., 2006). Many species out of all those mentioned above are given "qualified presumption of safety" (QPS) status by the European Food Safety Authority (EFSA, 2016).

2.6 Properties and functions of probiotics

Probiotics benefits were first reported by Metchinkoff in 1907 when he suggested that fermented milk can have a beneficial effect on gut (Kumar *et al*., 2015). Production of bioactive metabolites like bacteriocins, biogenic amines, exopolysaccharides and proteolytically released peptides during fermentation is one of the main properties of LAB (Hill *et al*., 2017).

2.6.1 Health related benefits

Prominent health benefits of probiotic enriched foods are highlighted in figure 2.3. Probiotics show antagonistic activity towards pathogens. They are capable of sticking and colonizing to gut mucosa. They suppress inflammatory effect and enhance immuno-stimulation. Different probiotic strains are helpful for the treatment of diarrhea including rotavirus diarrhea, reduction of colon tumors, cholesterol, intestinal pathogens and toxic compounds. Probiotics increase humoral immune response, lactose tolerance and nutrient bioavailability. They maintain intestinal flow by enhancing the absorption of iron, magnesium, and calcium. They are also involved in carcinogens detoxification and vitamin B production (Granato *et al*., 2010).

Anticarcinogenic (Marteau *et al*., 2001), antimutagenic, antimicrobial (Lourens-Hattingh and Viljoen 2001) and antihypertension are the major properties of probiotics. They have a health impact on mineral metabolism along with providing bone stability. They prevent from bowel disease and Crohn's syndrome (Marteau *et al*., 2001). *Lactobacillus* strains are reported to show anti-pathogenic activity against *Salmonella enteritidis, E. coli, Shigella sonnei*, and *Serratia marcescens*. Probiotics are proven to prevent from atopic dermatitis and food allergies. "*S. thermophilus, bifidobacteria, L. acidophilus, L. plantarum, L. casei, L. delbrueckii ssp. bulgaricus,* and *E. faecum*" are reported to reduce Hepatic encephalopathy (HE) (Solga, 2003).

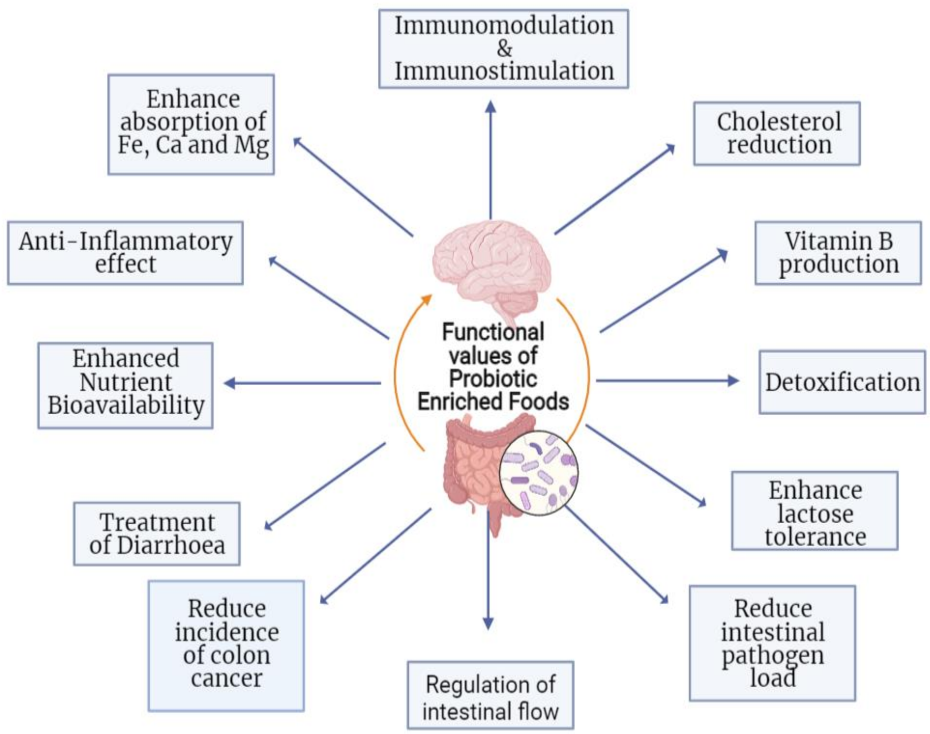


Figure 2.3: Health benefits of probiotic enriched foods (Granato *et al*., 2010).

Lactobacillus species are reported to show resistance to aminoglycosides, such as gentamycin, kanamycin, and streptomycin (Ouwehand *et al*., 2016). Until date tetracycline resistance is the most common among LAB (Zoumpopoulou *et al*., 2017). LAB produced bacteriocins with broad spectrum antibacterial activity against *Salmonella derby*, *Listeria monocytogenes*, *Salmonella enterica*, *S. typhimurium*, *S. napoli*, *S. enteritidis*, *E. coli and Staphylococcus aureus* (Losio *et al*., 2015).

2.6.2 Metabolite production

During food fermentation probiotics due to their metabolic activity produce certain biologically active molecules and enzymes. These healthy bio-functional metabolites add additional health value to the final food product. Hence, probiotics act as microbial food factory to enrich food stuff. The main bioactive compounds produced by LAB during dairy fermentation are vitamins, gamma-aminobutyric acid, bioactive peptides, bacteriocins, enzymes, conjugated linoleic acid, and exopolysaccharides (Table 2.1).

Probiotic Strain	Bioactive Metabolite	Food Product	Health Effect	Reference
L. casei ***B. infantis*** ***L. plantarum***	Thiamine/ Riboflavin (Vitamin B1/B2)	Fermented milk Fermented Soymilk	Vitamin enrichment	Drywien *et al*., 2015 Tamime, 2006 Levit *et al*., 2016
L. helveticus	Biotin (Vitamin B7)	Fermented milk	Vitamin enrichment	Patel *et al*.,2013
P. freudenreichii ***B.animalis*** ***L. reuteri***	Cobalamin (Vitamin B12)	Kefir Fermented milk Soy yogurt	Vitamin enrichment	Van Wyk *et al*., 2011 Patel *et al*.,2013 Gu *et al*.,2015
L. amylovorus ***S. thermophilus*** ***L. bulgaricus*** ***B. lactis***	Folic Acid (Vitamin B9)	Yogurt Fermented milk	Vitamin enrichment	Laiño *et al*.,2013 Crittenden *et al*.,2003
L. casei Shirota ***S. salivarius*** ***L. brevis***	GABA	Fermented milk Fermented soya milk	Antidiabetic, Blood pressure	Inoue *et al*., 2003 Chen *et al*., 2016 Park and Oh, 2007
L. bulgaricus ***L. helveticus***	Bioactive Peptides	Yogurt Fermented milk	Anti-hypertensive	Qian *et al*., 2011 EFSA, 2008
L. lactis ***P.acidilactici*** ***L.acidophilus***	Bacteriocins	Cheese Yogurt	Pathogen inhibition	Arques *et al*., 2015 Ahmed *et al*., 2010

L. rhamnosus ***B. bifidum***	Conjugated linoleic acid	Buffalo Cheese	Cholesterol lowering	Van Nieuwenhove *et al*., 2007a
L. mucosae ***L. bulgaricus***	Exopolysaccharides	Yogurt	Hypocholesterolemic Immunostimulatory	Rayan *et al*., 2015 Makino *et al*., 2016
B. longum			Immune modulation	Prasannaa *et al*., 2013

Table 2.1: Health-promoting metabolites produced in fermented dairy products (Linares *et al*., 2017).

Probiotics produce functional metabolites with distinct roles for example bacteriocins which exhibit antimicrobial activity against food-borne pathogens and thus play a role in food preservation. Probiotics synthesize vitamins (Folate, Riboflavin, Vitamin B12 & k2) which provide antioxidants. Other than this, the vast array of enzymes produced by probiotics have positive effect on organoleptic properties such as aroma, texture, and appearance resulting in the improved nutritional quality of food products (Chugh and Kamal-Eldin, 2020).

2.7 Sources of probiotics

Probiotics are found both in dairy and non-dairy products (Figure 2.1). However, dairy remains the main source of probiotics, and yogurt is the main carrier medium to transfer probiotics in the human diet (Granato *et al*., 2010).

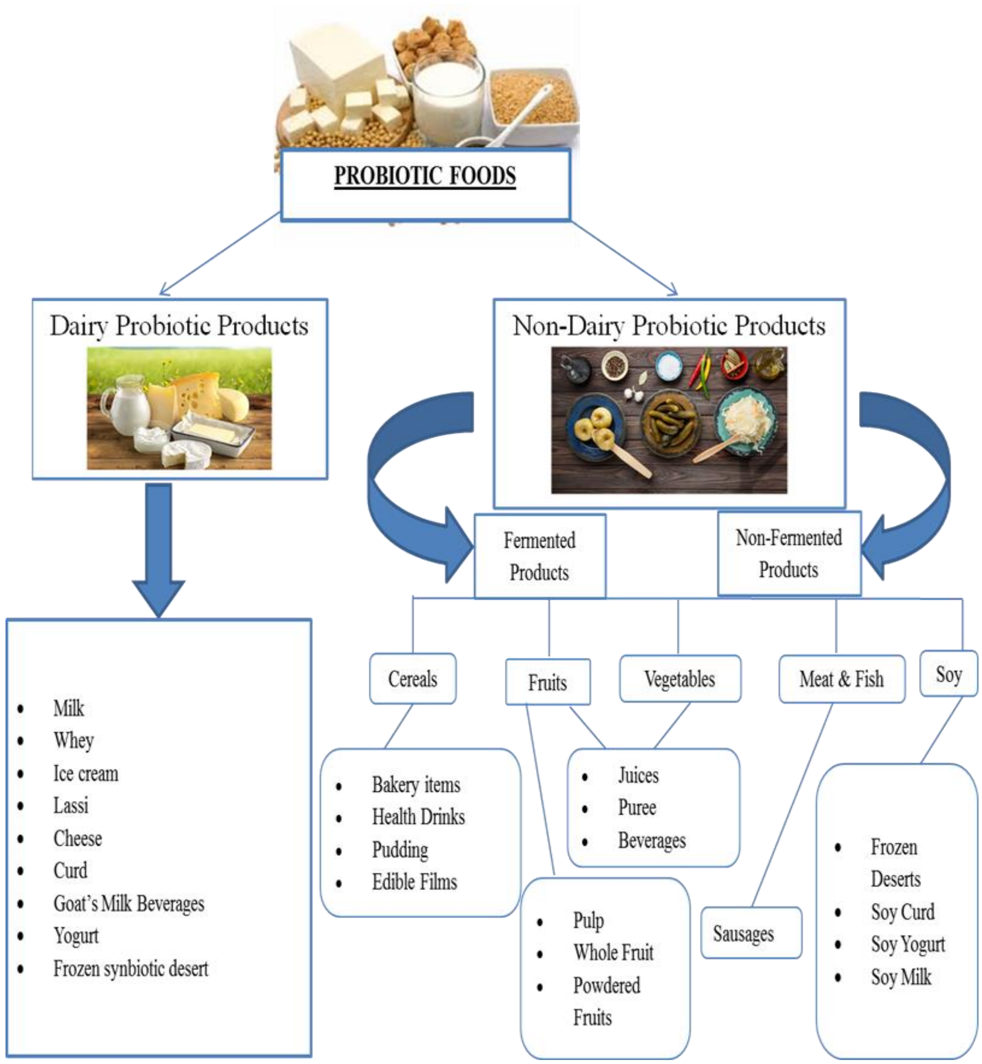


Figure 2.4: Classification of probiotic sources (Kumar *et al*., 2015).

2.7.1 Nondairy sources

Allergy to milk proteins, lactose intolerance, and high cholesterol content are some of the factors due to which people consume non-dairy sources of probiotics. Cereal-based fermented foods are widely being accepted among non-dairy sources. Cereals are a good source of dietary fiber. Fermentation of cereals increases the bioavailability of zinc and iron (Gawkowski and Chikindas, 2013; Gupta and Abu-Ghannam, 2012).

Fruit and vegetable juices being rich in nutrients are also a good non-dairy source of probiotics. They have a refreshing taste and are consumed by all age groups. The presence of sugar also supports probiotic growth. Additionally, as juices stay for a less time in the stomach, so the probiotic strain is exposed to the stomach acids for a limited time. *Lactobacillus* being resistant to an acidic environment is preferably used in juices rather than *Bifidobacterium* (Granato *et al*., 2010; Kumar *et al*., 2015).

2.7.2 Dairy probiotic sources

Even though fermented dairy products have a short shelf life, they are the most popular probiotic products on the market. Artisanal curds, such as Dahi, Matzoon, Kefir, and Lasi, are the most often consumed probiotic products. They contain a significant level of *Lactobacillus* that has health advantages when taken. According to recent studies, different *Lactobacillus spp*., such as *L. fermentum* and *L. rhamnosus*, have been isolated from fermented milk and yogurt (Ali *et al*., 2021).

Yogurt, fermented milk products, ice creams, and cheese are reported dairy probiotic sources. According to a study, mango and strawberry yogurts have a great number of *Lactobacillus* compared to plain yogurt. This shows the effect of pH on probiotic viability (Ranadheera *et al*., 2010). When eating frozen sweets and ice cream, a high concentration of probiotics is guaranteed due to the low storage temperature (Cruz *et al*., 2009). Probiotics are naturally present in milk which has a great nutritional value.

2.8 Lactic acid bacteria

The major group of probiotic microorganisms used in the food industry is lactic acid bacteria (LAB). They play a major role in food preservation and modification, by enhancing the aroma, texture, and quality of food products. *Lactobacillus rhamnosus*, *Lactobacillus delbreuckii*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus plantarum* and *Streptococcus thermophillus* are some of the numerous LABs that play a substantial role in the food industry (Amund, 2016).

LAB are characterized as fastidious, non-spore-forming, gram-positive aero-tolerant anaerobes. They are divided based on their morphology and glucose fermentation profile. The conversion of carbohydrates into lactic acid is the main end product of LAB fermentation. Homo-fermentative bacteria exclusively create lactic acid as a byproduct, but hetero-fermentative bacteria also produce carbon dioxide, ethanol, and other byproducts in addition to lactic acid. LABs are capable of synthesizing homopolysaccharides and heteropolysaccharides. Homopolysaccharides are composed of either glucans or fructans. Whereas heteropolysaccharides are made from a combination of glucose, galactose, and rhamnose (Prado *et al*., 2015). Characteristically LAB belongs to the *Lactobacillales* order which includes genera of *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragonococcus,* and *Weissella* (Duar *et al*., 2017).

2.8.1 *Lactobacillus* as probiotics

Lactobacillus is the most commonly used and researched probiotic genus. They are a crucial component of the human gastrointestinal flora, and they have a positive impact on the GIT environment. That is why, in general, they are considered safe. Many medical diseases have been linked to the usage of *Lactobacillus* as part of a regular dietary regimen, ranging from cancer to infantile diarrhea, antibiotic-related diarrhea, inflammatory bowel disease, and urogenital infections (Gomes and Malcata, 1999). They are also known for their production of antimicrobial compounds such as bacteriocins. Hence, *Lactobacilli* have a long history of use as an effective therapy for the amelioration and treatment of various pathological conditions. Mainly commercial probiotics include microorganisms from the *Lactobacillus* genus *L. rhamnosus*, *L. acidacidophilus plantarum*, *L. delbrueckii*, *L. helveticus*, *L. reuteri*, and *L. casei* (Raveschot *et al*., 2018).

2.8.2 *Streptococcus* as probiotics

The term *streptococcus* was first used by Rosenbach (1884) to refer to coccus-shaped, chain-forming bacteria that cause wound infections. *Streptococcus spp*. have complex nutrient requirements and ability to survive in environments with abundant carbohydrate and protein sources, such as animal digestive tracts, vegetables, and dairy products (Schleifer and Kilpper-Bälz, 1987). In this genus, *Streptococcus thermophilus* is an exception, as it is used as a starter in the manufacture of cheese, yoghurt, and other fermented foods. Because of its ease of growth and simple substrate environment, it has a long history of use as a starter culture. As a result, it has the potential to be employed as a solitary microbe for therapeutic purposes, in addition to its current use in dairy products (Cui *et al*., 2016).

2.9 Prerequisite of a probiotic candidate

Microorganisms that are or will be used as probiotics must meet specific selection criteria based on biosafety, technical factors, and functional features (Sanders, 2000). Survival, safety, and functionality are the most crucial characteristics for a strain to be considered probiotic and provide health benefits to the host. To provide the functional characteristics, probiotic candidate must be resistant to the effects of the upper gastrointestinal system such as acid, bile, saliva, and gastric juices to survive and metabolise there. It should have adhesion to the intestinal epithelium along with the ability to transiently colonize the gut in order to have antagonistic activity against pathogens. It must be able to control immune response, provide clinically demonstrated health benefits, and confer disease resistance either by enhancing immunity or by inducing the creation of an antimicrobial component in the gastrointestinal tract (de Melo Pereira *et al*., 2018).

With regard to technological aspects probiotics must remain viable during storage by tolerating stress conditions during processing. It is suggested that probiotic strains shall be abundant at the level of at least 10^7 cfu/g in the product to the date of minimum durability (Terpou *et al*., 2019). For biosafety the probiotic microorganism must be non-pathogenic, nontoxic and there must be no transferable antibiotic resistance genes. Moreover, it must be of human origin, genetically stable and capable of remaining viable for long periods in field condition. Lastly, probiotic microbes should have technologic properties for commercial viability such as stability of desired characteristics during processing, storage and transportation (Tripathi and Giri, 2014)

2.10 Probiotics food applications

Probiotics have long been employed as a starter culture in fermented foods due to their ability to boost nutritional content. LAB strains have been reported to improve the texture, aroma, flavor, and organoleptic qualities of the final food product. They cause rapid acidification of the raw material, resulting in the production of end product metabolites such as bacteriocin, lactic acid, exopolysaccharides (EPS), and a variety of enzymes that provide a product a variety of useful properties. Hence, starter culture is considered to be the microbial preparation which is introduced in the raw material to accelerate the process of fermentation (Linares *et al*., 2017).

Previously, the presence of microflora on the surface of the raw material was employed to cause spontaneous fermentation of food products. Then, because of the advantages of high control over the fermentation process and standardization of the end product, starting culture as a direct addition to food was favored. By growing in a media, a starter culture must be able to increase the number of cells. Synergism and antagonism, such as bacteriocins, which confer antimicrobial qualities, allow starter culture and auxiliary probiotic strain to interact. Starter cultures are now accessible commercially as freeze dried or lyophilized cultures (Leroy and De Vuyst, 2004).

Lactobacilli employed as commercial starter cultures have a number of metabolic characteristics like as acid tolerance, proteolytic activity, bacteriocin production, bacteriophage resistance, and exopolysaccharide formation, all of which promote the survival of LAB environmental stress conditions. These characteristics also influence the flavor and texture of the food products in which they are utilized as a starter culture (Alan and Yildiz, 2021).

The benefits of starter culture, aside from boosting the nutritional content of the food, have recently been investigated, and it was discovered that it is the functionality of starter culture that offers the host with health benefits. It's important to keep in mind while choosing a starter culture that it shouldn't create racemate, D-lactic acid, or biogenic amines (Vinicius De Melo Pereira *et al*., 2020). LAB probiotic cultures are among the most popular starter cultures. Dairy products are thought to be the best and safest way to get probiotics into the human intestine. Probiotic cultures are now employed in a variety of goods, including infant formulae, yoghurt, fermented milk, and nutritional supplements (Vijaya Kumar *et al*., 2015).

2.11 Yogurt

Yogurt is one of the most important functional diary product and is widely consumed all over the world (Abdel-Hamid *et al*., 2020). Historically, yoghurt has been acknowledged as "a healthful food" with medicinal benefits and hence, it can be the most suitable probiotic carrier. The word yogurt comes from the Greek phrase 'Yogurmak' which means to coagulate, thicken, or curdle. Milk products are thought to have been introduced in human diets between 10,000 and 50,000 BC. In 1932, the first yogurt laboratory was established in France. Yogurt is now defined as fermented milk containing live bacteria capable of acidifying a product with nutritional value (Fisberg and Machado, 2015).

Yogurt is a semi-solid food, produced by lowering the pH of milk proteins to their isoelectric point by fermentation of lactose using LAB. During yogurt formation milk lactose is transformed to lactic acid and a variety of other substances resulting in drop of pH from 6.5 to 4.5. Acidifying conditions inhibits the growth of undesirable microbes in yogurt, and the entire process breaks down the nutrients in the milk into a more digestible form, improving the availability of nutrients to consumers (Osorio-Arias *et al*., 2020). Potential pathogens such as *Salmonella enteritidis* and *Listeria monocytogenes* have been found to be inactivated in commercially made yoghurt.

Probiotic LAB have been reported to produce abundant bactericidal proteins in dairy foods (Guerra *et al*., 2001). Yogurt is made up of a unique structure. It has a high moisture content, similar to milk. After gelation, however, it becomes a solid-like structure because of casein micelles in milk aggregating to form a three-dimensional network (Bai *et al*., 2020).

There has been a substantial increase in the popularity of yogurt especially probiotic yogurt in recent years due to enhanced sensory characteristics and consumer acceptance. The conventional yogurt starter culture comprises of a symbiotic relationship between two LAB strains, *S. thermophilus* and *L. delbreukii subsp. bulgaricus* the addition of adjunct cultures such as *L. acidophilus* and *B. bifidum* into yogurt can further add nutritional and physiological values (Nguyen *et al*., 2014).

Yogurt is a well-known fermented product that is high in minerals such as calcium, phosphorus, and vitamins. Yogurt is not only beneficial for those with gastrointestinal problems and lactose intolerance, but it also helps to improve the immune system. The immune response of yogurt on animals have been investigated by many scientists. The impact of yogurt was checked on the incidence and duration of various forms of diarrhea (Kaur Sidhu *et al*., 2020). Several cohort studies have found strong links between yoghurt consumption and the incidence of Type 2 Diabetes. Higher consumption of yogurt and yogurt-based beverages has been linked to a reduction in body fat, a lower risk of cardiovascular disease, and improved cardio-respiratory fitness (Sarkar, 2019).

2.12 Probiotic market

Due to consumer awareness and more interest in natural nutrients and health promoting food, demand of functional probiotic food products has increased. Because of the bidirectional link between the gut microbiota and the lung, the gut–lung axis, nutritional status and food have a significant impact on the COVID-19 disease process hence increasing consumer demand of probiotics. Probiotics are a nutrient booster to keep consumer immune and strong. U.S. has seen a rapid increase of 33% in probiotic sales since the pandemic period (Batista *et al*., 2021).

By 2027, functional food market size is expected to reach around 268 billion U.S. dollars (Statista Inc., 2021). The global market for probiotics reached a value of about 49.4 billion U.S. dollars in 2018 and is expected to reach about 69.3 billion dollars by 2023 (Statista Inc., 2020). The global probiotic market is projected to reach USD 91.1 billion by 2026, at a compound annual growth rate (CAGR) of 8.3% (Polaris market research report, Jul 2021 (FB 2269).

The largest probiotic market is in Asia Pacific with European and North American markets taking the lead. Most commonly probiotics are available in capsules or sachet form. Top five probiotic products of 2022 available in market are listed in Table 2.2.

Product	Probiotic Concentration	Manufacturer
BlueBiotics	Mixture of 11 *Lactobacillus* strains with *S. boulardii* (61 Billion CFU)	BlueBiology
Ultimate Flora	10 probiotic strains (50 Billion CFU)	RenewLife
Raw Probiotics	34 probiotic strains (100 Billion CFU)	Garden of Life
Multi-Strain Probiotic	Misture of 31 strains including *Lactobacillus*, *Bifidiobacterium* complexes and 2 variants of *S. thermophillus*	Innovix Labs
Pro-25	13 probiotic strains (25 Billion CFU)	Vitamin Bounty

Table 2.2: Top five probiotics product of 2022 (Consumer's health report, 2022).

Other common commercially available probiotic products include; PRO-Kids, Ultimate Care, Mega Flora, Nexa Biotic, PB 8, Digest Gold, Yakult, Sofyl, Chamyto, Activia, Actimel, Danito, Lective, Biofibras. Nestle, DuPont, Danone, ADM, General Mills, Chr Hansen, Kerry and Probi are top probiotic manufacturers in market.

CHAPTER 3

MATERIALS & METHODS

3.1 Probiotic strains:

Five potential probiotic strains *Lacticaseibacillus rhamnosus* FM-9, Y-59, *Limosilactobacillus fermentum* FM-6, Y-55 and *Lactobacillus delbreukii* I-17 that were previously isolated and evaluated for their cholesterol reducing ability and *in vivo* survival by lab fellows (A Muneera, 2017) (A Zaigum, 2018) (Zafar *et al*., 2022) (Noor ul ain, 2019) were selected for this study to assess their suitability as starter or adjunct culture in dairy products. All strains were revived and propagated in De Man, Rogosa and Sharpe (MRS) broth followed by streaking to obtain pure colonies. Presumptive conformation was done by gram staining and catalase test (Da Silva *et al*., 2016).

Table 3.1: Indigenous Probiotic strains used in study

Serial No.	Strain	Origin
1	*Lacticaseibacillus rhamnosus* FM9	Traditional fermented milk (lassi)
2	*Limosilactobacillus fermentum* FM6	Traditional fermented milk (lassi)
3	*Limosilactobacillus fermentum* Y55	Artisanal Yogurt
4	*Lacticaseibacillus rhamnosus* Y59	Artisanal Yogurt
5	*Lactobacillus delbreukii* I-17	Pickel

3.2 Assessment of technological potential

3.2.1 Tolerance to NaCl

Tolerance of probiotic strains to high salt concentration was determined according to the method mentioned by Reuben *et al*., 2019. Briefly, overnight grown LAB cultures were inoculated (1% v/v) into MRS broth (Merck Millipore, Germany) supplemented with increasing concentration of NaCl (Sigma-Aldrich, Germany) i.e. 0.5%, 2.0%, 4.0%, 6.5%, and 10% (w/v) followed by overnight incubation at 37 °C. Viability of strains was assessed in triplicates by measuring absorbance at 600 nm by spectrophotometer (Optima SP-300, Japan). MRS broth without addition of NaCl was used as control.

3.2.2 Heat tolerance

Overnight grown cultures (10 mLf) were harvested by centrifugation (HERMLE Z 326 K, Germany) at 8, 000 ×g, 4 ºC for 20 min. Followed by twice washing with PBS (pH 7.5) (Appendix B) and then the cells were re-suspended in 10% (w/v) skim milk (Oxoid, UK). Plating was done in triplicates after serially diluting. Then these cell suspensions were placed in a water bath at 60 ºC for 5 minutes followed by immediate cooling in an ice bath. After exposure to heat immediately plating was done in triplicates. Viable cells were counted after 24 hour of incubation at 37 ºC (Chait *et al*., 2021).

3.2.3 Autolytic potential

Probiotic strains were screened for their autolytic potential by method as described by Nieto-Arribas *et al.*, 2009. Cells were harvested from actively growing cultures by centrifugation (HERMLE Z 326 K, Germany) at 5, 000 ×g, 4 ºC for 20 min followed by washing and resuspension of pellets in 20 mM sodium phosphate buffer (pH 6.8) (Appendix A). Lysis was monitored during 4 h of incubation at 37 °C by recording the decrease in OD_{650} using a UV/VIS spectrophotometer A & E Lab, UK. Percentage of lysis was calculated by the following formula:

$$\% \boldsymbol{Lysis} = \mathbf{100} - (\frac{\boldsymbol{A1}}{\boldsymbol{A2}} \times \mathbf{100})$$

Where, $\mathbf{A_1}$ represents the lowest absorbance and $\mathbf{A_2}$ represents highest absorbance measured during incubation. Autolytic activity of the test isolates was classified as follows: 35–66, good; 24–34, fair and 0–22, poor (Ayad *et al*., 2004).

3.2.4 Proteolytic potential

Proteolytic activity was evaluated by following the method described by Mercha *et al.*, 2020, with some modifications. Briefly, skim milk agar was prepared by supplementing Plate Count Agar medium (Merck Millipore, Germany) with 10% (w/v) skim milk (Oxoid, UK). 20 mL of overnight grown probiotic culture was centrifuged (HERMLE Z 326 K, Germany) at 6, 000 ×g, 4 ºC for 20 min and their supernatant was obtained. On skim milk agar plates 8 mm wells were drilled by a sterilized cork borer and up to 200 µL of cell free supernatant was added in each well. The plates were then incubated at 37 ºC for 18-24 h after which presence or absence of zone was observed. *Staphylococcus aureus* strain was taken from Integrative Biology and Genomics Lab, ASAB (NUST) and was used as control.

3.2.5 Diaceytl production

For the evaluation of diaceytl production overnight grown cultures were centrifuged at 4, 000 ×g, 5 ºC for 15 min. Obtained pellets were re-suspended in peptone water. 1% (v/v) of this bacterial suspension was inoculated in 10 mL of UHT whole milk (Nestle, Pakstan) and incubated at 37 ºC for 24h. Next day 1 mL of milk microbial culture was taken in sterile test tube followed by addition of 0.5 mL of 1% (w/v) α-naphthol solution and 16% (w/v) KOH solution. Cultures were incubated at 37 ºC for 10 min. Diacetyl production was indicated by the formation of a pinkish ring in tubes, which was further classed as faint, medium, or strong depending on the intensity of the ring color (Margalho *et al*., 2020). *Klebsiella pneumonia* was taken from Microbiology and Virology Research Lab, ASAB (NUST) and used as positive control.

3.2.6 Acidification potential

3.2.6.1 Acidification potential in MRS supplemented with 3% sucrose

2% (v/v) of overnight grown culture was inoculated in MRS broth supplemented with 3% (v/v) of sucrose (Sigma-Aldrich, Germany) solution. With the help of a pH probe meter (Hanna instruments HI 2211 pH/ORP meter, USA) drop in pH was noted after incubation of 37 °C at 0 h, 2 h, 4 h, 6 h and 8 h.

3.2.6.2 Acidification potential in skim milk supplemented with 3% sucrose

Acidification profile was also observed in skim milk media. Briefly, 2% (v/v) of overnight grown culture was inoculated in 10% (w/v) reconstituted skim milk (Oxoid, UK) supplemented with 3% (v/v) of sucrose (Sigma-Aldrich, Germany) solution. With the help of a pH probe meter (Hanna instruments HI 2211 pH/ORP meter, USA) drop in pH was noted after incubation of 37 °C at 0 h, 2 h, 4 h, 6 h, 8 h and 24 h (Mercha *et al*., 2020).

3.2.6 Viability in milk acidified with lactic acid

Overnight grown bacterial cultures were centrifuged (HERMLE Z 326 K, Germany) at 6, 000 ×g, 4 ºC for 15 min followed by twice washing of cells with PBS pH 7.4. The pellets were then resuspended in 500 µL PBS. This cell suspension was further transferred to 10 g/100 mL of skim milk (Oxoid, UK) acidified with lactic acid (Duksan Pure Chemicals Co. Ltd., South Korea) to a final pH of 4 and 5. Skim milk without lactic acid was used as control. Prepared cultures were stored at 5 °C and colony counts were performed on day 0, 15 and after 30 days in duplicates (Vinderola *et al*., 2008).

3.3 Yogurt production

3.3.1 Bacterial strains

Commercial yogurt starter culture containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was obtained by the courtesy of Dr. Muhammad Usman (Dairy Section Manager) University of Veterinary & Animal Sciences (UVAS), Lahore. Commercial yogurt starter culture was activated in both MRS and M17 broth followed by plating on same agar for obtaining pure cultures. For adjunct culture on basis of technological characterization best performer *Lacticaseibacillus rhamnosus* Y-59 was selected.

3.3.2 Preparation of yogurt inoculum

Initial yogurt inoculum was prepared according to the method of Mohan *et al*., 2020 with some modifications. Briefly, all cultures (commercial and adjunct) were grown in their respective broth. 2% of overnight grown cultures was inoculated in 10 mL of pasteurized milk (Prema´, Pakistan) and incubated at 37 ºC overnight. These initial yogurt cultures were stored at - 20 ºC and - 80 ºC for future use.

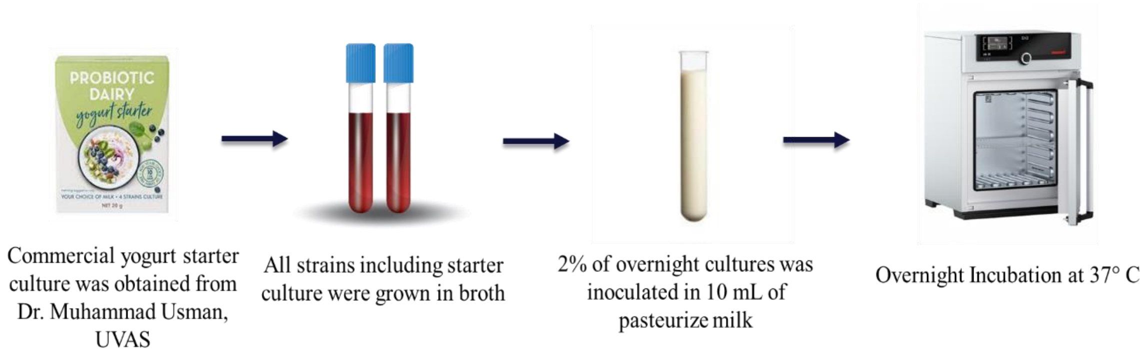


Figure 3.1: Preparation of initial yogurt inoculum.

3.3.3 Yogurt formation

Pasteurize milk (Prema´, Pakistan) was tempered at 45 ºC for about 15-20 minutes before inoculation was done at the rate of 2% v/v for each strain followed by incubation at 45 ºC until pH reached between 4.4 – 4.8. 0.5% w/v sucrose (Sigma-Aldrich, Germany) was used as sugar source and 0.25% w/v starch was use as stabilizer. Yogurt samples were prepared by using two different combinations of culture and coded as sample A: Commercial yogurt starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) and sample B: Commercial yogurt starter culture + *Lacticaseibacillus rhamnosus* Y-59. Sample A was used as a positive control, sample B was formulated to evaluate the potential of *L. rhamnosus* Y-59 for suitability as adjunct culture. Coagulation was observed and pH was recorded (Sarwar *et al*., 2019). Prema´ natural yogurt (C) was used as reference commercial control.

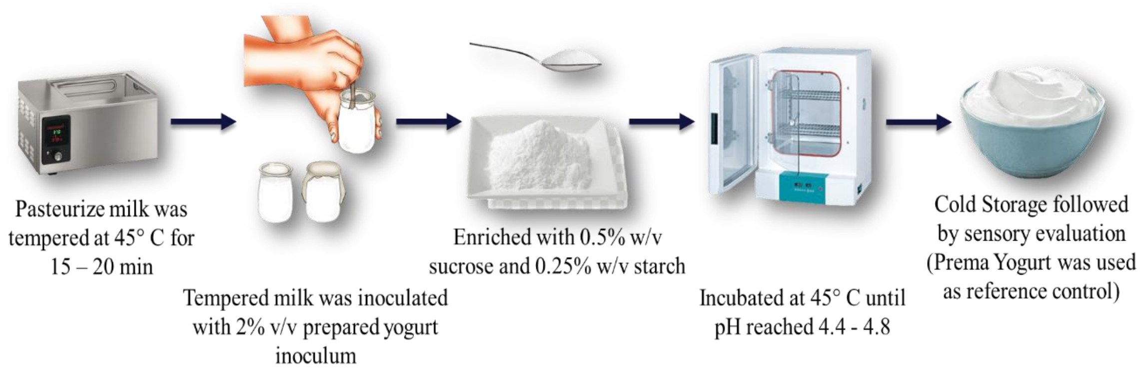


Figure 3.2: Steps for yogurt formation.

3.4 Sensory evaluation

Forty people were selected randomly and provided with the yogurt samples to taste. They were asked to note the appearance, aroma, taste, texture, odor and overall acceptability of yogurt samples which were randomly coded (Appendix C). Sensory evaluation was applied using a 9- point hedonic scale (1 Very Bad, 9 Excellent) (Abdel-Hamid *et al*., 2020).

3.5 Statistical analysis

One-way ANOVA comparison test was applied followed by Duncan's test through Graph Prism Pad.

CHAPTER 4

RESULTS

4.1 Tolerance to NaCl

All tested strains were resistance to NaCl and showed significant growth in tested conditions. All *Lactobacillus* isolates showed high tolerance to salt there growth decreased gradually with increasing concentration of salt (Figure 4.1). Most of the strains showed normal growth pattern however *Lactobacillus delbrueckii* I-17 showed exceptionally well growth even in presence of 10% NaCl and remained most viable with least growth reduction followed by *Lacticaseibacillus rhamnosus* Y 59. Hence, they can be used in high salt concentration food products.

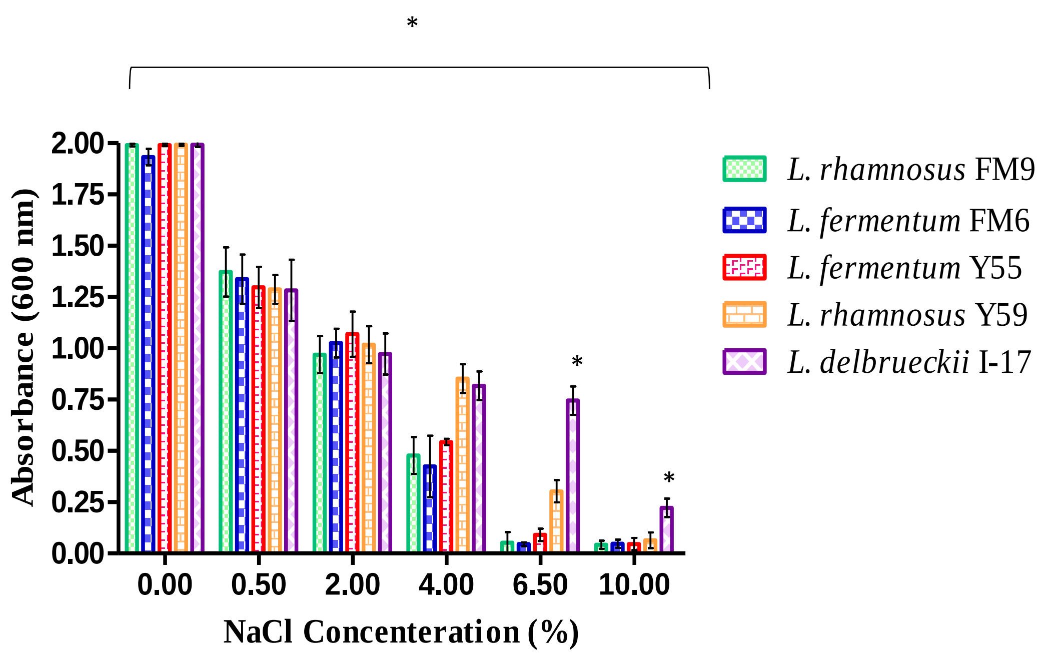


Figure 4.1: Survival of selected probiotic strains in increasing salt concentration. *Superscripts differ significantly ($P \leq 0.05$)

4.2 Heat Tolerance

The selected isolates were able to survive after 5 minutes of heat shock at 60 ºC (Table 4.2). Reduction in viable cell counts was measured in heat death and ranged from

0.11 Δlog (*L. delbrueckii* I-17) to 2.86 Δlog (*L. fermentum* Y 55). *L. delbrueckii* I-17 and *L. rhamnosus* Y 59 being most heat tolerant could be used in heat processed food.

Table 4.2: Effect of heat on viability of probiotic strains in terms of heat death.

Strain	Heat death (Δlog)
L. fermentum **FM 6**	0.45 ± 0.07^{AB}
L. rhamnosus **FM 9**	0.89 ± 0.54^{BC}
L. delbrueckii **I-17**	0.11 ± 0.09^{A}
L. rhamnosus **Y 59**	0.27 ± 0.05^{AB}
L. fermentum **Y 55**	2.86 ± 0.15^{E}

Values are means (±SD) of 3 repetitions. Different superscript letters (A-E) within a column indicate significant ($p < 0.05$) differences among mean observations

4.3 Autolytic Potential

Although based on reported classification (Ayad *et al*., 2004) the tested isolates show poor autolytic activity (Figure 4.3). However, among these strains *L. rhamnosus* Y-59 showed highest autolytic potential of about 14.58% after 4h of incubation followed by *L. delbrueckii* I-17 (13.58%) and *L. fermentum* FM-6 (12.62%). Hence, *L. rhamnosus* Y-59 represents a promising application in fermented foods in terms of modification of the aroma and flavor.

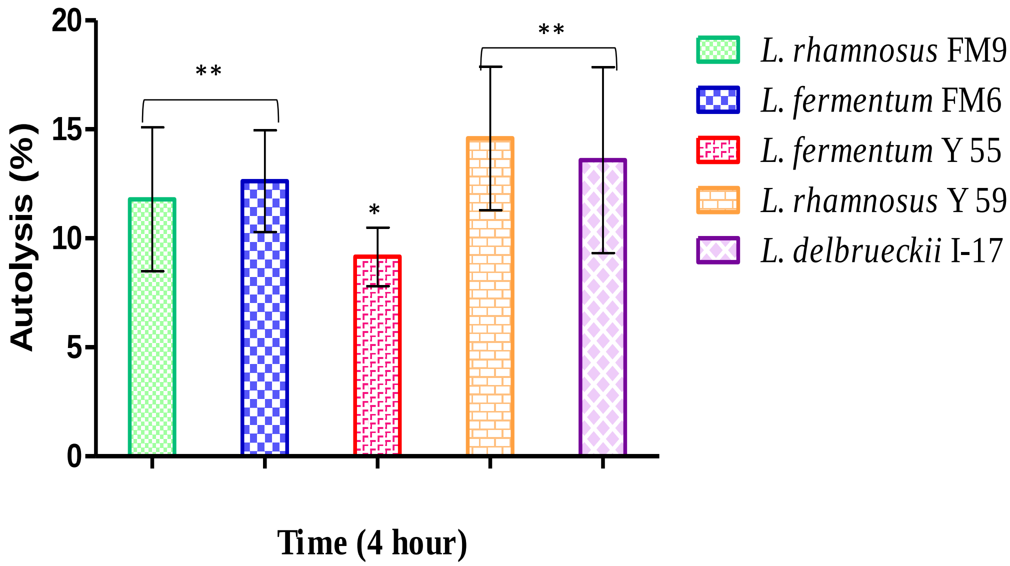
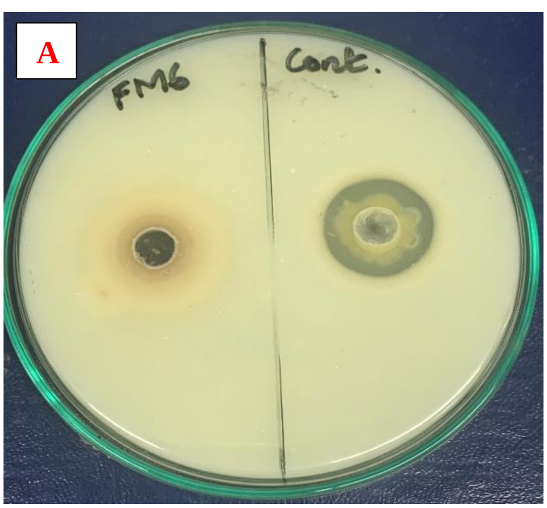
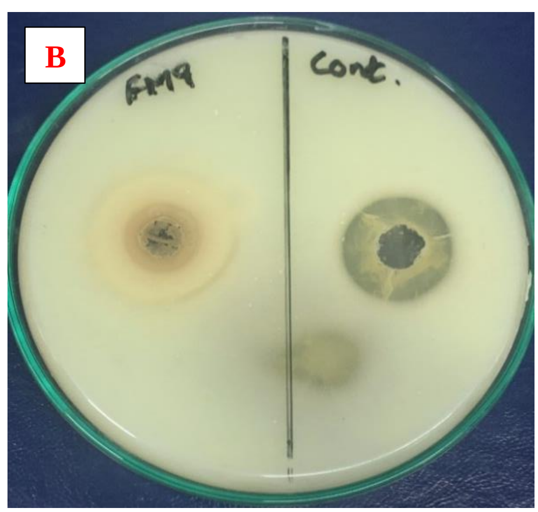


Figure 4.3: Autolytic activity of isolates. Standard error (n = 3 independent experiments) is indicated in error bar. *Superscripts differ significantly ($P \leq 0.05$) **Superscripts non-significant ($P \leq 0.05$).

4.4 Proteolytic Potential

Tested strains didn't show prominent proteolytic activity and hence they can be characterized as weekly proteolytic and *L. delbrueckii* I-17 as non-proteolytic (Figure 4.4). This indicates that they can't be used as starter culture but can be use as adjunct culture.





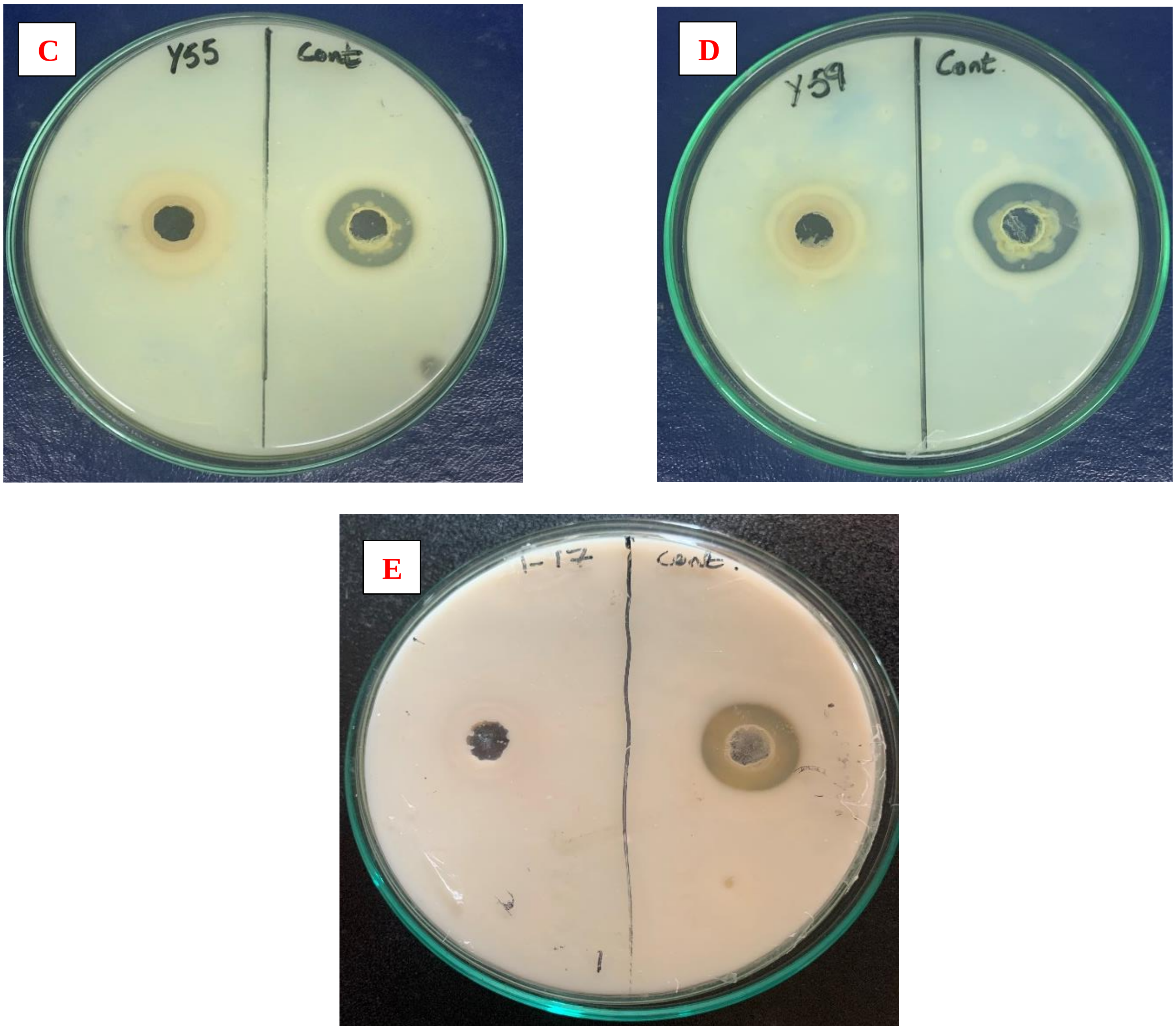


Figure 4.4: Proteolytic activity on skim milk agar plates (A) *L. fermentum* FM 6 (B) *L. rhamnosus* FM 9 (C) *L. fermentum* Y 55 (D) *L. rhamnosus* Y 59 (E) *L. delbrueckii* I-17.

4.5 Diacetyl production:

The ability of selected probiotic strains to produce aromatic compound such as diacetyl was evaluated by Voges Proskauer (VP) test by distinguishing red/pink ring on top (Figure 4.5). Only *L. fermentum* FM 6 and *L. rhamnosus* Y 59 were found to have metabolic potential to metabolize citrate and hence, they can be used as adjunct culture in dairy products to impart distinct flavor and buttery aroma due to its diacetly production potential overall contributing to the flavor enrichment of fermented dairy products.

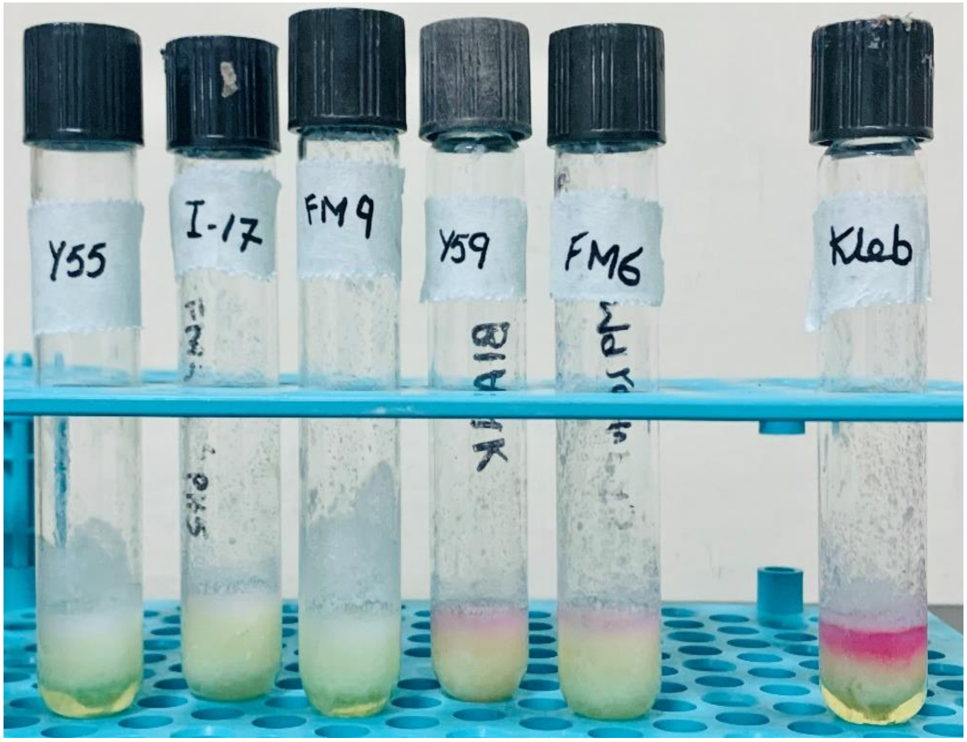


Figure 4.5: Qualitative analysis of diacetyl production.

4.6 Acidification Potential

All strains were evaluated for their acidification potential both in MRS broth and skim milk.

4.6.1 Acidification potential in MRS broth supplemented with 3% sucrose

All isolates showed good acidification profiles in MRS broth within 8 h of incubation. *L. delbrueckii* I-17 showed highest acidification rate followed by *L. rhamnosus* Y 59 and *L. fermentum* FM 6 (Figure 4.6.1). By reducing the pH of the MRS medium to 5.0, the strains demonstrated considerable acidification characteristics.

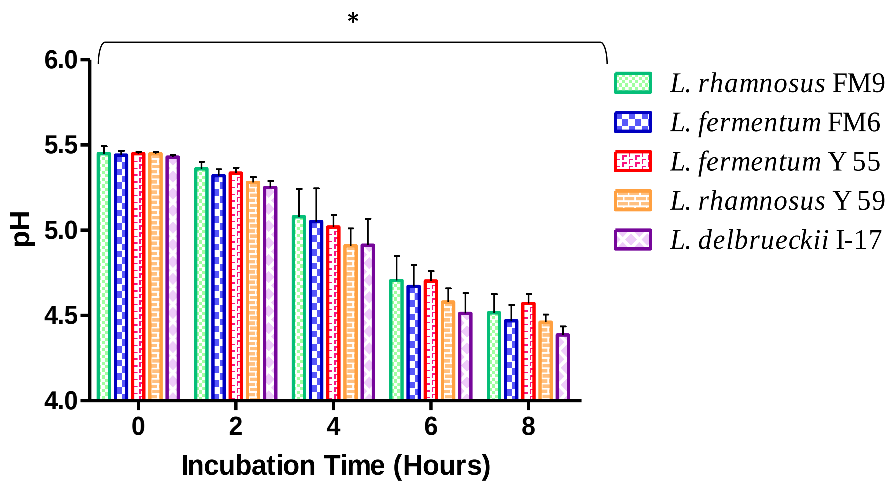


Figure 4.6.1: pH kinetics in MRS broth supplemented with 3% sucrose. *Superscripts differ significantly ($P \leq 0.05$)

4.6.2 Acidification potential in skim milk supplemented with 3% sucrose

Rate of acidification in skim milk was slow as compared to MRS broth (Figure 4.6.2). However, after 24h of incubation *L. delbrueckii* I-17 and *L. rhamnosus* Y 59 showed significant results with a drop in pH from 6.30 to 3.75 and 6.29 to 3.89 respectively. Hence, they can be used for yogurt production. Overall, results show our isolates are suitable candidates for use in the dairy fermentation process, where a rapid pH reduction is a critical step for milk coagulation and limiting the formation of unwanted microflora.

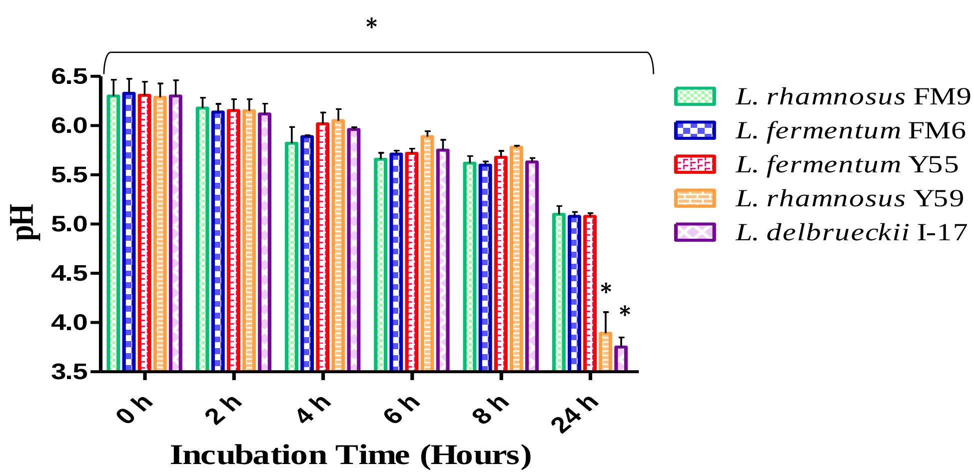


Figure 4.6.2: pH kinetics in skim milk supplemented with 3% sucrose. *Superscripts differ significantly ($P \leq 0.05$).

4.7 Viability in milk acidified with lactic acid

All probiotic strains remained viable and showed normal growth rates in milk acidified with lactic acid for upto 30 days of storage at 5 °C.

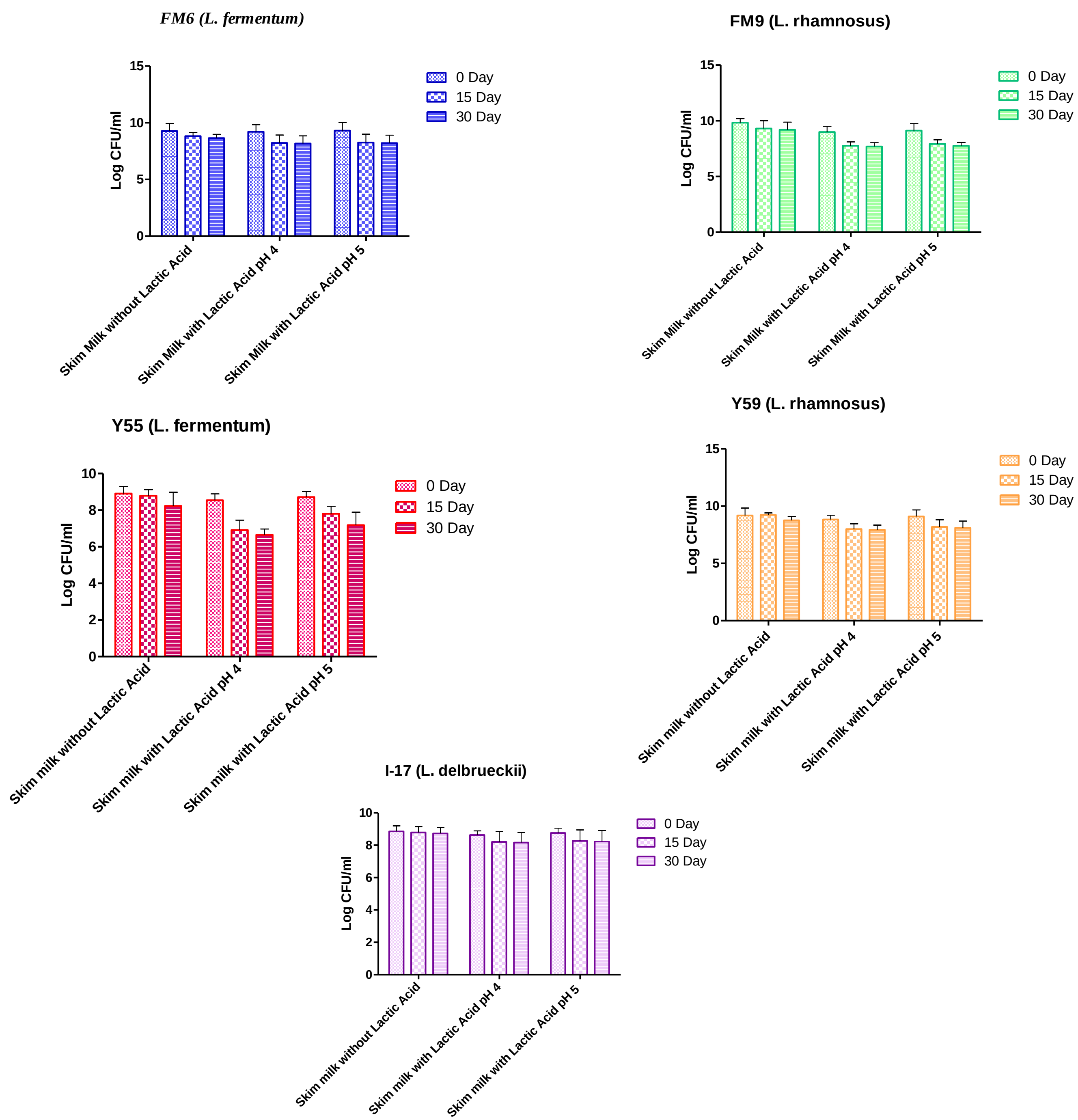


Figure 4.7: Viability profiles in milk acidified with lactic acid.

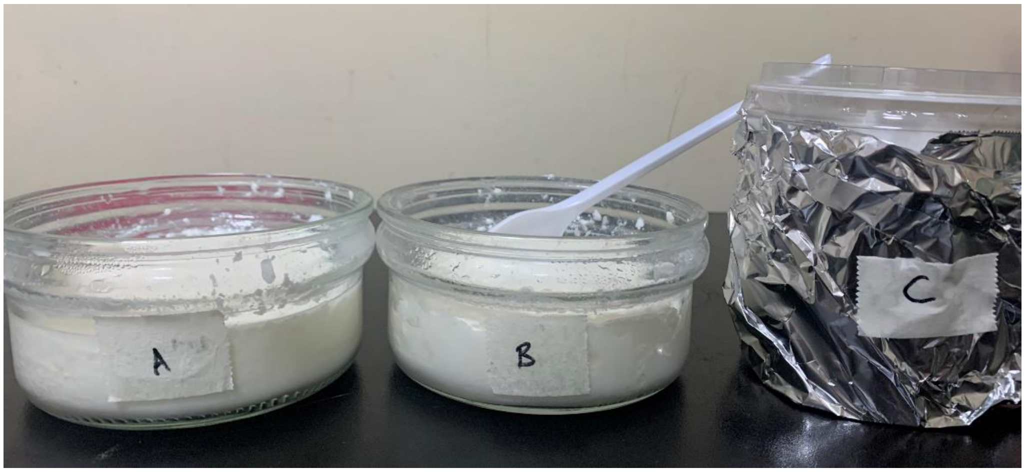
4.8 Yogurt formation

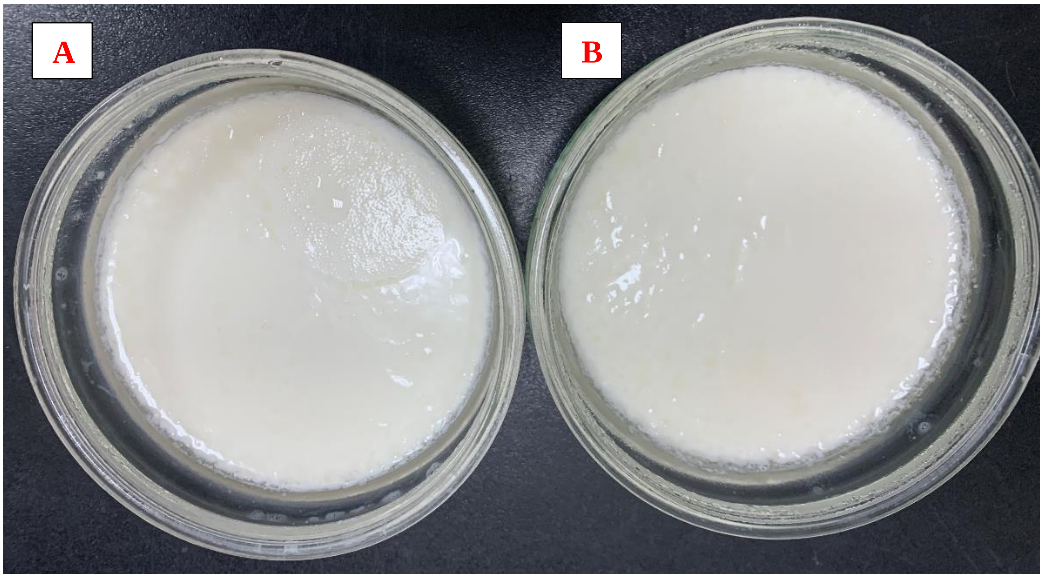
2 yogurt samples were prepared in independent triplicates and stored at 4 °C for 1 hour prior sensory evaluation. PH of each combination was recorded (Table 4.8). Prema´ natural yogurt (C) was used as commercial control.

Table 4.8.1: Combination of bacterial culture for yogurt formation.

Yogurt Code	Culture Combination	Incubation Temperature (°C)	Incubation Time (h)	pH
A	Commercial starter culture (*Streptococcus thermophillus* and *Lactobacillus bulgaricus*)	45	4	4.6
B	Commercial starter culture + *Lacticaseibacillus rhamnosus* Y59 (indigenous)	45	4	4.67

The three yogurt samples were compared for their consumer acceptance by sensory evaluation. Sample B showed stand out results in all sensory attributes evaluated i.e. appearance, taste, smell, texture and overall acceptability as compared to other two yogurt samples (Figure 4.8). Thus, proving that *L. rhamnosus* Y 59 can be used as adjunct culture for production of yogurt as it not only improves taste, organoleptic properties but can also reduce serum cholesterol level.





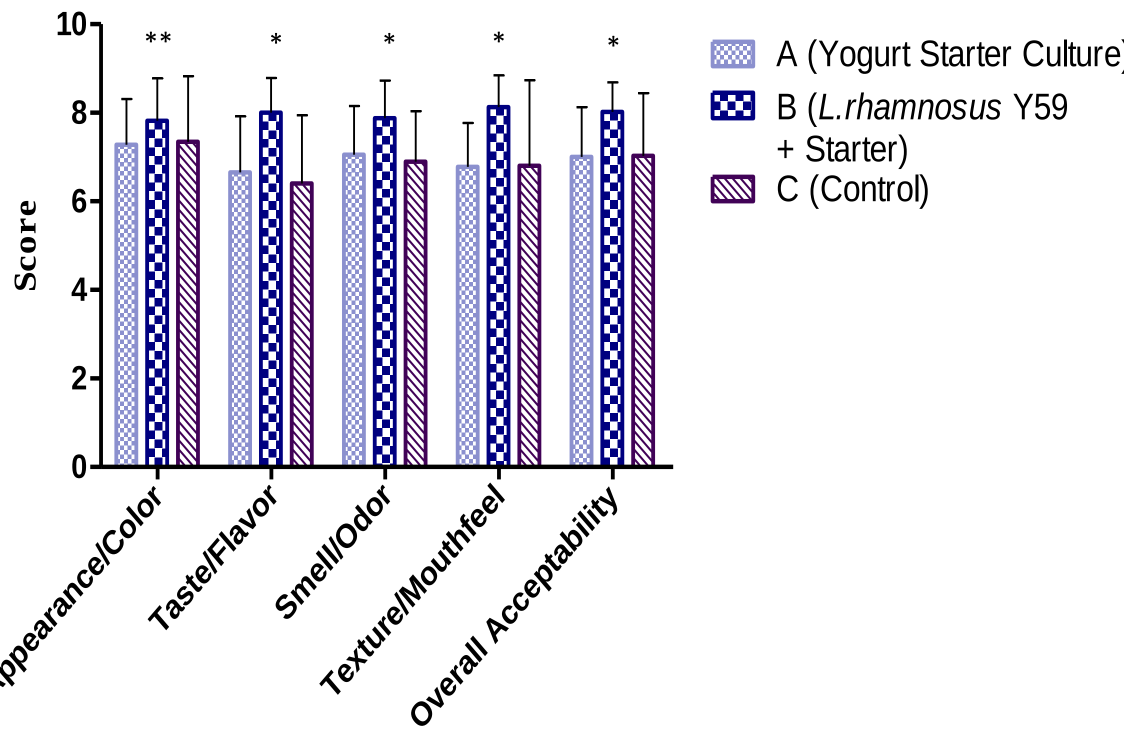


Figure 4.8: Sensory evaluation of Yogurt. Standard deviation indicated by mean error bars.
*Superscripts differ significantly ($P \leq 0.05$) **Superscripts non-significant ($P \leq 0.05$)

Table 4.8.2: Sensory score of yogurt samples

No. of Participant	Appearance/Color			Taste/Flavor			Smell/Odor			Texture/Mouthfeel			Overall Acceptability		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	9	7	8	9	7	4	7	7	8	5	9	4	8	8	4
2	9	8	9	6	9	7	9	9	7	9	8	8	8	8	8
3	7	7	5	7	8	7	8	8	7	6	8	7	7	8	7
4	7	7	9	7	8	7	6	9	8	7	8	8	8	8	8
5	7	8	7	7	8	6	7	8	7	7	8	7	7	8	7
6	6	8	7	5	8	5	7	8	6	6	9	5	6	8	5
7	7	9	9	8	8	7	9	9	7	7	8	9	8	9	8
8	7	7	6	6	8	5	6	8	6	7	8	5	8	8	6
9	6	8	4	5	8	4	6	6	5	5	8	6	3	8	6
10	7	8	7	5	8	3	7	8	5	7	8	1	7	8	4
11	6	8	8	5	8	8	7	8	7	6	8	8	5	8	8
12	8	7	8	9	8	7	8	9	8	8	7	9	8	7	8
13	7	9	9	7	9	8	7	9	8	8	9	8	7	9	8
14	5	8	7	8	7	8	8	7	7	6	7	8	6	7	8
15	5	7	8	4	6	6	6	7	6	4	8	7	5	7	7
16	8	4	7	7	6	6	9	7	6	8	7	6	8	7	6
17	8	8	6	5	9	7	8	8	7	8	8	6	6	8	7
18	8	8	8	6	9	8	7	8	8	7	8	7	7	8	9
19	7	8	9	7	9	7	7	8	8	7	8	8	7	8	8
20	7	7	9	7	8	9	7	7	9	7	8	9	7	8	9
21	7	8	7	8	8	7	7	9	7	8	9	7	9	8	7
22	7	7	5	7	7	5	6	7	4	8	8	6	7	8	6
23	9	8	9	7	7	9	9	7	9	7	9	9	6	8	9
24	7	9	7	8	8	7	8	9	6	7	9	8	7	9	8
25	7	8	5	7	9	7	6	9	7	5	9	8	7	9	8
26	7	7	8	5	7	4	6	6	8	6	6	4	6	7	4
27	7	7	9	6	8	7	8	8	7	6	8	8	7	8	8
28	8	9	5	5	8	7	3	7	7	7	8	7	8	9	7
29	7	9	9	7	9	7	7	9	8	6	9	8	7	9	8
30	9	8	7	8	8	6	7	8	7	7	9	7	7	9	7
31	6	7	7	5	8	5	7	7	6	7	7	5	6	7	5
32	9	8	9	8	9	7	8	7	7	7	8	9	9	8	8
33	8	9	6	9	8	5	7	8	6	8	8	5	8	8	6
34	7	9	4	7	8	4	7	9	5	7	9	6	7	9	6
35	7	8	7	7	8	3	7	8	6	7	8	1	7	8	4
36	6	8	8	6	8	8	6	8	5	6	8	8	6	8	8
37	7	7	8	6	8	7	6	8	7	6	8	9	7	8	8
38	9	9	9	6	7	8	7	7	8	7	7	8	7	7	8
39	8	9	7	8	9	8	7	8	8	7	9	7	8	9	8
40	8	8	8	6	9	6	7	8	7	7	9	6	8	7	7
Mean=	7.275	7.825	7.35	6.65	8	6.4	7.05	7.875	6.875	6.775	8.125	6.8	7	8.025	7.025
SD=	1.037	0.958	1.477	1.272	0.784	1.549	1.108	0.853	1.137	1	0.723	1.9375	1.132	0.66	1.423

Sensory evaluation by volunteers using commercial starter culture sample A (control: *S. thermophillus* and *L. bulgaricus*), experimental yogurt sample B (Commercial starter culture + indigenous *Lacticaseibacillus rhamnosus* Y 59 and reference commercial yogurt sample C. Score index ranging from 1-9, 1 = Very bad and 9 = Excellent.

CHAPTER 5

DISSCUSION

The purpose of the present study was to evaluate the technological potential of previously isolated *Lactobacillus* strains for their suitability as adjunct or starter culture. Five indigenously isolated potential probiotic strains of dairy origin were selected for their suitability as starter or adjunct culture for yogurt production. Selected strains; *Lactobacillus delbrueckii* (I-17), *Lacticaseibacillus rhamnosus* (Y-59 and FM-9) and *L. fermentum* (FM-6 and Y-55) were previously evaluated for their probiotic and cholesterol reducing potential. The protocols for technological characterization were optimized. The results of the study confirmed the suitability of protocols for intended purpose.

Probiotics have traditionally been used in dairy products being optimum vehicles for probiotic transfer. Probiotic strains must initially meet a number of technological requirements, including maintaining viability during food processing and storage, product feasibility, and food physicochemical processing resistance. Proteolytic activity on milk caseins, starter culture compatibility, fermented food pH tolerance, and stress during packaging should all be taken into account. Moreover, final probiotic dairy products must have good sensory properties, while retaining their functionality (Peirotén *et al*., 2019).

For a functional probiotic strain in food products it must be able to survive in stress conditions like salt and heat tolerance. It is important for stability of strain in the final product. Sodium chloride is a key element in the food industry, as it improves the sensory qualities of products while also meeting the recommended daily NaCl consumption for humans. Furthermore, NaCl is commonly employed as a preservative, especially in long-term storage cheeses (Meng *et al*., 2018). Results suggest that all strains were able to tolerate 10% NaCl which is in accordance to previous studies. Reuben *et al*., 2019 reported LAB isolates to be tolerant to 6.5% NaCl with OD > 0.500 however minimum OD of 0.310 was also noted. Further at 10% NaCl very weak growth was observed ranging from 0.115 to 0.177. Whereas, Prabhurajeshwar and Chandrakanth (2017) reported OD ranging 0.5 to 2.5 at 2% Nacl, 0.5 to 1 at 4% NaCl and at 6% ≤ 0.5. LAB tolerate salt by a variety of mechanisms, including the uptake or production of a restricted number of solutes (de Almeida Júnior *et al*., 2015).

Resistance to heat is an important technological aspect. High temperature during processing can hinder the cell growth. Hence, probiotic strains which are able to adapt to stress conditions during processing and storage along with being thermotolerant are of immense interest in the potential functional food industry (Saarela *et al*., 2004). The ability of probiotic strain to survive in high temperature not only ensures their viability in the end product but also enhances their performance during food processing (Desmond *et al*., 2002). Hence stress tolerant probiotic strains may flourish the future of probiotic technology. The reduction in growth 0.9 to 2.5 log CFU/ml of LAB isolates was noted when they were exposed to heat treatment at 60 ºC for 5 min (Abushelaibi *et al*., 2017). The results of this study verifies with outcomes reported by Abushelaibi *et al*. (2017). Whereas Santos *et al*. (2016) reported heat death of *Lactobacilli* ranging 1.94 to 3.39 log CFU/ml which is slightly more than these results. However du Toit *et al*. (2013) noted a higher death reduction rate for probiotics ranging from 0.95 to 4.91 log CFU/ml.

Autolytic potential is defined as the ability of self-produced enzymes to break down all or part of a cell or tissue. Certain bacteria release intracellular enzymes during autolysis, which modify the aroma and flavor of fermented foods and could be a beneficial characteristic in some food mediums (Shivangi *et al*., 2020). Autolysis is characterized as a strain dependent property and is a desirable trait during cheese ripening. In our study autolysis of *Lactobacillus* strains increased by 9.15%-14.58% within 4 h of incubation. Although our strains are characterized as weekly autolytic but still the presence of autolytic activity represents a promising application in fermented foods. Nieto-Arribas *et al*., 2009 reported 20% of its tested *Lactobacillus* strains to be poorly autolytic with percentage lysis up to 14% which is in agreement to the present study. Moreover, Meng *et al*., 2018 reported less than 10% autolytic activity for 1 strain of *L. rhamnosus* even after 24 h of incubation. Hence, all these results validates our findings.

Proteolytic system is essential for the optimal growth in milk and also contributes to flavor development in milk products. LAB use their proteolytic activity to degrade peptidase and proteins in order to generate different metabolites that contribute to flavor, antimicrobial activity, and structure of different food products. Good proteolytic activity results in higher levels of soluble proteins and delivery of free amino acids in food matrices (Sharma *et al*., 2018). Tested strains didn't show prominent proteolytic activity and hence they can be characterized as weekly proteolytic. This indicates that they can't be used as starter culture but can be use as adjunct culture.

Different flavors emerge from the microbial synthesis of aromatic compounds in fermented foods like yogurt and cheese. For example, diacetyl is a byproduct of citrate metabolism, has antimicrobial properties against food-borne infections but isn't found in all types of LAB. It is an essential component of many dairy products as it imparts a distinct flavor and a buttery aroma even at low concentration (Melo *et al*., 2021). In our study, only two strains *L. fermentum* FM 6 and *L. rhamnosus* Y 59 were found as diacetyl producers. Our results are in agreement to previous studies. Meng *et al*., 2018 reported 67% of *L. rhamnosus* as diacetyl producers. de Albuquerque *et al*., 2018 characterized five of its *L. fermentum* strains as medium diacetyl producers.

The capacity of LAB strains to produce more acid by reducing the pH of the growing medium more effectively qualifies them as potential candidates for starter culture fermentation (Banwo *et al*., 2013).. The *Lactobacillus* strains tested in our study were good acidifiers with pH reduction in the range of 4.57 to 4.386 after 8 h of incubation in MRS broth. Acidifying potential in skim milk with a pH lowering to about 3.75 after 24 h of incubation was observed. Hence, our results compiles with the findings already reported. According to Zalán *et al*., 2010 *Lactobacillus* strains exhibited faintest fermentation pH profile (6.2 to 5.5) in reconstituted skim milk after 18 h of incubation and a maximum decrease of pH 3.7 from initial control pH of 5.5 ± 1 after 18 h of incubation in MRS broth. For LAB strains Ribeiro *et al*., 2014 reported pH ranging from 5.39 to 4.90 after 6 h and 5.36 to 4.64 after 24 h in skim milk. After 24 h of incubation Briggiler-Marcó *et al*., 2007 classified *L. rhamnosus* and *L. delbrueckii* as fast milk acidifying strains with maximum and minimum pH of 5.78 ± 0.19 and 3.73 ± 0.03 respectively. Many factors affect the acidifying kinetic parameters such as probiotic strain, the food matrix, and milk supplementation. Difference between acidifying properties of *Lactobacillus* strains may be attributed to the specificity of each strain to breakdown the milk constituents, genetic makeup of bacteria, fluctuation in incubation temperature, long lag phase which is due to transfer of bacteria from broth to milk or might be deficiency in the nutrient transport system of fermentable sugars (Soomro and Masud, 2008).

The survival of strain in food product and its shelf life during storage depends on many factors such as food matrix attractions, medium, pH, additives and lactic starter metabolites. The basic requirement for the assumption of any microbe to be probiotic is its ability to tolerate pH because it should be able to survive in the highly acidic stomach environment along with maintain high growth rate during fermentation in fermented food products like yogurt. All the tested strains showed good viability in milk acidified with lactic acid. The similar results were also reported by Vinderola *et al*., 2008. pH tolerance is a specie and strain specific property (Montville and Matthews, 2013). The difference in stimulation of H^+-ATPase activity which results in the removal of protons (H^+), external environment alkanization and varying composition of cell envelope contributes to acid tolerance (Cotter and Hill, 2003). The reduction in growth may be due to production of H^+ ions by acid which directly affects the strains' cell wall and metabolism (Doyle and Buchanan, 2012). *Lactobacillus* spp. are reported as the most tolerant species of probiotics to acidic conditions and this property attributes to their reasonable growth in food fermentation (Devirgiliis *et al*., 2009). Probiotics being gram positive uses F_0F_1-ATPase activity to survive under low pH (Corcoran *et al*., 2005). They also produce certain polysaccharides which act as buffer and reduce the effects of decreasing pH on cell (Barakat *et al*., 2011).

Suitability of *L. rhamnosus* Y 59 as adjunct culture was evaluated by making yogurt with the commercial starter culture and resulting yogurt was subjected to sensory evaluation (Figure 4.8). The produced probiotic yogurt was better in evaluated organoleptic attributes such as taste, flavor, aroma and texture. Hence, according to our results *L. rhamnosus* Y 59 can be used as adjunct for yogurt production. Previously, probiotic yogurt containing *L rhamnosus GR-1* was reported for its consumer acceptability therefore validates our study (Hekmat and Reid, 2006).

CONCLUSION

Fermented foods have been considered as one of the first processed food products consumed by humans, for their superior functional and nutritional properties. In conclusion, this study reveals the tested five *Lactobacillus* strains have high potential for probiotics application. Tested traits exhibited by these LAB strains are of industrial and technological relevance as well as for preservation.

Considering all the screening results, *Lacticaseibacillus rhamnosus* Y 59 was considered technologically active and showed the best probiotic potential among the strains and was further evaluated for yogurt production. Our results show that *L. rhamnosus* Y 59 could be used as adjunct culture for yogurt production with potential probiotic functional properties and it has no adverse effects on the quality parameters of the yogurt as verified by the sensory evaluation. *L. rhamnosus* Y59 could be used as adjunct culture for yogurt production. The strain has no adverse effects on the product (yogurt). The yogurt prepared with *L. rhamnosus* Y 59 has good consumer acceptance and all the sensory attributes of the product are better or comparable to industrially prepared yogurt.

However, subsequent in vitro and clinical studies should be performed to verify any potential health benefits. Further in vivo tests must be realized to guarantee its safety and probiotic effects prior its use in food products. Moreover, this probiotic yogurt can be used to study its hypocholesterolemic effect in vivo trails. EPS production, bacteriocin extraction and its characterization can be evaluated. Different metabolites produced by the strains can be evaluated. The effect of multiple strains in combination can be evaluated. Further in silico genome-based analysis can be done to further validate our findings. This indigenous probiotic yogurt can be used as a delivery vehicle for *L. rhamnosus* Y 59 for clinical trials. The effect of multiple strains in combination can be evaluated. The potential of *L. rhamnosus* Y 59 to manufacture cheese and other fermented food products can also be investigated.

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APPENDICES

Appendix A

20 mM Sodium Phosphate Buffer pH 6.8

Sr. No.	Ingredients	Amount/L
1.	$Na_2HPO_4.7H_2O$	2.625 g
2.	$NaH_2PO_4. H_2O$	1.409 g
pH adjusted by 1 N HCl and 1 N NaOH		

Appendix B

Phosphate Buffered Saline (PBS) pH 7.4 - 7.5

(Mishra and Prasad, 2005)

Sr. No.	Ingredients	Amount/L
1.	NaCl	8.06 g
2.	KCl	0.22 g
3.	Na_2HPO_4	0.85 g
4.	KH_2PO_4	0.20 g
5.	Sodium Acetate	1.0 g
pH adjusted by 1 N HCl and 1 N NaOH		

Appendix C

SENSORY EVALUATION CONSENT FORM

Project Title: Technological characterization of cholesterol-lowering probiotic *Lactobacillus* strains for the synthesis of dairy products

You are invited in a study involving indigenous probiotic yogurt sensory evaluation. The overall objective of this study is to develop a yogurt supplemented with potential probiotic strain *Lacticaseibacillus rhamnosus* (Y-59). Probiotics have beneficial health effects on consumer ranging from being antimutagenic, anticarcinogenic, enhancement of lactose metabolism, prevention against diarrhea, decreased cholesterol level in the body and competitive activity against pathogenic microbes but most important for a probiotic strain is to be nonpathogenic and attain a status of generally regarded as safe (GRAS). *L. rhamnosus* is being used worldwide to produce probiotic products like fermented milk, yogurt, ice-cream, cheese, pickles etc.

The strain used in this study, previously have been characterized safe in pre-clinical in vivo trials along with exhibiting excellent ability to reduce serum cholesterol level. In this context, you will be asked to taste the yogurt samples supplemented with *L. rhamnosus* and rate them for intensity of each characteristic. There are no risks or discomforts expected as a result of your participation. If you have prior experience of any allergic reactions to dairy products, you should not participate in this study.

Your participation in this research is confidential. Responses are coded to be confidential and any publications or presentation of the results of the research will only include information about group participation. Names or other identifiable information will not be disclosed or published.

I ______________________ understand the above information and voluntarily consent to participate in the study described above. I have been given a copy of this consent form.

Signature ____________________ Date ____________________

<u>SENSORY EVALUATION PERFORMA</u>

Name: __________________ **Date of Evaluation: __________________**

Sample Code: ____

INSTRUCTIONS: You are presented with 3 yogurt samples. Kindly taste and evaluate them for the following characteristics given below. Finish evaluating a sample before proceeding to the next. Wash your mouth with tap water in between evaluations. You can taste the sample more than once.

	Appearance/Color	**Taste/Flavor**	**Smell/Odor**	**Texture/Mouthfeel**	**Overall Acceptability**
Very bad					
Bad					
Imperfect					
Sufficient					
Mediocre					
Satisfactory					
Good					
Very good					
Excellent					

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