ASSESSMENT OF INDIGENOUS *LACTOBACILLUS* STRAINS FOR PROBIOTIC DAIRY FOODS

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A thesis submitted in partial fulfillment of the requirements for the degree of MS

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DEDICATION

TO MY FAMILY; YOU ARE MY WINGS AND MY ROOTS

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Abbreviations

PBS	Phosphate buffer saline
%	Percentage
CFS	Cell free supernatant
DPPH	2,2-diphenyl 1-1 picrylhydrazyl
EPS	Exopolysaccharides
LAB	Lactic acid bacteria
GABA	Gamma amino butyric acid
ATCC	American type culture collection
EFSA	European Food safety Authority
MRS	De Man, Rogosa and Sharpe
GIT	Gastro intestinal tract
SCFA	Short chain fatty acids
BSH	Bile salt hydrolase
IL	Interleukin

ABSTRACT

Lactobacillus species used in fermented foods, they contribute to the aroma, flavor, texture and shelf life of the product. Several studies reported the fermented food products with health benefits such as the ability to decrease the serum cholesterol level. The objective of this study was the suitability assessment of previously isolated *Lactobacillus rhamnosus* I-2 and *Lactobacillus delbrueckii I-22* strains as the starter and adjunct culture for the use in probiotic dairy food. The antibacterial activity of the *Lactobacillus rhamnosus* I-22 was assessed against the different foodborne pathogens. The antioxidant potential was showed by *Lactobacillus rhamnosus* I-2 and *Lactobacillus delbrueckii I-22*. Suitability analysis of strains as adjunct or starter assessed by the formation of yogurt. The *L. rhamnosus* I-2 showed the potential of adjunct culture and *L. delbrueckii I-22* and *S. thermophilus* I-5 lack the ability to form yogurt and have low acidification rate. So, *L. delbrueckii I-22* and *S. thermophilus* I-5 cannot be used as starter culture. Invitro evaluation revealed that the *L. rhamnosus I-2* have the good technological potential to lower the cholesterol level, act as antioxidant and can be used as adjunct culture in dairy foods.

CHAPTER 1: INTRODUCTION

Greek philosopher and the father of medicine introduced the idea thousands year ago that food can be used as medicine. Hippocrates, the father of the medicine once wrote 'Let food be thy medicine and medicine be thy food.' It is clearly not a new or innovate idea that food can be used as medicine or provide the therapeutic effects to the host. In the 20th century scientist was focusing on the identification of the essential elements and their role in the treatment of diseases (Ka & Lugasi, 2008).

The link was found between the disease and excessive nutrition, which shifted the whole attention of the scientist towards the development of food with health benefits. They began to identify the bioactive components from animals and plants which are termed as zoo chemicals to phytochemicals respectively. These bioactive compounds having potential benefits reduce the risk of various diseases, then the term functional foods was introduced (Heller, 2015).

The idea of using food as therapeutic agent or medicine has been emerged and the term functional food was introduced in the food industry. All foods are considered somewhat functional because of the natural nutritive value of the food. Nowadays, food are being examined to add some nutrition compounds that could confer positive impact on the human health. Gut is one of the main targets for the functional foods because there is a direct link between the food and the human health. Other than that gut that is the reservoir of the microbes they are also present on the mucous layer of the humans (Hasler, 2002).

Most emerging field in the development and research is functional foods with probiotics and the prebiotics. In the present era of science and technology, consumer are well aware with the importance of dietary habits and good life style. They are now more concerned with the food products available in the market and their impact on health (Stiles & Holzapfel, 1997).

Scientific progress in understanding the relation between health and nutritional value has a profound impact on the consumers approach towards the healthy diet with

health benefits. There is an increasing demand towards the functional food in developed countries. Functional foods are virtually being developed in all categories of the food. Products of functional foods have been launched mainly in dairy probiotic products (Lindgren & Dobrogosz, 1990). Probiotics are defined as living microorganisms which confer health benefits upon digestion of the adequate amount of bacteria. The term probiotics was firstly introduced by the Metchnikoff. A bacteria is termed as probiotic when it meets the criteria of the selection for probiotic. Prerequisite for probiotics are tolerance to gastrointestinal tract stress i.e. acid tolerance, bile tolerance, low pH and phenol tolerance. A probiotic should have adhesion ability and colonization in the gut of the host. It should modulate the immune system in a positive way. The most desirable characteristics for probiotic is the colonization in the gut (Gilliland, 1990).

Most of the strains for probiotics are belong to the lactic acid bacteria i.e. *Lactobacillus* and *Streptococcus;* the two important members for the development of the probiotic food products. LAB are gram positive, non-spore forming, facultative anaerobic microorganism. The main product of lactic acid bacteria is lactic acid and they start the rapid acidification of the raw material. *Lactobacillus* are found in fermented meat and meat products, vegetables, milk, yogurt and in the gastrointestinal tract of the humans and animals. They are also present on the mucosal surfaces. They are non-respiratory and lack the catalase enzyme. They are divided on the base of their morphology as rods and cocci and glucose fermentation as homo-fermentative and hetero-fermentative (Stiles & Holzapfel, 1997).

Streptococcus thermophilus also used as a starter organism for the production of cheese, yogurt and various fermented products. It grows at 45°C to 50°C. It is widely used as starter in various fermented dairy products. Probiotic attributes are often consider as secondary and it has rare use in clinical studies as a sole live microorganism in the product. It has long history of use as starter culture, ease of growth and simple substrate requirement, it has the potential to be used as sole microorganisms for therapeutic use beyond its use only in dairy products (Colakoglu & Gursoy, 2016). *Lactobacillus* have a symbiotic relation with the host and provide beneficial effects. They are used as starter culture in many products which provide additional benefits other than the nutritional value such as yogurt (Amund, 2016).

Lactic acid bacteria used as singly or in the combined form with some other bacteria to form a starter culture. They rapid up the process of fermentation and responsible for the flavor, aroma, texture and other benefits of the product e.g. yogurt. Lactic acid bacteria with other starter culture help to increase the flavor and improve the aroma profile by producing diacetyl and acetaldehyde. LAB has stimulatory or inhibitory effect towards the other bacterial strains which are used as starter culture. The most common stimulatory effect of the lactic acid bacteria is towards the *S. thermophilus* together with *L. delbrueckii*. This combination result in the increase production of acid in the product and hence improved flavor of the product e.g. set type yogurt (Hasler, 2002).

Yogurt is considered worldwide as one of the fermented milk product by most of the regulatory agencies. It is an ancient food having essential nutrients like vitamin B2 and B12, phosphorus, calcium and proteins. It is fermented milk product containing *Streptococcus salivarius spp. thermophilus* and *Lactobacillus delbrueckii spp. bulgaricus* which convert lactose into lactic acid. There are many types of yogurt such as set type, stirred yogurt etc. (Fisberg & Machado, 2015). A typical method for the production of set type yogurt is given below (Karam, Gaiani, Hosri, Burgain, & Scher, 2013).

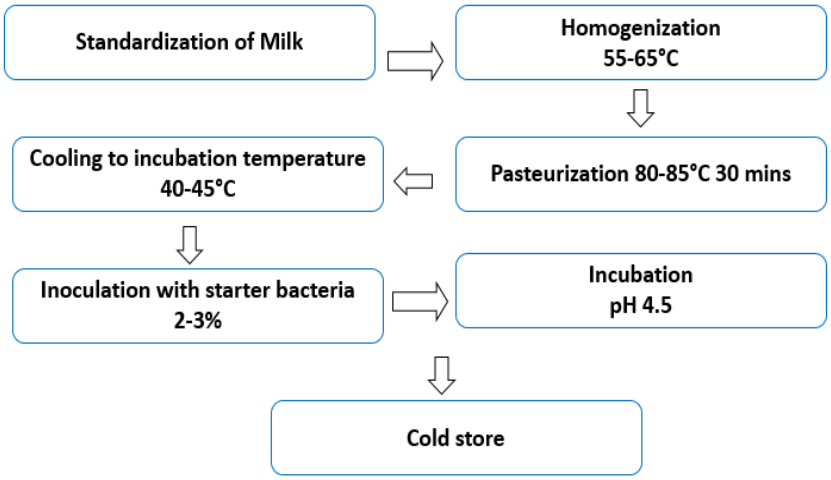

Figure 1: Main processing steps for set type yogurt

Lactobacillus produce heat stable proteins called as bacteriocin. Bacteriocin are used in the food industry as preservative because of its antimicrobial nature. They have the efficacy to protect the product from pathogens and also it degrade easily within the body by the proteolytic enzymes. There is a least chance of interaction between the bacterial target strain and the bacteriocin so it minimize the chance of resistant strain development (Lindgren & Dobrogosz, 1990).

Lactobacillus also produce exopolysaccharides (EPS) which helps in the adhesion and colonization of the bacteria in the gut. EPS also plays an important role in the dairy industry and improves texture and taste of the product for example yogurt. EPS are polysaccharides with repeating units of sugar derivatives or sugar such as galactose or glucose in different ratios. During the growth process they are secreted into their surrounding environment. They are temporarily attached to the microbial surface which distinguish them from capsular polysaccharides (Di Cerbo, Palmieri, Aponte, Morales-Medina, & Iannitti, 2016).

Lactobacillus having bile salt hydrolase activity possess a potential role in lowering the serum cholesterol level. It is very important role of probiotics in the treatment of hyperlipidemia. Conventional treatment are available for the high cholesterol level in blood but there are some drawbacks i.e. stomach pain, neurological disorders, liver damage and muscle pain (Pigeon, Cuesta, & Gilliland, 2002).

Research has already conducted on the use of *Lactobacillus* and *Streptococcus* strains for the production of bio-yogurt. Different potential *Lactobacillus* have been tested in vitro and in vivo for the safety assessment and their potential for the food industry product with health benefits. In the present study, previously isolated Lactobacillus strains from different food source was used. They were already evaluated for the probiotic potential included tolerance to phenol, acid, bile and ability to adhere small and large intestine. Safety assessment was also conducted by using rat model In vitro cholesterol lowering ability was checked by comparing it with the commercial culture. Indigenous *Lactobacillus* strains and *S. thermophilus* was used in the study, on the basis of previous evaluations. Strains were assessed for the potential of starter or adjunct culture in dairy foods with potential health benefits. Following were the objectives of the research.

RESEARCH OBJECTIVES:

1) Assessment of suitability of indigenous *Lactobacillus rhamnosus* I-2 as adjunct culture.

2) Assessment of suitability of indigenous *Lactobacillus delbrueckii* I-22 together with *Streptococcus thermophilus* I-5 as starter culture.

CHAPTER 2: LITERATURE REVIEW

2.1 LACTIC ACID BACTERIA (LAB):

Lactic acid bacteria (LAB) are fastidious gram positive, non-spore forming bacteria. LAB are aero-tolerant anaerobes and grow best under anaerobic conditions. They are non-respiratory and lack catalase with DNA base composition of less than 53%G+C content. Variation in genome range from 1.2 to 4.9 MB (megabases) for lactic acid bacteria. They are divided on the base of their morphology as rods and cocci and glucose fermentation as homo-fermentative and hetero-fermentative. The major end product of Lactic acid bacteria is the conversion of carbohydrates into lactic acid as its major end product (Stiles & Holzapfel, 1997). Homo-fermentative bacteria provides only lactic acid as the major end product while hetero-fermentative produce other products such as carbon dioxide, ethanol etc. other than lactic acid. The optimal growth temperature for LAB ranges from 30 to 45°C and pH ranges from 5.5 to 6.2.There are countless genera of microorganisms having lactic acid as an end or auxiliary finished end product but typical lactic acid bacteria are those of *Lactobacillales* order, which comprises following genera:

Aerococcus,Carnobacterium,Enterococcus,Lactobacillus,Lactococcus,Leuconostoc,Oenococcus,Pediococcus,Streptococcus,Tetragonococcus,Weissella (Duar et al., 2017).

The most group of microorganisms used in food industries are lactic acid bacterial strains. LAB plays an important role in food industry providing preservation and modification of the characteristics of the food, providing best aroma, texture and quality. There are many lactic acid bacteria which plays an important role in food industry (Stiles & Holzapfel, 1997). Few of them are; *Lactobacillus rhamnosus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus reuteri, Lactobacillus plantarum* and *Streptococcus thermophilus* (Amund, 2016).

2.2 HABITATS OF LACTOBACILLI:

Lactic acid bacteria having fastidious growth requirements occupy nutrient rich environments such as animal feed, spoiled foods, surface of animals and plants, body of vertebrates and invertebrates (Yadav, Puniya, & Shukla, 2016).

2.2.1 PLANT SOURCE:

Lactobacilli are not mostly occur on plant surfaces except the decaying surfaces of the plants where simple and complex carbohydrates support their growth .The occurrence of lactobacilli on plant is sporadic and not as plant symbionts. So, the role of plant associated lactobacilli is not fully understood. They are also found in soil and sewage due to fecal contamination (Stiles & Holzapfel, 1997).

2.2.2 FOOD SOURCE:

Lactobacilli occur as food spoilage microorganisms and dominate the microbiota of the vast majority of fermented foods such fermented milk, yogurt and fermented meat. *Lactobacillus* are rich in meat and meat products and in fermented food products, they help to keep the nutritive value of the food ,increase shelf life and helps to inhibit the growth of deteriorating microorganisms which cause spoilage of the food (Access, 2016).

2.2.3VERTEBRATE AND INVERTEBRATE HOST:

Lactobacillus isolated from a variety of different insects such as flies, bees. Food storage organs are the primary habitat for LAB such as forestomach and these organs are found in animal hosts. Vertebrates is also rich source for the isolation of Lactobacilli particularly from birds, rodents, humans and other animals, Humans is rich source of lactobacilli found in oral cavity and gastrointestinal tract(GIT) (Amund, 2016).

2.3 GUT MICROFLORA

The mammalian intestinal tract is colonized by microbial community that is composed of one hundred trillion distinct bacterial species. This complex collection of bacteria is mostly composed of intestinal microflora. Microbiota plays an important role in the contribution of human health and physiology. Intestinal microbiota enhance digestive efficiency and degrade dietary polysaccharides. Function of intestinal microbiota is consider as the main factor or driving force behind the evolution of the mammalian-host interactive relationship (Gilliland, 1990).

Gram negative *Proteobacteria* and *Bacteriodetes,* Gram positive *Firmicutes s*uch as *Lactobacillales* and *Clostridiales* are among the eubacteria represent the major phyla in the intestine of a healthy individual. Commensal microorganisms adapt themselves

according the intestinal environment and acquire nutrients by other microorganisms through complex networking. For example; *Lactobacillus* specie deficit in the ability to synthesize certain amino acids so they obtain it from intestinal habitats (Balakrishna, 2013). The number of lactobacilli in human intestine is low and species which are able to adapt the acidic environment can cause ecological niche. Most of the ecological studies indicate that the Lactobacillus specie found in human GIT are likely to be evanescent, originating from the food or the oral cavity (Gilliland, 1990).

The presence of protective viscoelastic mucous layer over the surface of gastrointestinal tract helps the adhesion of L*actobacillus* and protects against harsh luminal environment. Resident microbes are able to inhibit the growth of pathogenic microbes by competing with them for the nutrients and by inhibiting their adherence (Mishra et al., 2015).

The resident microbiota have the ability to oppose the growth of pathogenic microbes by various type of competition, e.g. they compete for; nutrients, site of attachment and by producing antimicrobial peptides .*Bifidobacterium* and *Lactobacillus* have the ability to block the disease causing mechanism of enteropathogenic microorganisms that are usually involved in diarrhea. Various type of *Lactobacillus* are found to intervene with such type of pathogens and confer health benefits to the host (Burton, Chanyi, & Schultz, 2017).

2.4 CLASSIFICATION OF *LACTOBACILLUS*

Lactobacillus is classified on the basis of morphology, mode of glucose fermentation and sugar utilization. Lactic acid bacteria have close phylogenetic relationship with their members, share small AT rich regions (2.5Mb) and similar metabolic pathways. LAB are phylogenetically linked but there is diversification relating to the niche of the microorganisms and this niche diversity is linked to the evolution of the microorganism. LAB consist of mainly 4 genera i.e. *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus* (Amund, 2016).

According to the latest taxonomic genera revisions and the new genera include: *Alloiococcus,Aerococcus,Carnobacterium,Dolosigranaulum,Globicatella,Enterococcus, Lactococcus, Tetragenococcus, Oenococcus* and *Weisella* (Stiles & Holzapfel,

1997). Lactobacilli and *Carnobacteria* are rods all the other genera are coccus except *Weisella w*hich may be cocci or rod shaped.

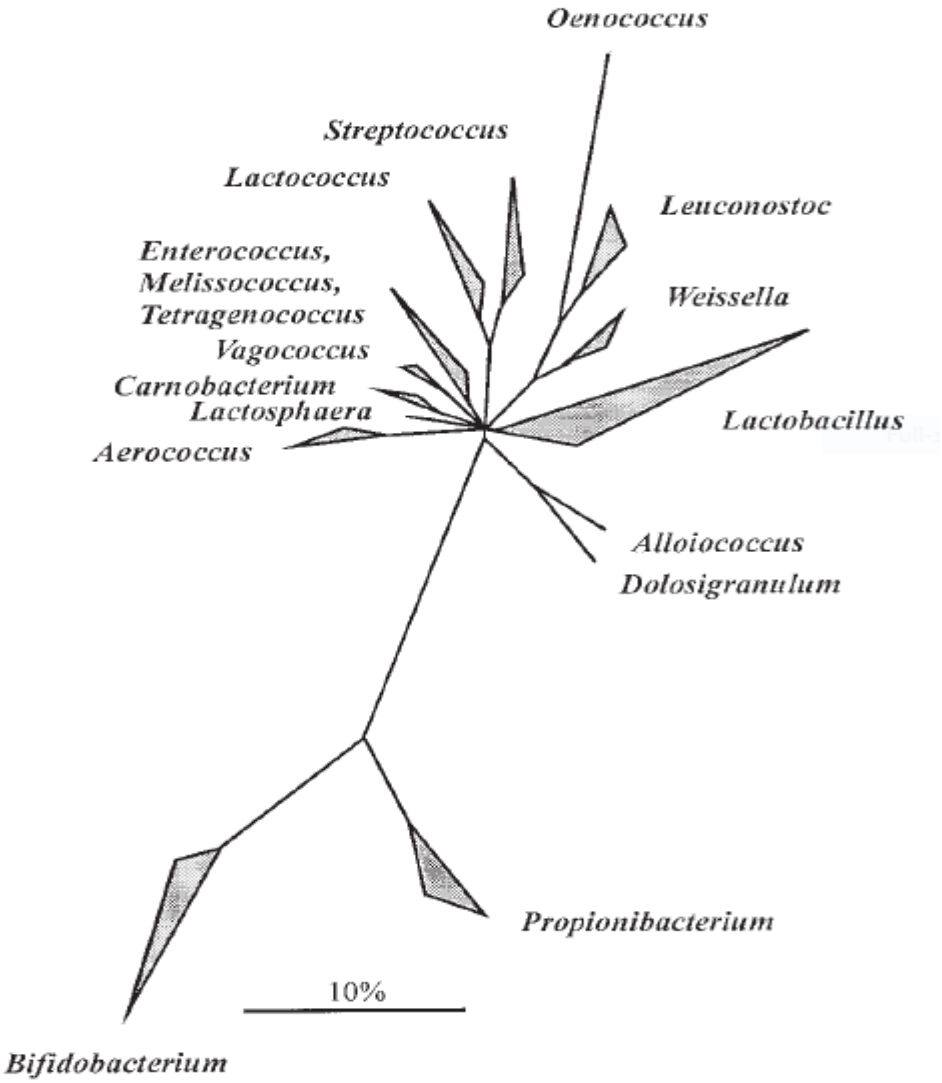

Figure 2 the major phylogenetic groups of lactic acid bacteria and their relatives among Gram- positive bacteria

(Schleifer & Ludwig, 1995)

2.5 METABOLITES OF LACTOBACILLUS:

Lactobacilli possess the more variation in peptidase activity other than any lactic acid bacteria. It is a focal metabolic activity of Lactic acid bacteria to transform peptides into free amino acids (Heller, 2015). Fermented dairy products are listed below with the bioactive compounds to promote health status.

Table 1: Fermented dairy products with health promoting compounds

Probiotic strain	Bioactive	Food product	Health effect	Reference
L. acidophilus ***L. plantarum*** ***L. lactis***	Bacteriocins	Cheddar cheese Yoghurt	Inhibition of pathogens	(Lindgren & Dobrogosz, 1990)
S. salivarius ***L. plantarum*** ***L. brevis***	GABA	Fermented milk	Blood pressure, Antidiabetic	(Qian et al., 2011)
L. helveticus	Biotin (Vitamin B7)	Fermented milk Soy-yogurt Kefir	Vitamin enrichment	(Patel, Shah, & Prajapati, 2013)
S. thermophilus ***L. bulgaricus*** ***B. lactis***	Folic acid (vitamin B9)	Yogurt Fermented milk	Vitamin enrichment	(Patel et al., 2013)
L. helveticus ***L. bulgaricus***	Bioactive peptides	Fermented milk	Anti-hypertensive	(Qian et al., 2011)
L. lactis ***L. plantarum*** ***L. bulgaricus***	Exopolysaccharides	Yogurt Cheddar cheese	Hypercholesterol-emic Immune modulation	(Makino et al., 2015)

Adapted from (Linares et al., 2017)

Lactobacillus also can catabolize amino acids by the hydrolysis. In fermented food products *Lactobacillus* exhibit many important roles e.g. antimicrobial activity which

inhibits the growth of the pathogens. Production of bacteriocin not only act as bactericidal but also helps in the preservation of food. *Lactobacillus* when used as starter culture have the better potential to ferment lactose and it's beneficial for the people with lactose intolerance ability (Access, 2016).

Lactic acid bacteria produce some vitamins (Vitamin B12, folate, riboflavin vitamin k2, etc.) which helps in the essential functions of human body e.g. metabolism and antioxidant activities. They also possess an extensive collection of enzymes. Lactic acid bacteria have an extensive collection of enzymes many of which have the potential to modify or influence the properties of the product like aroma, texture, appearance etc. All these properties of lactic acid bacteria produce products with improved nutritional qualities (Duar et al., 2017).

2.6 PROBIOTICS:

Probiotics defined by the FAO/WHO as Live microorganisms which when administered in adequate amount confer health benefits to the host. The term probiotics was derived from the Greek word ''probios'' which means for life.

Probiotics can colonize in the GIT and exert beneficial effects on the host. Probiotics have a long history of use in the treatment of mucosal infections. They are being used nowadays in industries for food and pharmaceutical applications (Di Cerbo et al., 2016).

2.6.1 HISTORY OFPROBIOTICS:

The term probiotics was first used by Lilly and Stillwell as those substances which when secreted in by one organism helps or stimulate the growth of another microorganism. Fuller defined probiotics as live microbial feed supplements which maintain the intestinal microbiota of the animal host. Elie Metchnikoff (1990) introduce the concept of health benefits of the microbes on human health after finding that microbes are responsible for fermentation (Burton et al., 2017).

Bulgarian physician discovered a Bulgarian bacillus and suggest the counteract effect of gastrointestinal metabolism that contributes towards aging and illness. It was claimed by the Hippocrates that bad digestion is the root cause of every evil (Stiles & Holzapfel, 1997).

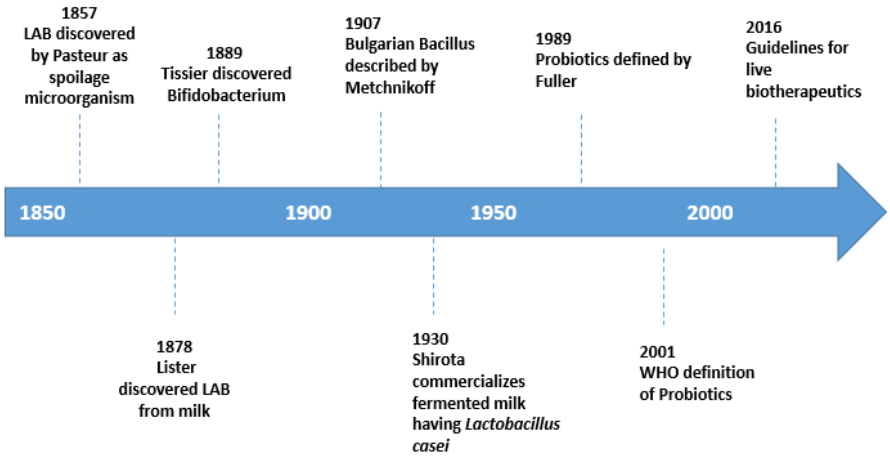

Figure 3: Timeline of Probiotics

Adapted (O'Toole, Marchesi, & Hill, 2017)

Metchnikoff also claimed that toxins of the bacteria are responsible for aging these bacteria which were called putrefying bacteria are now termed as proteolytic clostridia. He state that intestinal microbes dependent on food and it makes them more adaptive according to their environment and replace harmful microbes. Probiotics have a history as old as the human history because of the use of fermented food. Nowadays, probiotics plays crucial role in food and pharmaceutical industry (Burton et al., 2017).

2.7 *LACTOBACILLUS* AS PROBIOTIC:

The ecosystem of gut microbiome is not silent but cross-talk with the immune system of the body and exert many physiological functions. Lactobacilli have a long history of use as an effective therapy for the amelioration and treatment of various pathological conditions and displayed a proper safety profile (Di Cerbo et al., 2016).

Lactobacilli have the longest history of use as probiotics because of its beneficial effects. They are common for the consumption of farm animals especially for poultry and pigs because the microflora of these animals are rich in lactobacilli (Tannock, 1997).

Lactobacilli is resident of normal microflora of the gut and perform very useful functions for body. But, they are lack in some abilities, for that reason it is logically required to ingest or take probiotic strains which provide some characteristics to promote health. Population of lactobacilli ingested should be enough so that they could compete for the nutrients and adhesion sites with the resident lactobacilli to exert a positive effect on health (Tannock, 1997). Mainly commercial probiotics include microorganisms from the *Lactobacillus* genus *e.g. L.rhamnosus, L. acidophilus, L. plantarum, L. delbrueckii, L. helveticus, L. reuteri, L. casei* are generally recognized as safe (GRAS) organisms.

Lactobacillus are sensitive towards many type of stresses such as oxygen, low pH, temperature etc. For them to act as probiotics they must fulfil some aspects related to functional aspects such as ability to adhere intestinal mucosa, production of antimicrobial compounds, immunomodulation etc. (Access, 2016).

2.8 STREPTOCOCCUS AS PROBIOTICS:

Rosenbach (1884) was the first who used the generic name *Streptococcus* to describe the coccus shaped, chain forming bacteria associated with the infections of wounds. Wide range of bacteria are included in this genus which are associated with highly pathogenic diseases. They have complex nutritional requirements and survive in the environment with good supply of carbohydrate and proteins such as intestinal tract of animals, vegetables, dairy products, milk and different food products (Stiles & Holzapfel, 1997).

Streptococcus thermophiles is an exception and is used as a starter organism for the production of cheese, yogurt and various fermented products. It grows at 45°C to 50°C (Heller, 2015). It is widely used as starter in various fermented dairy products. Probiotic attributes are often consider as secondary and it has rare use in clinical studies as a sole live microorganism in the product. It has long history of use as starter culture, ease of growth and simple substrate requirement, it has the potential to be used as sole microorganisms for therapeutic use beyond its use only in dairy products (Burton et al., 2017).

Table 2 : The most commonly used species of lactic acid bacteria for probiotics preparations

Lactobacillus sp.	*Bifidobacterium sp.*	*Streptococcus* sp.
L. casei	*B. bifidum*	*S. salivarius*
L. plantarum	*B. adolescentis*	*S. intermedius*
L. delbrueckii ssp. (bulgaricus)	*B. animalis*	*S. cremoris*
L. fermentum	*B. longum*	
L. acidophilus		
L. reuteri		

(Adapted from (Vafaeie, 2015)

2.9 PREREQUISITE OF A PROBIOTIC CANDIDATE:

Number of aspects are related to probiotics that should be consider for a strain to be called or recognized as probiotics. Prerequisites or characteristics features of a probiotic include the survival, safety and functionality. These three features are important to exert a health benefit to the host (Di Cerbo et al., 2016).

Probiotic only exert its effect when they are enable to colonize and survive in the gut. Their number should be enough to compete or exclude a pathogen. Safety profile of a probiotic stain is also a crucial parameter for the assessment of a strain. The strain should have no antibiotic resistance genes to transfer and exert no toxic effects to the host. Strain must have ability to survive environmental stress and technological potential to provide some industrial benefits. Probiotic product should have 107CFU/g to elicit its functions. Human gastrointestinal system is quite tricky and product should design by considering it so that one could get better results. Microorganisms which are or meant intended to use as probiotics are required to meet certain criteria for selection based on ; biosafety, technological aspects and functional characteristics (Stiles & Holzapfel, 1997).

2.10 FUNCTIONAL CHARACTERISTICS OF PROBIOTIC STRAIN:

GIT related stress tolerance:

2.10.1 Acid tolerance:

Probiotic bacteria which are used as adjuncts in the food products should be able to tolerate and survive the stress faced from mouth to the intestinal tract. First of all, the probiotic microorganism should be resistant to the lysozyme present in the oral cavity and also resist the process of digestion. In vitro resistance to bile, acid and lysozyme is correlated with the in vivo survival.2.5 litre of gastric juice is secreted by the human body everyday.pH drops during fasting to 1.5 and during food intake it is usually 3 to 5. Food transit time is 90 minutes, so probiotic bacteria should able to cope with this low pH and digestion process in order to exert its effects on the host (Amund, 2016).

2.10.2 Bile Tolerance:

Lactobacillus is widely used as probiotic for humans. They face a lot of stress in their way from gastrointestinal tract to colon. In order to exert their effect they must have a specific number of viable cells. Bile is one of those stress challenges which are faced by a probiotic microorganism. It is in the upper part of the small intestine and its main component is bile acid. Bile acid produce and conjugate bile salts and helps in the digestion of fibres. Bile helps to sharpen the innate microbial community of gut. But, to those microbes which are not adapted to the intestinal environment are toxic. So, a probiotic organism should have a defensive mechanism in order to resist the deleterious effect of these compounds (Ruiz, Margolles, & Sánchez, 2013).

2.10.3 Phenol tolerance:

Tolerance to phenol is also another important selection parameter for probiotic. A probiotic strain has to survive the toxic effects of the metabolites like phenols. It is produced during the process of digestion. Aromatic amino acids produced endogenously or derived from dietary proteins deaminate in the gut by the bacteria and could have bacteriostatic effects (Shehata, El Sohaimy, El-Sahn, & Youssef, 2016).

2.10.4 Cell adhesion and Immunomodulation:

Adhesion of a probiotic microorganism to the intestinal mucosa is considered important and basic for its selection criterion. Viability and colonization both are in the list of primary features for a strain to be called as probiotic. Colonization is followed by the adhesion of the probiotic bacteria which is a complex process and based on the non-specific physical interaction between the cell surface proteins and complementary receptors. The efficiency of adherence reduce the chances of adherence of pathogenic strains (Balakrishna, 2013).

Strains of probiotics influence the innate and acquired immunity by playing an important role in human disease. The cross-talk between immune system of the host and the probiotic bacteria affects the dendritic cells, epithelial cells, monocytes and T-cells. B-lymphocytes stimulated or effected by probiotic bacteria produce antibody and enhance the response against a vaccine (Access, 2016). So, in this way a probiotic bacteria helps to reduce the severity of an infection by the activation of receptors to release anti-inflammatory cytokines (Amund, 2016).

2.10.5 Antagonistic Activity:

Antagonistic or antimicrobial activity against the pathogenic strains is very crucial for probiotics. They block the colonization of the pathogenic bacteria by altering the environment of the intestine so that the colonization of the probiotic strain improved (Amund, 2016). Probiotic stain adhere to the epithelial cells b blocking the adhesion of the pathogenic microbe and decreasing the luminal pH. Other than that factors probiotics produce some defending compounds such as bacteriocin and organic acid e.g. hydrogen peroxide which hamper pathogens growth (Access, 2016).

2.11TECHNOLOGICAL POTENTIAL OF PROBIOTICS:

2.11.1 Antioxidant Activity:

Oxidative stress is caused through the oxidation process by the over activity of the reactive oxygen species (ROS).ROS generate in two ways either endogenously or exogenously (Ou et al., 2009). ROS is very detrimental for humans and is responsible for many diseases like heart diseases, diabetes etc. LAB have the potential to scavenge the oxygen free radicals. They possess a signalling pathway by which the downregulate the ROS producing enzymes, also have chelate ions and their own antioxidases to ameliorate the effect of ROS (Wang et al., 2017).

2.11.2 Bile Salt Hydrolase Activity:

Lactobacilli have multiple beneficial properties like bile salt hydrolase activity which makes it a valuable candidate for probiotics organism's (which is also known as choloyglycine hydrolase), hydrolyses bile salts to form taurine. Hydrolysed bile salt less absorbed by the human body (as compared to non-hydrolysed bile salt) and excrete via faeces. In this way the amount of bile salt decrease in the human body and body fulfil their demand through the de-novo synthesis from the cholesterol. Thus decreasing the amount of serum cholesterol on the human body (Technology, Prague, Republic, & Science, 2015).

2.12 SAFETY ASSESSMENT OF PROBIOTICS:

2.12.1Antibiotic resistance:

Assessment of the antibiotic resistance is one of the main safety concerns for a probiotic candidate organism. Concern regarding to resistance depends on the type of resistance element whether it is transmissible or not. Bacteria that contain specific drug resistance genes should not be used in products because they might transfer it to other microbes and made them more harmful (WHO and FAO, 2001).

Identification of antibiotic resistance profile for probiotics could be achieved by the selection of minimum inhibitory concentration , PCR and microarray analysis (Stiles & Holzapfel, 1997).

2.13 BENEFITS OF PROBIOTICS:

Probiotics possess many beneficial effects including various application in food industry and pharmaceutical industry. Health benefits include the treatment of various diseases and functional food helps to prevent the disease and maintain a healthy homeostasis of gut microbiota.

2.13.1Health benefits of Probiotics:

Probiotics possess functional properties which have been demonstrated for various application of therapeutics.

Table 3: Positive effects of *Lactobacillus* on human health

Lactic Acid Bacteria	Effects on human health	Reference
L. rhamnosus GG	Helps to ameliorate the symptoms of atopic dermatitis and ulcerative colitis	(Gill & Prasad, 2008)
L. reuteri	Reduction in the duration diarrhoea	(Itsaranuwat, Al-Haddad, & Robinson, 2003)
L. plantarum	Inhibition of carcinogenic agents by the reduction of enzyme activities	(Shah, 2007)(Cavallini, Bedani, Bomdespacho, Vendramini, & Rossi, 2009)
L. acidophilus	Inhibits the development of pathogen, Reduce level of blood cholesterol	(Balakrishna, 2013)
L. casei	Stimulation of the immune system and reduce the symptoms of Crohn's disease	(Gill & Prasad, 2008)
L. johnsonii	Inhibit *H.pylori* infection	(Reid et al., 2003)

Adapted from (Itsaranuwat et al., 2003)

Health benefits that are provided by the probiotics are strain specific and no strain possess all the attributes for the health benefits to the host.

Probiotics have the strongest efficacy in the management of various diseases such as lactose intolerance, diabetes, cancer, hyperlipidaemia etc. Single dose of probiotic must contain 106-107CFU/g of food which is considered as adequate amount (WHO and FAO, 2001).

In animal nutrition dietary probiotics helps to maintain the healthy intestinal microflora. This healthy microflora helps to maintain and improve the health status of the animal. Improve microflora also suppress the growth off food borne pathogens e.g. *Salmonella* and *Compylobacter*. This application of probiotics is very important for healthy meat and meat products (Access, 2016).

Lactobacillus as probiotics have the potential health benefits in many disease conditions. For example (1) diarrheal diseases; antibiotic associated diarrhoea, infective diarrhoea, radiation diarrhoea, clostridium difficle diarrhoea and traveller's diarrhoea (2) Irritable bowel syndrome (3) *H. pylori* infection (4) prevention of colon cancer (5) Metabolic disorders (Di Cerbo et al., 2016).

LAB have positive effect on the mobility of the intestine and relieve constipation by the reduction of intestinal pH. Probiotics such as *L. rhamnosus GG*, *L. acidophilus* helps to treat and reduce the risk of pouchitis by decreasing the mucosal inflammation.

Diarrhoea is the most treated acute infectious disease by the strains of probiotics. *L. reuteri*, *L. rhamnosus*, *L. delbrueckii* are the most important probiotics that support the efficacy of probiotics in the treatment and prevention of the diarrheal diseases by the direct or indirect interaction with enterotoxins. *L. acidophilus* is very effective in the treatment of antibiotic associated diarrhoea. They not only treat the disease but also helps in the reduction of risk for the development of disease. They are also efficient in the prevention and treatment of various type of diseases such as traveller's diarrhoea, clostridium difficle diarrhoea etc. (Hojsak & Shamir, 2013).

The anti-carcinogenic effect of probiotics include a combination of mechanism e.g. induction of various types of pro-inflammatory or anti-inflammatory compounds such as cytokines which inhibit the carcinogenesis. The studies related to the anti-carcinogenic effect of probiotics against colon cancer is very basic and further

research is required. The hypothesis behind the anti-carcinogenic activity of probiotics is the reduction in the activity of the enzyme B-glucuronidase (Balakrishna, 2013).

Lactose intolerance is the most common metabolic disorder in many people. People with this disorder lack the ability to metabolize the lactose, so the probiotic strains having the lactase enzymes helps to improve the lactose digestion and reduction of intolerance symptoms (Access, 2016).

Majority of the probiotic disease are related to the treatment of gut disease but there are some studies that elucidate the effect of probiotics in the treatment of various diseases e.g. atopic dermatitis also known as eczema. *L. rhamnosus GG* reduce the risk of and symptoms of this allergic reaction (Di Cerbo et al., 2016).

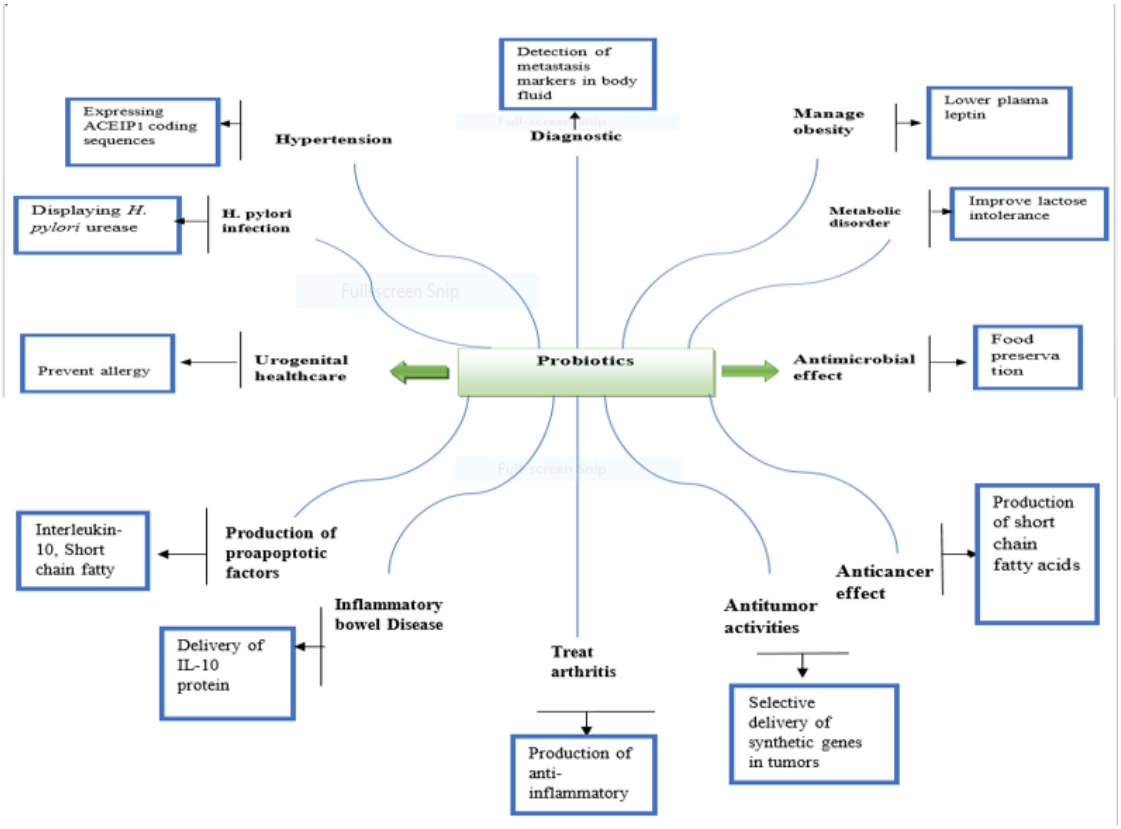

Figure 4 : Role of probiotics in the prevention of different diseases

Adapted from (Mooventhan, 2017)(Chua, Kwok, Aggarwal, Sun, & Chang, 2017)

Hyperlipidaemia: There is an association of coronary heart diseases and the level of serum cholesterol level. Hyperlipidaemia is one of the leading cause of death and

cause almost double number of deaths than any other disease. It was reported that the fermented dairy products have the positive effect on the serum cholesterol level. Selective strains of lactic acid bacteria are able to increase the utilization of cholesterol to synthesize bile acids and thus decreasing the serum cholesterol level. During the metabolism of lipid micelles formation aids in the absorption of cholesterol in the intestine. *Lactobacilli* deconjugate the bile salts to form bile acids and inhibit the formation of micelles. *L. plantarum and L. acidophilus* have the potential to reduce the serum, cholesterol level.

2.13.2 Food applications of Lactic acid Bacteria

LAB have a long history in the food industry and used as a starter culture in fermented food and beverages. They are used because of the potential to improve the nutritional quality of the product. LAB improve the aroma, flavour, texture and organoleptic properties of the final food product. They initiate rapid acidification of the raw material and produce bacteriocin, lactic acid, exopolysaccharides (EPS) and many enzymes which render a product many beneficial characteristics (Linares et al., 2017).

Starter culture is the microbial preparation which is introduced in the raw material to accelerate the process of fermentation. They are in the form of freeze dried culture or in lyophilized form. Spontaneous fermentation method was used earlier for the fermentation of the food product by the presence of microflora on the surface of the raw material. Then, starter culture as the direct addition to the food was preferred because the advantage of high degree of control over the process of fermentation and standardization of the final product (Contribution, 1978).

Recently the benefits of the starter culture other than improving the nutritional quality of the product was explored and it was the functionality of the starter culture that provides the health benefits to the host. During the selection of the starter culture it must be noted that it should not produce the racemate or D-lactic acid (DL) and biogenic amines. LAB probiotic cultures are in the category of the successful starter cultures. The beneficial effects on the human health by the lactic acid bacteria was firstly discovered by the Metchnikoff. He mentioned the health benefits of the fermented milk and fermented products. Nowadays probiotic cultures are used for a

number of products like infant formulas, yoghurt, yoghurt drinks and dietary supplements (Heller, 2015).

Yogurt is worldwide recognized fermented product that is a rich source of nutrients like calcium, phosphorus, vitamins etc. The name comes from the Greek word 'yogurmak' which means to coagulate, to thicken or curdle. It is believed that around 10000-5000BC, the product of the milk were included into the human diet. The first yogurt laboratory was opened in France in 1932.Today yogurt is defined as fermented milk with viable bacteria having the ability to acidified a product with nutritional benefits. It is symbiosis of the two strains. Yoghurt is manufactured by the use of starter culture which is a combination of *S. thermophilus and L. delbrueckii subsp. Bulgaricus* (Fisberg & Machado, 2015).Lactose present in the milk is converted into lactic acid and many other products. There is decrease in pH in the final product 6.5 to 4.5.This decrease in pH delayed the growth of the undesired microorganisms in the yogurt. The whole process breakdown the nutrients present in the milk in more digestible form hence increase the availability of the nutrients to the consumer (Karam et al., 2013)

A strain to be used as starter culture must meet the following requiremnets.1: Should be able to increase the number of cells by growing in a media.2: Ability of microorganisms to withstand the stress during preparation like freeze drying.3: Able to tolerate the gastro intestinal related stress (Linares et al., 2017). Species of LAB that are particularly used as probiotics culture belong to the species of lactobacillus and it include *L. acidophilus, L. reuteri, L. rhamnosus, L. plantarum, L. lactis* etc. Dairy products are ideal and safest way to deliver the probiotics to the human gut. Interaction between starter culture and probiotic organism is possible by the synergism e.g. yoghurt and by antagonism e.g. bacteriocins which exhibits antimicrobial properties. A product having probiotic must have viable number of cells 106 CFU/g to confer health benefits to the host (Access, 2016).

Table 4: Probiotic used in the production of fermented food products

Genus	Application in dairy food	Reference
B. lactic, L. rhamnosus, L. paracasei	Cheddar cheese	(Access, 2016) (Philips et al,2006)
Leu. mesenteroids	Cheese, sour cream, buttermilk	(Burton et al., 2017)
L. rhamnosus and L. acidophilus	Yoghurt, fermented milk	(Maragkoudakis, Miaris, Rojez, Manalis, et al., 2006)
S. thermophilus, L. delbrueckii	Yoghurt enriched with skim milk	(Cogan et al., 2007)

CHAPTER 3: MATERIALS AND METHODS

3.1 Strains used in Study:

Two potential probiotic strains *L. rhamnosus I-2* and *L. delbrueckii* I-22 were selected that were previously isolated by Lab fellows (K Hafsa, 2016) (A Muneera, 2017). *L. rhamnosus I-2* used in the study to assess it potential as adjunct culture in dairy foods which was evaluated previously for cholesterol reducing ability and vivo survival by lab fellow (A Zaigum, 2018).

Table 5: Bacterial strains used in study

Serial no.	Strain Name	Origin
1	*Lactobacillus rhamnosus* I-2	Milk
2	*Streptococcus thermophilus* I-5	Yogurt
3	*Lactobacillus delbrueckii* I-22	Yogurt
Pathogenic strains		
1	*P. aeruginosa* ATCC 9027	
2	*S. aureus* ATCC 6538	
3	*E. coli* ATCC 8739	
4	*K. pneumonia* CC2	
5	*S. enterica* ATCC 14028	

Technological potential and safety assessment of indigenously isolated *L. delbrueckii* I-22 strains were already evaluated in vitro and this strain have good antimicrobial activity against gut pathogens. The strain of *S. thermophilus I-5* was provided by Dr.

Muhammad Imran from Department of Biological Sciences, Quaid-i-Azam University. *L. delbrueckii* I-22 were selected because of its history of antimicrobial activity against gut pathogens. *S. thermophilus I-5* and *L. delbrueckii* I-22 was assessed for their potential as starter culture for making yogurt.

3.2 ASSESSMENT OF TECHNOLOGICAL POTENTIAL:

3.2.1 Antimicrobial activity:

Antimicrobial activity of he selected strain were assessed against five pathogenic strains using agar well diffusion method. Test microorganisms were five pathogens i.e. *P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Salmonella* ATCC 14028, and *Klebsiella* CC2. Pathogenic strains were grown overnight in LB broth. Pathogenic culture (100µL) was spread on the plate MHA agar plate. Wells of 8mm diameter were created in the plates. CFS 75µL of overnight grown *Lactobacillus* culture were poured into the wells. Plates were incubated at 37°C after dry for 24h.Cell free supernatant (CFS) of the selected strains were prepared after overnight growth in MRS broth by centrifugation at 10000xg for 10mins. The pH of the CFS maintained at 6.5 by suing 1Mol/L NaOH. Zone of inhibition after incubation would indicate bacteriocin like product (Yadav et al., 2016).

3.2.2 Assessment of antioxidant potential:

The ability of Lactic acid bacteria to scavenge radicals of 2, 2-diphenyl-1-picryhydrazyl (DDPH) was assessed by the overnight culture of strains in MRS broth. Overnight grown bacterial culture centrifuged (6000xg for 10 mins). Intact cells were washed three times by phosphate buffer saline (PBS) (pH 7.0). DPPH solution 0.2mM was prepared in 90% ethanol. Solution of 0.8mL intact cells and 1mL of DPPH was prepared and vortex for 10 seconds. Incubate the reaction mixture for 30 mins at room temperature. The level of scavenge DPPH was then measured by spectrophotometer. Optical density was taken at 517nm using UV vis spectrophotometer (Ou et al., 2009).

The level of scavenged DPPH was measured by using the formula:

[1-A517 (sample)/A517 control)] x100%

3.2.3 Screening of BSH activity:

The qualitative bile salt hydrolase activity was assessed by using MRS agar supplemented with bile salt (0.3% w/v) and $CaCl_2$ (0.03% w/v). Bacterial strains were cultivated overnight in MRS broth (pH 5.6) separately followed by their inoculation on MRS agar plates that were punctured at different spots and fresh culture were inoculated in the wells. Plates then incubated at 42°C for 72 hours. Plates were then analyzed for halo or precipitation zone. Stains of Lactobacilli were used as negative control and grown on MRS agar without bile salt (Technology et al., 2015).

3.3 Safety Assessment:

3.3.1 Antibiotic susceptibility testing:

Agar disk diffusion method was used to assess the antibiotic resistance of the selected strain. Ten different antibiotics were used i.e. AMP-10 (Ampicillin), CIP-5 (Ciprofloxacin), C-30 (Chloramphenicol), CN-10 (Gentamicin), AMC-30 (Amoxicillin/ clavulanic acid), VA-30 (Vancomycin), and R-5 (Rifampicin). Antibiotic susceptibility of the strains was assessed on MRS agar plate for *Lactobacillus* and M17 agar for *S. thermophilus I-5*. Bacterial cultures grown freshly in MRS broth. Fresh culture 100µL was spread on MRS agar plate and allowed to dry. Antibiotic disk were placed on the plates and incubated at 37°C for 24 hours. Bacterial susceptibility to antibiotics was shown by the inhibition zone (Yadav et al., 2016).

3.4 DETECTION OF BACTERIAL INTERACTION:

Bacterial interaction was detected by well diffusion agar assay. Strains were cultivated in MRS and M17 broth for *Lactobacillus and Streptococcus thermophilus I-5* respectively at 42°C. Fresh cell free supernatant (CFS) was prepared by removing the cells of the overnight culture at 10,000 x g for 15 mins at 4°C. The pH of the CFS was adjusted at 6.5 by using 1M NaOH. MRS agar plates were inoculated with the indicator strains (1% v/v). Wells of 8mm diameter was created by puncturing the agar. CFS 50µL was added into the wells followed by the incubation at 42° C for 24 hours. Zone of inhibition was examined after 24 hours (Maragkoudakis, Miaris, Rojez, & Manalis, 2006).

3.5 ACIDIFICATION POTENTIAL OF STRAINS IN DIFFERENT MEDIA:

Acidification potential of the strains was determined at different time intervals by using a pH probe meter. Bacterial strains were grown in M17 for *S. thermophilus* I-5 and MRS broth for *Lactobacillus rhamnosus I-2, Lactobacillus delbrueckii I-22*. Media was enriched with sucrose solution (3% v/v) and strains were inoculated (2 % v/v) and incubated at 42° Drop in pH was determines at 0h,2h,4h,6h and 8h by using pH probe.

Acidification profile was also observed in skim milk media by drop in pH. Skim milk was enriched by sucrose(3% v/v) solution and strains were inoculated in skim milk separately and in combination at the rate of 2% v/v. Milk was incubated at 42°C and pH was recorded at 0h, 2h, 4h., 6h and 8h by using pH probe (Biosci et al., 2016)(Maragkoudakis, Miaris, Rojez, & Manalis, 2006).

3.6 PRODUCTION OF YOGURT:

3.6.1 BACTERIAL STRAINS AND GROWTH CONDITIONS:

Potential probiotic *L. delbrueckii I-22* and *L. rhamnosus* I-2 strains were selected that were previously isolated by lab fellows (A Muneera, 2017) (K Hafsa, 2016). They were grown in MRS broth at 42°C. *S. thermophilus* I-5 was obtained from Biological Sciences Department Quaid-i-Azam, University. *S. thermophilus I-5* was cultivated in M17 broth at 42°C. Commercial starter culture (vital natural yogurt) was used and culture was incubated according to the manufacturer recommendations.

3.6.2 PREPARATION OF YOGURT INOCULUM:

L. rhamnosus I-2, L. delbrueckii I-22 and *S. thermophilus* I-5 were propagated in MRS broth and M17 broth respectively. Wet biomass inoculum was prepared by centrifugation (10000x g, 10 min, and 4°C) of 18 h old culture of *L. rhamnosus I-2, L. delbrueckii I-22* and *S. thermophilus* I-5 and washed with PBS, pH 7.2 (Maragkoudakis, Miaris, Rojez, & Manalis, 2006).

3.6.3 YOGURT FORMATION:

Skim milk (10% w/v) was used for the formation of yogurt samples by sterilizing at 105°C for 10 mins. Sucrose solution (3% v/v) was added to enrich the milk .Skim milk was tempered at 45°C before the inoculation of the culture. Yogurt samples were prepared by using three different combinations of culture and coded as sample A;

Commercial starter culture (*Streptococcus thermophilus and Lactobacillus bulgaricus)*, sample B; Commercial starter culture (*Streptococcus thermophilus and Lactobacillus bulgaricus) +L. rhamnosus I-2* (indigenous*)*, sample C *L. delbrueckii I-22* and *S. thermophilus* I-5 (Indigenous). Sample A was used as positive control, sample B was formed to assessed the potential of *L. rhamnosus I-2* for adjunct culture and sample C was used to assess the potential of *L. delbrueckii I-22* and *S. thermophilus* I-5 as starter culture (Yilmaz-Ersan & Kurdal, 2014).

Details of the combination, culture conditions and name are given in the table.

Table 6: Combination of bacterial culture for yogurt formation

Code	Culture	Incubation Temperature (°C)	Incubation Time (h)
A	Commercial starter culture(*Streptococcus thermophilus and Lactobacillus bulgaricus)*	42	4
B	Commercial starter culture(*Streptococcus thermophilus and Lactobacillus bulgaricus)+L. rhamnosus I-2 (indigenous)*	42	4
C	*L. delbrueckii I-22* and *S. thermophilus* I-5 (Indigenous)	42	10

Indigenous *L. delbrueckii I-22 and S. thermophilus* I-5 was used in combination (1:1) (18 hours old culture) to assess their potential as starter culture. They were inoculated in milk with 3% sucrose solution. Skim milk was incubated after inoculation at 42°C for 8 to 10 hours until pH 4.6 reached. *L. rhamnosus* I-2 was inoculated with

commercial starter culture at the rate of 1%v/v for 4 hours. Coagulation was observed and organoleptic properties of the yogurt was analyzed (Elbashiti, n.d.).

Twenty students were randomly selected and provided with the yogurt samples to taste. They were asked to note the taste, aroma and texture of the yogurt samples which were randomly coded. Sensory evaluation were applied using a five-point score system (1 unacceptable, 5 excellent).

CHAPTER 4: RESULTS

4.1 ASSESSMENT OF TECHNOLOGICAL POTENTIAL:

4.1.1 Antibacterial Activity:

Antagonistic activity of the *L. rhamnosus* I-5 was assessed against five different pathogenic strains. Antibacterial activity of the isolate was observed only against three pathogens i.e. *Salmonella, E. coli* and *P. aeruginosa.*

Table 7: Antibacterial activity of the *L.rhamnosus* I-2 against various pathogenic strains

Isolate	*E. coli*	*S. enterica*	*P. aeruginosa*	*S. aureus*	*K. pneumonia*
L. rhamnosus I-2	**6±0.2**	**4.8±0.3**	**14±0.2**	**-**	**-**

CFS (pH 6.5) against five different pathogens (ATCC) measured by zone of inhibition (ZOI) in mm. - = No zone, ±=standard deviation

4.1.2 Assessment of Antioxidant Potential:

Anti-oxidative effect of the isolates was assesses by using DPPH. All the strains showed antioxidant potential against DPPH free radicals.Highest potential was observed for *L. plantarum* ATCC 14917 .

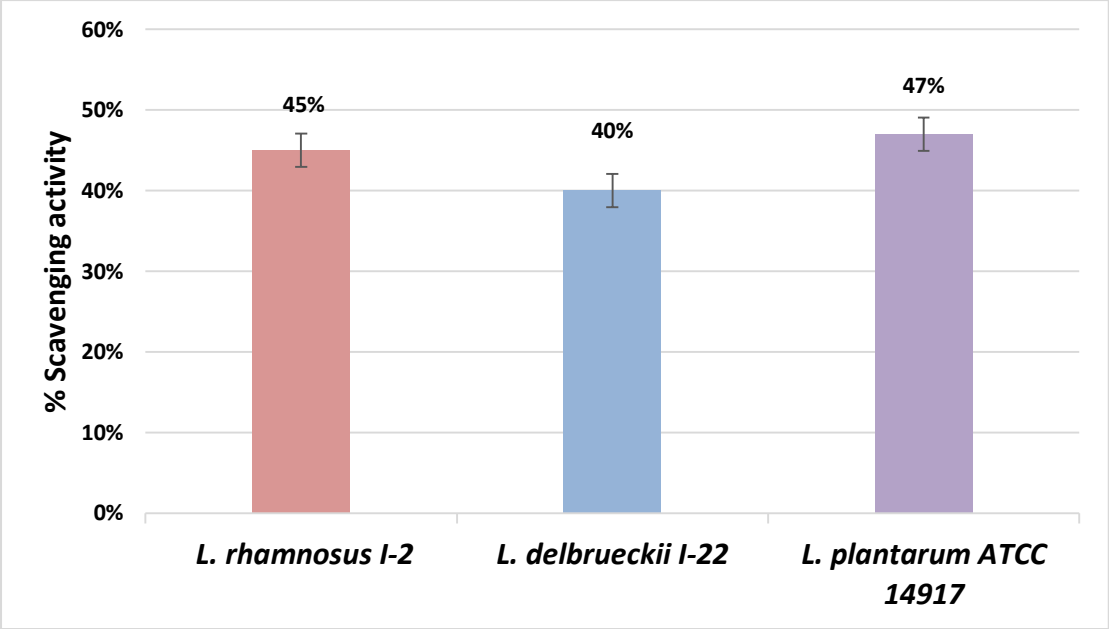

Figure 5: Antioxidant potential of the strains (% Scavenging effect)

Error bar represents the standard deviation (n=3)

4.1.3 Screening of BSH potential:

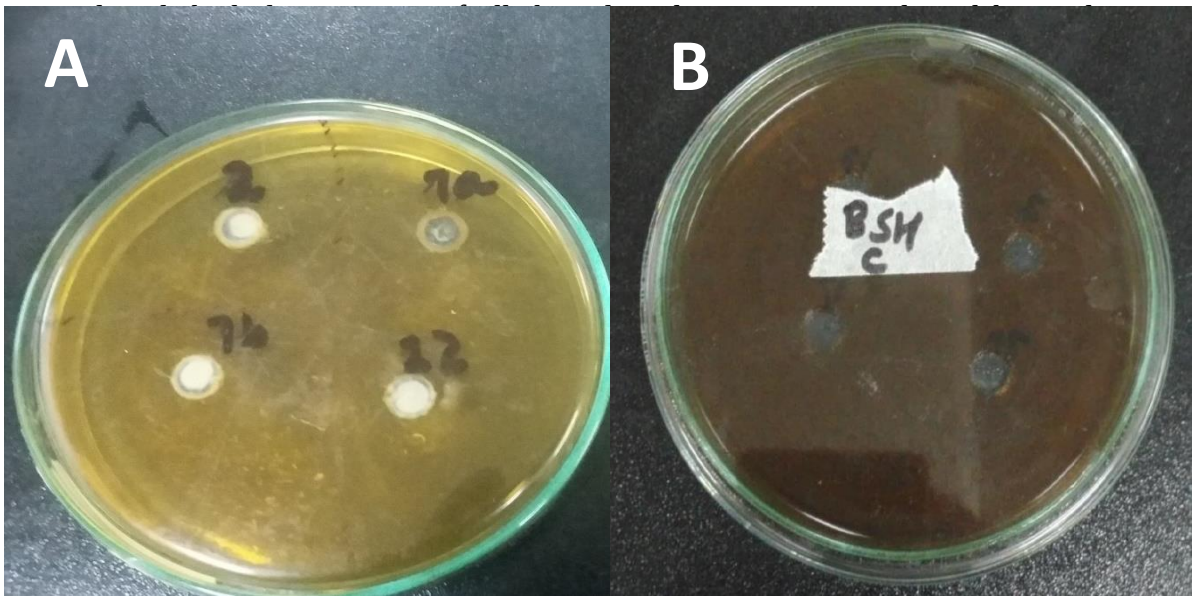

Figure 6: BSH activity of the isolates

MRS media supplemented with bile salt. A: Formation of precipitation around *L.rhamnosus* I-2 showed BSH positive activity. B: Media without bile salt was used as control.

Only one isolate i.e. *Lactobacillus rhamnosus* I-2 showed significant BSH activity. Strains of *Lactobacillus delbrueckii* I-22 and *Streptococcus thermophilus* I-5 showed no significant BSH activity. Media without bile salt was used as control and no activity was observed in that plate.

4.2 SAFETY ASSESSMENT

4.2.1 Antibiotic susceptibility testing:

Antibiotic susceptibility of the strains were evaluated by using seven different antibiotics. All the selected strains were tested against all these antibiotics. Isolates which were resistant to antibiotic showed growth on the plate and susceptible isolate was not able to show any growth.

Table 8: The antibiotic susceptible/resistance profile of the isolates against different antibiotics.

Isolate	AMC-30	C-30	CN-10	CIP-5	RD-5	S-10	VA-30
Lactobacillus rhamnosus I-2	S	I	I	R	S	R	R
Lactobacillus delbrueckii I-22	S	S	S	S	S	S	S
Streptococcus thermophilus I-5	S	S	S	I	S	S	R

AMP-10 (Ampicillin), CIP-5 (Ciprofloxacin), C-30 (Chloramphenicol), CN-10 (Gentamicin), AMC-30 (Amoxicillin/ clavulanic acid), VA-30 (Vancomycin).RD-5 (Rifampicin) S= Sensitive, R = Resistant, I=Low resistance

4.3 DETECTION OF BACTERIAL INTERACTION

Bacterial interaction was tested by using the supernatant of the fresh cultures of *L. delbrueckii* I-22 *and S. thermophilus* I-5. No inhibition was observed against which depicts that the coexistence of the selected strain is possible.

Table 9: Co-culture suitability assessment of *S. thermophilus* I-5 and *L. delbrueckii* I-22

Indicator strain	Test strain	Effect on growth
L. delbrueckii I-22	*S. thermophilus* I-5	No visible antagonistic activity
S. thermophilus I-5	*L. delbrueckii* I-22	No visible antagonistic activity

Effect of CFS of *S. thermophilus* I-5 on the growth of *L.delbrueckii* I-22, and vice versa (Well-diffusion agar assay)

4.4 ACIDIFICATION POTENTIAL OF STRAINS IN DIFFERENT MEDIA

All the selected strains exhibited fast acidification in MRS broth for *Lactobacillus* and M17 media for *S. thermophilus*. The value of pH in the above mentioned media is 4.6 after 8h of incubation. But the rate of acidification in skim milk media was very slow, and the value of pH was almost 5.7 after 8h of incubation.

As a result their application as starter culture for yogurt is not possible but they could be used as starter or adjunct culture for other fermented food products.

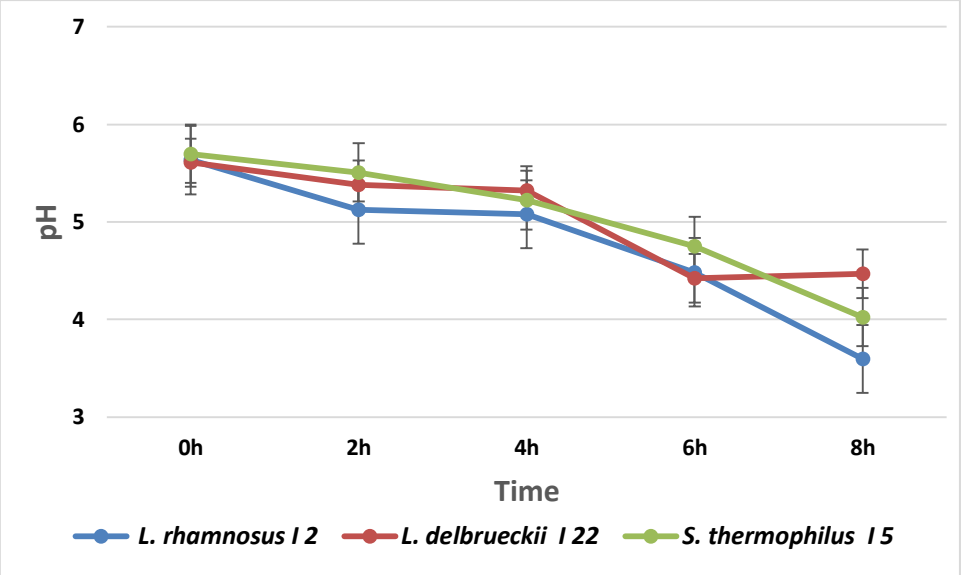

Figure 7: pH Kinetics of *L.rhamnosus I-2 and L.delbrueckii* I-22 in relevant media

Bacterial strains were grown in M17 for *S. thermophilus* I-5 and MRS broth for *L. rhamnosus I-2, L. delbrueckii I-22*. Error bars represents standard deviation based on three replicates

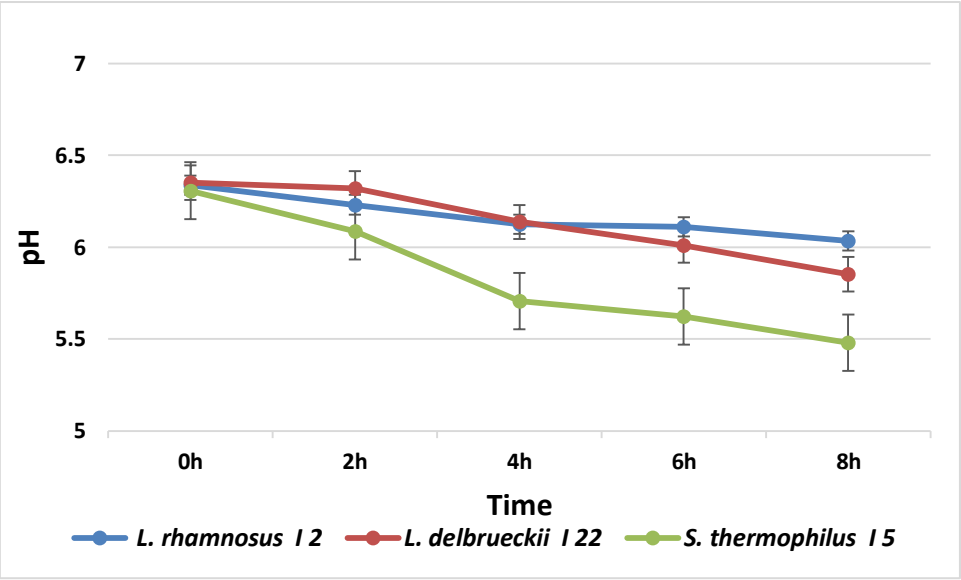

Figure 8: pH Kinetics of *L. rhamnosus I-2, L. delbrueckii* I-22 and *S. thermophilus* I-5 in skim milk

Error bars represents standard deviation based on three replicates

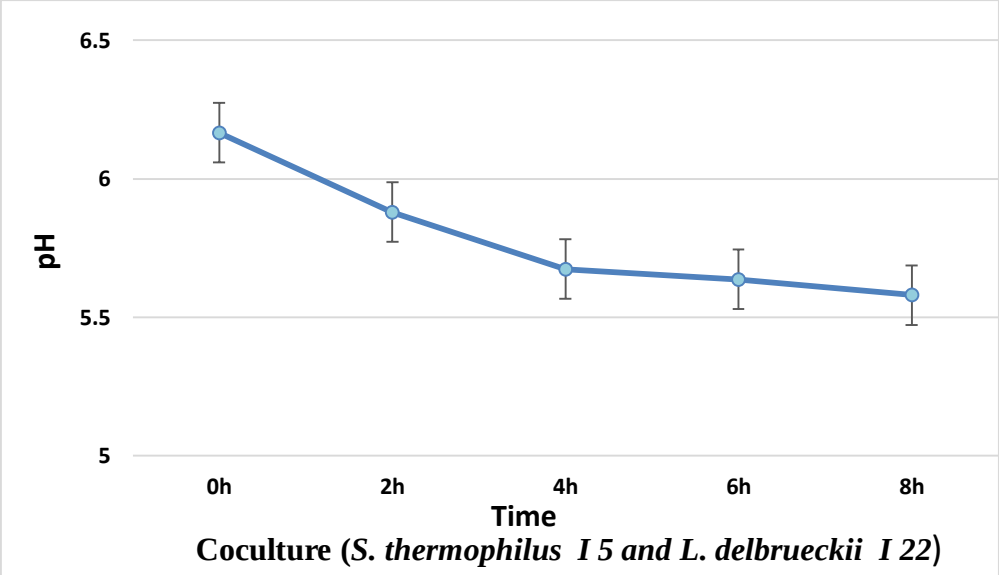

Figure 9: pH kinetics of co-culture; *S. thermophilus* I-5 and *L. delbrueckii* I-22 in skim milk media

Error bars represents standard deviation based on three replicates

4.5 YOGURT FORMATION:

Yogurt samples were produced with different combinations in three independent replicates and stored at 4°C for sensory evaluation after the fermentation completed. The result obtained showed that the incubation period was 4h for sample A (control yogurt; commercial starter culture (*Streptococcus thermophilus and Lactobacillus bulgaricus)* and sample B (control yogurt and indigenous *L. rhamnosus I-2*) was not significantly different. Sample B in which indigenous *L. rhamnosus* I-2 was evaluated for the use in dairy industry as adjunct culture showed fermentation time quite closer to the commercial product which indicates that it could be used in yogurt as adjunct culture.

The incubation period was differed for sample A (control yogurt) i.e. 4 h and sample C (*L. delbrueckii I-22 and S. thermophilus I-5*) which reached the target pH after 10-12h.This difference of incubation time to reach target pH between sample A (control yogurt) and experimental sample C showed that the application of these indigenous strain as starter culture for yogurt is not possible.

It was found that the incubation period to reach target pH is dependent on the type of culture and their concentration. Aroma, texture, taste and overall acceptability of the

two samples A and B was quite close to each other and overall score was 16 for sample A and 15.95 for sample B. So, the *L. rhamnosus* I-2 can be used as adjunct culture. Total score for sample A is 16.05 and sample B is 15.95 which is considered good according to the hedonic scale.

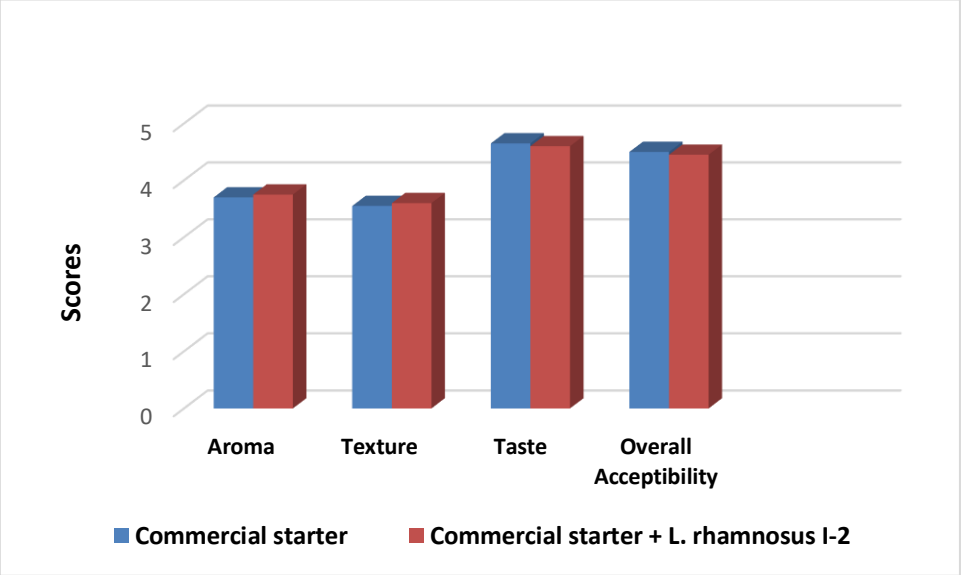

Figure 10: Sensory evaluation of yogurt

Sensory evaluation by volunteers using commercial culture (control) and experimental yogurt (commercial culture and indigenous *L. rhamnosus* I-2. Score index ranging from 1-5, 1=extreme dislike and 5= extreme like

Table 10 Sensory score of samples

No. of Participants	Aroma		Texture		Taste		Overall Acceptability	
	A	B	A	B	A	B	A	B
1	3	4	3	4	5	5	4	4
2	4	4	3	4	3	4	4	5
3	4	4	3	4	4	5	5	4
4	3	4	4	4	5	4	3	3
5	3	4	3	4	4	5	4	5
6	4	4	4	4	5	5	5	5
7	3	4	4	3	5	3	4	4
8	4	4	4	5	5	5	5	3
9	4	3	4	3	5	4	5	5
10	3	4	4	3	5	5	5	4
11	4	3	3	3	4	4	5	5
12	4	4	3	3	5	5	5	3
13	4	3	3	3	5	5	4	4
14	3	3	3	3	4	4	5	3
15	4	4	3	3	4	4	5	4
16	3	4	4	3	4	4	5	4
17	4	4	4	3	5	4	4	4
18	4	4	3	5	5	5	4	5
19	4	4	3	5	5	4	5	5
20	3	3	3	3	5	4	3	5
Total score	**3.65**	**3.75**	**3.5**	**3.6**	**4.55**	**4.4**	**4.45**	**4.2**

Sensory evaluation by volunteers using commercial culture A (control; *S. thermophilus* and *L. delbrueckii*) and experimental yogurt B (commercial culture and indigenous *L. rhamnosus* I-2. Score index ranging from 1-5, 1=extreme dislike and 5= extreme like

CHAPTER 5: DISCUSSION

Probiotics are GRAS and have a long history of use in dairy and non-dairy products as starter or adjunct culture (Burton et al., 2017). The three indigenously isolated potential probiotic strains of dairy origin were selected for their suitability as starter or adjunct culture in the production of yogurt. Selected strains were *S. thermophilus I-5 L. delbrueckii I-22, L. rhamnosus I-2* and they were assessed previously for their probiotic potential. *L. rhamnosus* I-2 had a history of cholesterol reduction and it was used to assess its potential as adjunct culture in dairy food. *L. delbrueckii I-22* was selected due to its antimicrobial activity against food borne pathogens. *S. thermophilus* I-5 *L. delbrueckii I-22* were assessed for their suitability as starter culture for making yogurt.

Probiotics have a long history of use in dairy products but they need to meet certain criteria to be referred or called as probiotics. On the basis of this concept, selected strains were evaluated for their safety profile and technological potential (Heller, 2015). Strains were tested for their resistance or susceptibility profile against eight different antibiotics as safety assessment is a prerequisite for probiotics because the resistance against the antibiotics is becoming a major issue (Hojsak & Shamir, 2013). *L. rhamnosus* I-2 showed resistance only against ciprofloxacin, vancomycin and streptomycin (table 8) which indicates that strain is safe to use in food products. These results were supported by the fact that LAB exhibit intrinsic resistance to glycopeptides, fluoroquinolones and aminoglycosides (Zdolec, 2015). Antibiotic resistance profile of *S. thermophilus* I-5, *L. delbrueckii I-22* (table 8) showed that they are safe to use. *S. thermophilus* I-5 exhibit resistance against vancomycin and this resistance attributed to the increase use of that antibiotics in environment (Flórez et al., 2008).

L. rhamnosus I-2 was screened for its antimicrobial activity against different pathogens and growth inhibition was observed against *P. aeruginosa E. coli and S. enterica* (table 7). This antimicrobial activity is might be due to the presence of hydrogen peroxide, organic acids, diacetyl, and bacteriocin as well as by the competition for nutrients (Shehata et al., 2016). Probiotics which belong the

Lactobacillus showed a history of potential antioxidant mechanism which protects the cells from damage and such probiotics are used in functional food to confer health benefits. In the present study selected strains are assessed for their potential against the DDPH and they exhibited the scavenging activity against the free radicals of DPPH (Wang et al., 2017). *L. rhamnosus I-2* showed the antioxidant potential 45% when compared with the commercial strain *L. plantarum* ATCC 14917.

The cholesterol lowering potential of lactic acid bacteria has been studied for years. Hypercholesterolemia is associated with heart disease and its treatment include lowering the level of serum cholesterol in the body. Some probiotics strain have BSH activity by which they lower the serum cholesterol (Sedláčková, Horáčková, Shi, Kosová, & Plocková, 2015). *S. thermophilus I-5, L. delbrueckii I-22 and L. rhamnosus* I-2 were evaluated for their cholesterol lowering potential and data related to in vitro test showed that only *L. rhamnosus* I-2 was BSH positive (figure 5). *L. delbrueckii* I-22 showed no BSH activity and it is supported by the previous studies that typically strains from dairy origin either have no BSH activity or a very low incidence. The diversity in the hypercholesterolemic action at both strain and species level highlighted the fact that each probiotic strain within each species may have different activities. The difference in the BSH activity might be due to absence of BSH gene, gene inactivation by mutation in regulation mechanism and it needs to be determined by different genetic methods (Albano et al., 2018).

Technological potential of *S. thermophilus I-5 L. delbrueckii I-22, L. rhamnosus I-2* were evaluated by pH kinetics for making yogurt. Acidification profile of the selected strains was observed in different media. *L. delbrueckii I-22, L. rhamnosus I-2* and *S .thermophilus* I-5 showed rapid acidification in MRS and M17 media. But the pattern of pH drop was different in skim milk from MRS or M17 media. It was observed on the basis of their growth at 42°C for 8h of incubation that the strain of *L. delbrueckii I-22* and *S .thermophilus* I-5 were slow acid producers and they cannot be used as starter culture for yogurt because of low acidification rate and long fermentation period to achieve pH 4.6. But they can be used for some other dairy products such as fermented milk.

Difference between the acidifying properties of *Lactobacillus* and *Streptococcus* may ascribed to the specificity of strain to breakdown the substances present in milk, genetic makeup of the bacteria, fluctuation in incubation temperature , long lag phase which is due to the transfer of bacteria from broth to milk or might be deficiency in the nutrient transport system of fermentable sugars (Soomro & Masud, 2008).

Suitability of *L. rhamnosus* I-2 as adjunct culture was evaluated by making yogurt with the commercial starter culture and the resulting yogurt was subjected to sensory evaluation (figure 6, 7). Fermentation time of experimental yogurt i.e. Combination of indigenous *L. rhamnosus* I-2 and commercial starter culture was quite close to the control yogurt (commercial culture). Sensory evaluation for both the yogurt samples was done and the score was not different. Our results in this study showed the fact that the *L. rhamnosus I-2* can be used as adjunct and the *L. delbrueckii* I-22 and *S. thermophilus* canto be used as starter culture for making yogurt (Figure 7).

CHAPTER 6: CONCLUSION & FUTURE PROSPECTS

The current study showed that among three indigenously isolated strains of *L. rhamnosus* I-2, *L. delbrueckii* I-22 and *S. thermophilus* I-5, only *L. rhamnosus* I-2 is BSH positive. These three strains were analyzed for pH kinetics and acidification potential and data indicates that *L. delbrueckii* I-22 and *S. thermophilus* I-5 are not a good candidates as starter culture for yogurt because an ideal starter culture should be steady and fast in acid production but use in other fermented dairy products could be possible as starter or adjunct such as in fermented milk. *L.rhamnosus I*-2 was assessed for their ability to use as adjunct culture and the results are positive as the sensory evaluation showed. *L. rhamnosus* I-2, *L. delbrueckii* I-22 *5* are good candidates for functional foods because of antioxidant, antimicrobial activities which are important for the development of functional foods. Safety profile was also assessed which showed that the strain is safe to use but further research is needed for in vivo results.

Overall result demonstrates that *L. rhamnosus* I-2 could be used as adjunct culture in the production of yogurt with potential probiotic properties and it has no adverse effects on the quality parameters of the yogurt as verified by the sensory score. However, subsequent in vitro and clinical studies should be performed in order to verify any potential health benefits.

In future these strains can be used for the assessment of safety and antioxidant potential in vivo. Whole genome sequencing of the strains can be done for safety analysis and further characterization. EPS production, bacteriocin extraction and its characterization can be evaluated. Different metabolites produced by the strains can be evaluated. Flavor profile, cell viability, pH drop during storage of yogurt can be studied.

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