Chapter-I

Introduction

Fermented dairy products have long been an essential component of nutritional diet. Dahi is a popular fermented dairy product in the Indian subcontinent, having a similar appearance to that of yogurt. It is prepared by fermenting milk from cows, buffalos or goats with mesophilic lactic cultures, and its preparation process and physico-chemical characteristics are well known (Rao et al., 2005). Yogurt, a western counterpart of dahi, in addition to its nutritive value, is believed to be effective in preventing and treating various illnesses, viz., gastrointestinal disorders, heart diseases and tumor development, both in human and animals (Deeth and Tamime, 1981). Several health benefits of dahi and yogurt have been reported. Enhancing the nutritional and therapeutic properties of traditional fermented milk can improve the health and physiology of consumers. Lactic acid bacteria are usually known as "milk souring organisms". Through their widespread use in the development of fermented foods, lactic acid bacteria have received considerable attention, which are characterized by hygienic safety, improved organoleptic properties and perhaps the probiotic qualities (Aly et al., 2006). Some of them also constitute a natural component of the intestinal microflora (Holzapfel et al., 2001).

Lactobacilli and *Bifidobacteria* are the most common bacteria considered as potential probiotics (Espirito Santo et al., 2003). Probiotics serve as supplement to the host microbes and provide protection against several enteric pathogens. The yogurt/dahi is a good source of probiotics. Probiotic yogurts are produced by the incorporation of other lactic acid bacteria such as, *Streptococcus thermophilus, Lactobacillus bulgaricus, Lb. acidophilus, Lb. casei, Lb. GG, Lb. plantarum, Lb. reuteri, B. bifidum, B. longum, Lb. acidophilus* and *Bifidobacterium* spp. and these probiotics are most widely used in dairy industry (Akın et al., 2006). Probiotics are known to establish promising results like improved gut barrier function; adding to their unique ability to compete with pathogenic microorganisms for adhesion to the gut and improve their colonization (Rao et al., 2016).

Probiotics have various physiological functions which contribute to the health of the host environment regulating microflora and are also helpful in combating overweight and obesity (Kobyliak et al., 2016). They boost the microbial population balance in the intestine, provide protection against potential pathogenic bacteria, and prevent and/or cure intestinal diseases (Schiffrin et al., 1997; Gionchetti et al., 2000) **(Figure 1).** Such effects are mediated by the synthesis of antimicrobial metabolites such as organic acids (e.g. lactate, acetate, and butyrate), hydrogen peroxide, bacteriocins and nutrients or adhesion receptors rivalry with harmful bacteria (Hudault et al., 1997).

Fermented milk products like Dahi might have a pretty good number of potential benefits for the humans, which include assimilation of cholesterol (Noh et al., 1997), destroying enteric pathogen, prevention of inflammatory bowel disease (Gomes and Malcata, 1999; Vasiljevic and Shah, 2008) and lowering blood pressure (Alhaj et al., 2007).

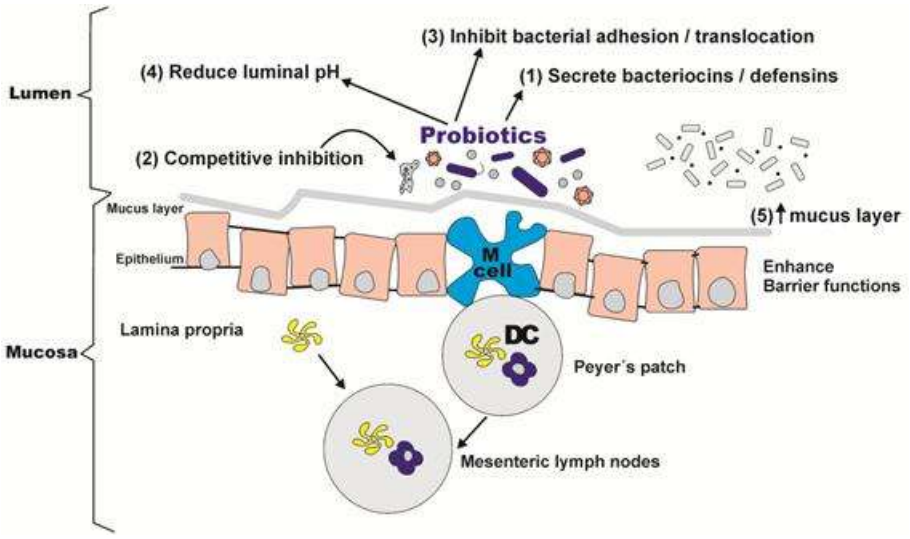

Figure 1. Mechanism of action of probiotics

(Source: Otutumi et al., 2012)

Besides, probiotic microorganisms are a good source of protein, calcium, phosphorus, potassium, vitamin B12, riboflavin (vitamin B2), thiamin (vitamin B1), folate, niacin, zinc and magnesium.

They also make sure the calcium is efficiently absorbed into the bloodstream so that the bones could receive optimum supply of the calcium (McKinley, 2005). Furthermore, Dahi is easily digestible and could reduce the risk of cardiovascular problems and cancerous tumors, including boosting the immunity of the body.

In view of above, it is clear that fermented dairy foods offer great potential to improve health and to prevent certain diseases when taken as part of a balanced diet and healthy lifestyle. The advantageous health effect of LAB is not understood well by the maximum people of Bangladesh so far. Around the developed world, milk and food companies have recently developed and sold many types of functional yogurts using selected probiotics including beneficial bacteria such as LAB and *Bifidobacterium* sp. The conventionally fermented dairy product could be considered a valuable resource for probiotic strain screening and starter culture application (Harun-ur-Rashid et al., 2007).

However, very limited work has been done in our country regarding the specific strain dahi production. The findings retrieved from the present study could encourage the people to consume more fermented dairy food products, as it could provide some important knowledge of functional properties of Dahi, which would play an important role for consumption of safe, sound and healthy food item. In addition, the result from this current study could encourage people to manufacture more fermented dairy products, which would boost the national economy as well as improve the health status of the general people of this country. So, the present study was conducted for the production of Dahi with the specific Lactic acid bacteria to meet the following objectives-

1. To isolate and identify *Lactobacillus* spp. and *Streptococcus thermophilus* from locally available dahi through phenotypic characterization and PCR method.
2. To preserve the isolated culture identified by PCR method.
3. To develop Dahi with identified specific culture.
4. To evaluate the physicochemical, microbiological and sensory properties of the developed Dahi.

Chapter-II

Review of Literature

Dahi is a gel-like fermented dairy food made by a natural acidification of the milk by the action of lactic acid bacteria. Probiotic lactobacilli are also recognized for their beneficial effect on human body (Nagpal et al., 2012). Dahi is the predominant food source containing probiotic microorganisms. The prime objective of the present study was to develop Dahi with specific LAB species identified by PCR. In order to conduct the study in an efficient manner, efforts were taken to understand the research works done on these aspects by the previous researchers through reviewing their studies and the details are given hereunder with appropriate headings. This chapter summarizes the background of Lactic acid bacteria, laboratory techniques currently used for isolation, identification and characterization of *Lactobacillus* spp. and *Streptococcus thermophilus*, yogurt composition with its manufacturing process and also describes the different health benefits of yogurt and future perspective in probiotics.

2.1 Background of Lactic acid bacteria

For bacteria that cause fermentation and coagulation of milk, the concept of the group name 'lactic acid bacteria' was created, and defines as those that produce lactic acid from lactose. Lactobacteriaceae, (the family name) was applied by Orla-Jensen (1919) to a physiological group of bacteria producing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide. The LAB are regarded as synonymous mostly with the family Lactobacteriaceae (Breed et al., 1957). They are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They are non-spore forming, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative. They are non-motile and do not reduce nitrite. *Streptococcus, Leuconstoc, Pediococcus,* and *Lactobacillus* are the four genera under 'lactic acid bacteria'. The term ‘lactic acid bacteria’ was used synonymously with “milk souring organisms”. Important progress in the classification of lactic acid bacteria was made when the similarity between milk-souring bacteria and other bacteria producing lactic acid from other environments were known (Axelsson, 1993).

Lactic acid bacteria are usually associated with habitats rich in nutrients, such as various food products (milk, vegetables, meat), but some are also members of the normal flora of the mouth and intestine of mammals. The genera that mostly fit the general description of the typical lactic acid bacteria are *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. The genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* largely remained largely unchanged, but some rod-shaped lactic-acid producing bacteria, formerly included in *Lactobacillus*, now form the genus *Carnobacterium* (Collins et al., 1987). Lactic acid bacteria have been widely used for the fermentation of many fermented product such as cheese, yogurt, sourdough, buttermilk, brined vegetables and sauerkraut (Ammor et al., 2006) **(Table 1)**. Several strains are regularly used as starter cultures to manufacture dairy products such as curd, cheese, whey and yogurt (Crow et al., 1993; Ayad et al., 2004). These bacteria produce organic acid, hydrogen peroxide and several enzymes during fermentation (Venema et al., 1993; Grobben et al., 1998). Growth of spoilage and pathogenic bacteria in the fermented food products are inhibited due to the production of antimicrobial substances by lactic acid bacteria as their competition for nutrients (Amézquita and Brashears, 2002).

Table 1. The main lactic acid bacteria associated with fermentation of dairy products

Species/subspecies	Their main uses in various dairy products	References
Lactococcus		
L. Lactis subsp. *Lactis*	Mesophilic starter used for many types of cheese, butter and butter milk	Wouters et al., 2002
L. lactis subsp. *Lactis biovar diacetylactis*	Used in Gouda, Edam, sour cream and lactic butter and butter milk.	Leroy and De Vuyst, 2004
L. Lactis subsp. *cremoris*	Mesophilic starter used for many types of cheese, butter and butter milk	Weerkam et al., 1996
Streptococcus		
S. thermophilus	Thermophilic starter used for yogurt and particularly hard and semihard high-cooked cheeses.	Beresford et al., 2001

Lactobacillus		
Lb. acidophilus	Probiotic starter culture used in cheese and yogurt.	Briggiler-Marcó et al., 2007
Lb. delbrueckii subsp. *bulgaricus*	Thermophilic starter used for yogurt and particularly hard and semihard high-cooked cheeses.	Slaterry et al., 2010
Lb. delbrueckii subsp. *lactis*	Used in fermented milks and high-cook cheese.	Giraffa et al., 2010
Lb. helveticus	Thermophilic starter used for fermented milk and particularly hard and semihard high-cooked cheeses.	Griffiths and Tellez, 2013
Lb. casei	Probiotic milk and cheese ripening adjunct culture	Kongo, 2013
Lb. plantarum	Cheese ripening adjunct culture.	Leroy and De Vuyst, 2004
Lb. rhamnosus	Probiotic additives used in cheese	Coppola et al., 2005
Leuconostoc		
Ln. mesenteroides subsp. *cremoris*	Mesophilic culture used for fresh cheese, Edam, Gouda, sour cream and lactic butter.	Weerkam et al., 1996; Slaterry et al., 2010

Lb. =Lactobacillus; L. =Lactococcus; Ln. =Leuconostoc; S. =Streptococcus, subsp. = subspecies

2.2 Probiotics

Probiotics were first introduced as a functional ingredient in dairy products to the world. Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host. Probiotics provide health benefits which go beyond the reach of the digestive health promotion. It has also been shown that probiotics regulate the fat storage and stimulate angiogenesis in the intestine. *Lactobacilli* are the most common bacteria considered as potential probiotics (Espirito Santo et al., 2003). Probiotics serve as supplement to the host microbes and provide protection against several enteric pathogens.

Probiotics are known to establish promising results like improved gut barrier function; adding to their unique ability to compete with pathogenic microorganisms for adhesion to the gut and improve their colonization (Rao et al., 2016) **(Figure 2)**. Probiotics have various physiological functions which contribute to the health of the host environment regulating microflora and are also helpful in combating overweight and obesity (Kobyliak et al., 2016).

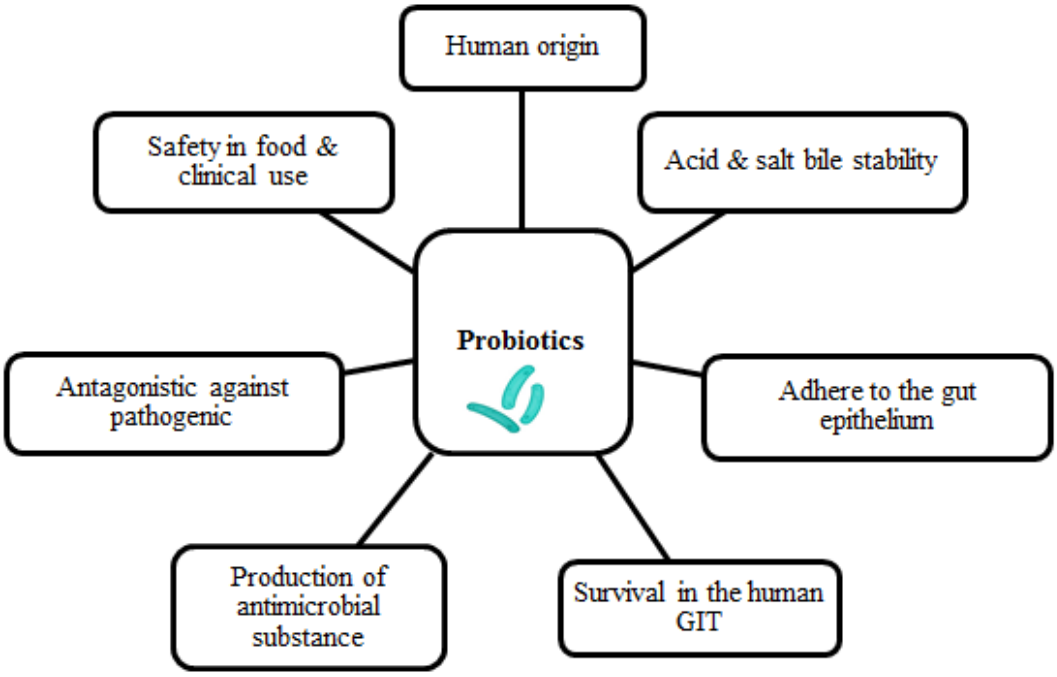

Figure 2. Various features of probiotics

Probiotics are usually consumed as preparations with active live cultures and contain bacteria, such as *lactobacilli*, *lactococci* or *bifidobacteria* (Bongaerts et al., 2016) **(Table 2)**. Probiotic foods may be defined as 'food products having live probiotic organism in adequate concentration, so that after ingestion, the obtaining postulated effect is beyond that of usual nutrient suppliers' (Saxelin et al., 2003).

Table 2. Current microorganisms used as probiotics

Probiotic bacterial genera	Species involved	References
Lactobacillus	*Lb. plantarum, Lb. paracasei, Lb. acidophilus, Lb. casei, Lb. rhamnosus, Lb. crispatus, Lb. gasseri, Lb. reuteri, Lb. bulgaricus*	Dixit et al., 2016
Propionibacterium	*P. jensenii, P. freudenreichii*	
Peptostreptococcus	*Peptostreptococcus productus*	
Bacillus	*B. coagulans, B. subtilis, B. laterosporus*	Nguyen et al., 2016
Lactococcus	*L. lactis, L. reuteri, L. rhamnosus, L. casei, L. acidophilus, L. curvatus, L. plantarum*	Eid et al., 2016
Pediococcus	*Pediococcus acidilactici, Pediococcus pentosaceus*	Sornplang et al., 2016
Streptococcus	*S. sanguis, S. oralis, S. mitis, S. thermophilus, S. salivarius*	Arora et al., 2013
Bifidobacterium	*B. longum, B. catenulatum, B. breve, B. animalis, B. bifidum*	Westermann et al., 2016
Bacteroides	*Bacteroides uniformis*	Kobyliak et al., 2016
Akkermansia	*A. muciniphila*	
Saccharomyces	*Saccharomyces boulardii*	Chen et al., 2013

2.3 *Lactobacillus*

Lactobacillus is a large and diverse group of gram positive, non-spore forming, catalase negative bacteria able to produce lactic acid as the main end product of the fermentation of carbohydrates (Pelinescu et al., 2009). They are rods, usually long and slender, that forms chains in most species. They are microaerophilic, but some are strict anaerobe. They ferment lactic acid as the main end product if they are homofermentative, with small amount of acetic acid, carbon-di-oxide and trace products, if they are heterofermentative, in addition to lactic acid they produce significant quantities of volatile products, including alcohol.

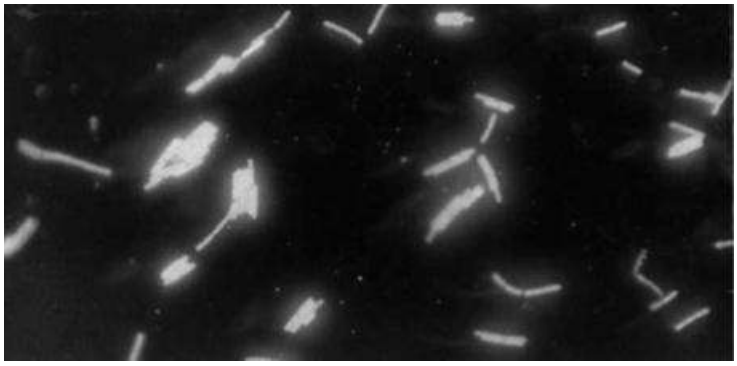

Figure 3. Acrydine orange staining of *Lactobacillus delbrueckii* subsp. *bulgaricus* at early stationary phase of growth
(Source: Teixeira, 1999)

Most species of *lactobacillus* ferment glucose into lactate hence the name *Lactobacillus* is the industrial production of fermented food production of fermented food products (Sozzi et al., 1980).

2.4 Isolation, identification and molecular characterization of *Lactobacillus*

2.4.1 Isolation of *Lactobacillus*

a) Enrichment

The pre-enrichment process may be needed for the samples with a small number of cells or cells of sub-lethal damage (heating, freezing, presence of inhibitory substances in the sample). These cells require a period of recovery in a medium, which permit repair of the damage and subsequently growth in an enrichment medium. As pre-enrichment media MRS broth can be used (Gebreselassie et al., 2016).

b) Culture

The De Mann Rogosa Sharpe (MRS) agar media (De Man et al., 1960) is specific media for detection of *Lactobacillus*. After inoculation, agar plates can be incubated aerobically at 30°C for 48 h (Gebreselassie et al., 2016) or at 37°C for 48 h in anaerobic condition (Ankita et al., 2015). The bacteria *Lactobacillus* spp. can be isolated from yoghurt samples by using modified MRS broth and MRS agar media (Hoque et al., 2010).

2.4.2 Preliminary identification of *Lactobacillus*

a) Staining

Lactobacilli are gram positive bacilli. During Gram's staining, they take color of primary stain crystal violet, as they possess a thick mesh-like cell wall which is composed of peptidoglycan layer (50–90% of cell envelope), and appeared as purple under light microscope. Gram stain morphology of *Lactobacillus* can vary, including as short, long, plump rods, slender rods, in chains or palisades.

b) Biochemical properties

The biochemical properties of *Lactobacillus* are -

- They are catalase negative, oxidase negative. They are also methyl red, Voges–prauskeur and citrate negative.
- Carbohydrate profile of *Lactobacillus* : All LAB utilizes galactose, maltose, glucose, fructose, mannose and lactose, which are the main fermenters (Gebreselassie et al., 2016).
- The *Lactobacillus* isolate exhibits negative pattern of H_2S formation, starch hydrolysis, nitrate reduction and urease activity. These are the common characters of *Lactobacillus* species.

2.4.3 Molecular identification

2.4.3.1 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is a common nucleic acid-based method for detecting microorganism. PCR includes thermocyclic enzymatic amplification of specific DNA sequences of the target bacteria. By using a pair of oligonucleotide primers that represent to DNA/cDNA regions of interest in the genomic sequence PCR is done. The genomic material of the target bacteria is first extracted by following any of the existing techniques. After that the sample is mixed with a heat-stable DNA polymerase (e.g., Taq DNA polymerase), dNTPs, primers, and PCR buffer. Amplification usually proceeds for 25 to 40 cycles in thermal cycler (Erlich et al., 1992). Molecular identification of *Lactobacillus* can be done by following the PCR assay developed by Gebreselassie et al., (2016). Molecular identification involves pure sequencing of 16s rRNA (LAB) genes. DNA extraction can be done by using DNA kit (GenEluteTM bacterial genomic DNA kit).

2.4.3.2 Agarose gel electrophoresis

After completion of the PCR reaction, the PCR products can be visualized on an agarose or acrylamide gel after electrophoresis and staining with ethidium bromide which binds to double-stranded DNA. Successful amplification of the target sequence is determined based on the PCR product's molecular size and/or sequence. The amplified products can be electrophoresed in 1.7% agarose gel and observed with gel documentation system (Alimolaei et al., 2016).

2.5 *Streptococcus thermophilus*

Streptococcus thermophilus is a type of lactic acid bacteria. Orla-Jensen was firstly recognized that *S. thermophilus* to the *streptococci* group of lactic acid bacteria in 1919 (Farrow et al., 1984). *Streptococcus thermophilus* is one of the commercially beneficial dairy bacterium which is considered as second most important starter culture in the manufacturing of dairy products (Yogurt). Basically *S. thermophilus* having following characteristics such as: homo-fermentative, gram positive, facultative anaerobic and non-spore forming and non-motile. Morphologically *S. thermophilus* is ovoid-spherical in shape with the range of diameter is about 0.7 to 0.9 μm and occurs in chains and in pairs. The optimum temperature is about 40-45°C for the growth of this bacterium, gives minimum growth at 20 to 25°C and maximum growth at 47 to 50°C. *S. thermophilus* can tolerate high rate of temperature like upto 60°C for 30 minutes (Tamime et al., 2007).

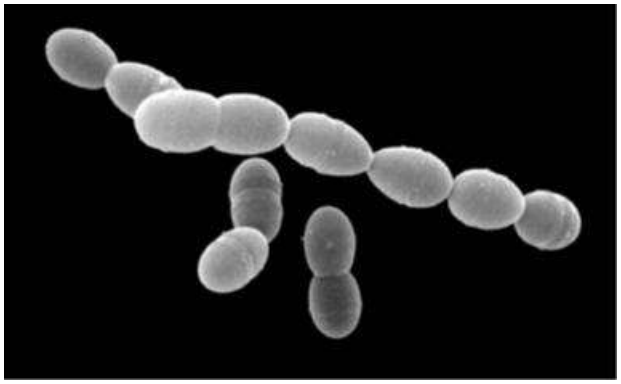

Figure 4. Electron microscopic image of *S. thermophilus*

(Source: Durso et al., 2003)

S. Thermophilus does not have an antigen of N group which is one of the unique characteristic of it among other streptococci. The DNA ranges between guanine and cytosine is about 37.2-40.3% (Zourari et al., 1992). Few strains of *S. thermophilus* have the ability to produce exopolysaccharides and for the enhancement of growth require some amino acids like histidine, cysteine, methionine, arginine, tyrosine, valine, leucine, isoleucine, tryptophan and glutamic acid as well. It can resist high temperature and it is mostly used in the production of yogurt and some different types of cheeses in association with one or more species of lactobacillus. *S. thermophilus* highly present in dairy products and *S. thermophilus can be* isolated from the dairy products by providing optimum incubation temperature.

The taxonomy of *Streptococcus thermophilus* is summarized below-

Phylum: *Firmicutes*

Class: *Bacilli*

Order: *Lactobacillales*

Family: *Streptococcaceae*

Genus: *Streptococcus*

Species: *Streptococcus thermophilus* (Bolotin et al., 2004)

They are fastidious bacteria which require simple carbohydrates as an energy source and preformed amino acids as a nitrogen source. It ferments lactose and gives L(+) lactic acid as the principal product. The cell membrane of *S. thermophilus* actively transports the lactose by means of a membrane located enzyme, galactoside permease. Then, the enzyme β-galactosidase hydrolyses the lactose to glucose and galactose inside the cell. The Embden-Meyerhof-Parnas (EMP) pathway converts the glucose to pyruvate and the pyruvate is then converted to lactic acid via lactic dehydrogenase. There are some strains of *S. thermophilus*, in which the galactose and lactic acid produced leave the cell and accumulate in the medium. But some strains can convert the galactose to galactose-1-phosphate by the help of galactokinase enzyme. The galactose-1-phosphate is then converted via the Leloir pathway to glucose-1-phosphate that is further metabolized via the EMP pathway (Robinson, 2000; Zirnstein and Hutkins, 2000).

2.6 Isolation, identification and molecular characterization of *Streptococcus thermophilus*

2.6.1 Isolation of *Streptococcus thermophilus*

a) Enrichment

S. thermophilus may be required a period of recovery in a medium, which permit repair of the damage and subsequently growth in an enrichment medium. *S. thermophilus* can be grown in M17 broth for 48 hours at 37 °C (Vanatkova et al., 2009).

b) Culture

M17 medium can be used for a good recovery of *S. thermophilus* from commercial yogurt. The M17 medium supplemented with lactose can be used for enumeration of *S. thermophilus* under aerobic incubation at 45 °C for 2-3 days (Van de Casteele et al., 2006).

2.6.2 Preliminary identification of *Streptococcus thermophilus*

a) Staining

S. thermophilus is a gram positive bacterium. It occurs in pairs or in long chains of 10-20 cells. It is spherical/ovoid in shape. It takes color of primary stain crystal violet and appeared as purple under light microscope.

b) Biochemical properties

The biochemical properties of *S. thermophilus* are –

- Catalase negative
- Homofermentative, L(+) lactic acid as the major end product. Lactose, glucose, fructose and sucrose are fermented by S. thermophilus.
- Lacks cytochromes
- Weak or no growth at 2 % NaCl
- Does not utilize arginine

2.6.3 Molecular identification

2.6.3.1 Polymerase chain reaction (PCR)

PCR amplification is considered as one of the important process for the detection of bacteria. A single copy of DNA can be amplified into several copies of the template DNA within one hour, which is rapid as compared to any other non-molecular methods.

Scientists have found several ways of identification of *S. thermophilus*, but the molecular biological methods are more time consuming and reliable than other methods.

Colmin et al., (1991) derived species-specific gene probes DNA fragment (4.2 kb) hybridizing to the closely related species *S. thermophilus* and *S. salivarius* and as well polymorphism of intraspecific restriction fragment. Ehrrmann et al., (1992) also developed a method to differentiate *S. salivarius* and *S. thermophilus* based on (23S rRNA sequence) by a gene probes. Lick and Teuber et al., (1992) published an oligonucleotide probe which is also Species-specific for *S. thermophilus.*

2.6.3.2 Agarose gel electrophoresis

Amplified products can be subjected to gel electrophoresis in 1.5 % gel and visualized by ethidium bromide staining (Vanatkova et al., 2009).

2.7 Yogurt and Dahi

Yogurt is 'the coagulated milk product obtained by the fermentation of lactic acid via the action of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Krasaekoopt et al., 2005). This dairy starters break down the sugar compound glucose and galactose that the lactose is composed of, under anaerobic conditions. The compound sugars are then processed leading to the formation of lactic acid and acetaldehyde, as shown in **Figure 5**. The composition of yogurt with its bacterial cultures determines the quality along with the nature of flavor and the way it appears. The characteristic flavor of a yogurt sample is due to the production of lactic acid, carbon dioxide, acetic acid, diacetyl, acetaldehyde and various other components from the milk fermentation process. Yogurt contains *Lactobacillus* and *Bifidobacterium* species at 10^6 viable cells per millilitre at the time of consumption (Arunachalam, 1999). According to the PFA regulations (1976), "Dahi or curd is the product derived from boiled or pasteurized milk by fermenting harmless lactic acid or other bacterial culture". It may contain added cane sugar. It should have same percentage of fat and solids-not-fat as the milk from which it is prepared. Although yogurt and dahi both are cultured or ripened dairy products still there are little differences between those. Yogurt is prepared by using starter organisms *Streptococcus thermophilus* and *lactobacillus bulgaricus* in a proportion of 1:1, whereas dahi is prepared by using mixed culture of *Streptococcus lactis*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Streptococcus citrophilus*, *Lactobacillus plantarum* etc.

Higher temperature (45°-50°C) and shorter (3-4 h) incubation period is required for yoghurt making. On the other hand, lower temperature (37-42°C) and long incubation period (8-15 h) is required for dahi preparation. Comparatively yoghurt is softer and dahi curd is reverse of that (Varnam and Sutherland, 1994).

Reagents End Products

Lactase Glycolysis+ Fermentation

Lactose ---------►Glucose + Galactose ----------►Lactic acid + Acetaldehyde

Figure 5. Enzymatic reaction in yogurt production

2.8 Composition of Yogurt

Yogurt is produced via the lactic acid bacterial fermentation of milk. Cow milk is commonly used for the development of yogurt. There may be slight difference in composition of yogurt and the milk from which yogurt is prepared **(Table 5)**.

Table 3. Chemical composition of different sources of milk used in yogurt production

Milk Source	Water (%)	Total Solids (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)
Cow	87.4	12.7	3.7-3.9	3.3-3.4	4.7-4.8	0.7
Goat	87.0	12.3	4.5	2.9-3.3	4.1-4.6	0.6-0.8
Sheep	81.7	19.3	7.4	4.5-5.6	4.4-4.8	0.9-1.0
Buffalo	82.1	17.9	8.0	4.2	4.9	0.8

(Source: Akin et al., 2006)

Table 4. Nutritional facts of Yogurt

Composition (%)	Yogurt	Low-fat yogurt	Non-fat yogurt
Fat	>3.25	>0.5 to <2.0	<0.5
Protein	4.4	5.7	5.2
Carbohydrate	7.5	7.5	6.9
Milk solids-not-fat	>8.25	>8.25	>8.25
Titrable acidity	>0.9	>0.9	>0.9

(Surono et al., 2011)

Table 5. Vitamin contents of milk and yogurt

Vitamins (Units/100 g)	Milk		Yogurt	
	Whole	Skim	Full Fat	Low Fat
Vitamin A (IU)	148	-	140	70
Thiamin (B_1) (µg)	37	40	30	42
Riboflavin (B2) (µg)	160	180	190	200
Pyridoxine (B6) (µg)	46	42	46	46
Cyanocobalamine (B12) (µg)	0.39	0.4	-	0.23
Vitamin C (IU)	1.5	1.0	-	0.7
Vitamin D (IU)	1.2	-	-	-
Vitamin E (IU)	0.13	-	-	Trace
Nicotinic acid (µg)	480	-	-	125
Pantothenic acid (µg)	371	370	-	380
Biotin (µg)	3.4	1.6	1.2	2.6
Choline (mg)	12.1	4.8	-	0.6

(Source: Tamime et al., 1980)

2.9 Types of Yogurt

Yogurt is primarily categorized according to its chemical composition, method of manufacture type of flavor or post-incubation process (Shah et al., 2000).

Table 6. Types of Yogurt (Based on fat content)

	Full-fat yogurt	Reduced-fat yogurt	Low-fat yogurt
Fat%	≥ 3	0.5-2	≤ 0.5
Solids-not-fat%	≥ 8.25	≥ 8.25	≥ 8.25
Titrable acidity %	≥ 0.9	≥ 0.9	≥ 0.9
pH	≤ 4.5	≤ 4.5	≤ 4.5

(Source: Shah et al., 2003)

- According to chemical composition, they are classified as full-fat, reduced-fat or low-fat yogurt **(Table 6)**.
- These can be grouped as set-type and stirred-type according to the production method. Set type of yogurt is incubated and then cooled in the final package which is characterized by a firm jelly like texture. It is fermented in a retail container, filled after milk inoculation and is incubated at 37°C for approximately 6 to 8 hours. On the other hand, for stirred-type yogurt, milk is incubated in a fermentation vat and the final coagulum is stirred before cooling and packaging stages. Stirred yogurt promotes the growth of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* at a mild temperature (between 40°C and 43°C) until a desired acidity level is reached (Tamime et al., 1980). Stirred yogurt texture will be less solid than a set yogurt.
- Yogurt can be classified, according to the type of starter culture used. Probiotic yogurts are produced by the incorporation of other lactic acid bacteria such as, *Streptococcus thermophilus, Lactobacillus bulgaricus, Lb. acidophilus, Lb. casei, Lb. plantarum, Lb. reuteri, B. bifidum, B. longum, Lb. acidophilus* and *Bifidobacterium* spp. and these probiotics are most widely used in dairy industry (Akın et al., 2006).

2.10 Yogurt Manufacturing Process

In the dairy industry, no matter which manufacturing process is applied, the fermented dairy product must be appropriate to national and international standard protocol. The flow diagram of manufacturing steps for yogurt production is given in **Figure 6** and the basic manufacturing steps for any types of yogurt are as follows:

2.10.1 Filtration

Filtration should be done to separate any cellular matter and other extraneous matter present in milk.

2.10.2 Standardization of milk

Standardization of milk refers to the adjustment of fat and solids not fat levels of milk by raising or lowering the levels. Standardization of milk is usually done in cases of market milk supply and also in the case of manufacture of milk products. The standardization of milk is one of the most important factors in obtaining good quality yogurt. Milk is fortified and mixed with skim milk and cream to adjust the fat content to the desired level. The minimum amount of milk solids not-fat specified in the requirements or regulations ranges from 8.2 to 8.6% in many countries. Also, stabilizers are applied to the milk to improve the correct yogurt properties including texture, mouthfeel, appearance, consistency and prevention of whey separation (Tamime et al., 1999).

2.10.3 Homogenization

Homogenization is a process of reducing a substance, such as the fat globules in milk, to extremely small particles and distributing it uniformly throughout milk. The cream will not rise to the top if milk is properly homogenized. Milk become whiter at the end of the homogenization process, and the yogurt formed from that milk is more viscous and also the flavor is homogeneously distributed all over the container (Tekinsen et al., 2000).

2.10.4 Heat treatment (Pasteurization)

The most commonly used heat treatment in the yogurt industry include 85°C for 30 minutes or 90-95ºC for 5 minutes (Tamime et al., 1999) as heating of milk greatly influences the physical properties and microstructure of yogurt. Nevertheless, sometimes very high temperature short time (100°C to 130°C for 4 to 16 s) or ultra-heat temperature (UHT) (140ºC for 4 to 16 s) are also used (Sodini et al., 2005).

There are several advantages of pasteurization such as:

- It helps to remove dissolved oxygen which promotes starter growth
- It leads to production of some aroma compounds

On the other hand, heat treatment also has some disadvantages because of the-

- Formation of certain by-products that have an inhibitory effect on the growth of bacteria in starter culture.
- Sometimes it causes reduction of pH, oxygen content of milk and denaturation of serum proteins, thus hydrophilicity of casein increases and syneresis decreases.

2.10.5 Effect of heat treatment on quality of dahi

Shekhar et al., 2012 have shown that, there is an effect of heat treatment of milk on the sensory and rheological quality of dahi. The texture depends primarily on the milk heat treatment. Among the other heat treatment, boiling treatment of milk resulted in least syneresis of whey in the dahi. Dahi prepared from boiled milk, showed the highest value in cases of firmness, consistency and index of viscosity. The study recommended that milk should be subjected to boiling treatment to produce best quality dahi. High heat treatment of milk increases gel firmness and reduces syneresis in the final product (Lucey et al., 1998; Vasbinder et al., 2004). Another study showed that better rate of gelation can be obtained via high heat treatment (Xu et al., 2008).

2.10.6 Inoculation

Following pasteurization, milk is allowed to cool to 40-45°C and generally inoculated in 1:1 ratio with the fresh starter culture bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Although inoculation level varies between 1-4%, the optimum level is 2%.

2.10.7 Incubation (Fermentation)

The quality of final yogurt production can be affected by the incubation time (Tamime et al., 1980). After inoculation, incubation takes place at 37ºC until the curd formation in the incubator (Vijayendra and Gupta, 2012) or may be incubated at 37ºC for 6 hours (Anukam and Olise, 2012).

2.10.8 Cooling and Storage conditions

Starter cultures continue to grow, if yogurts are not cooled immediately at the end of the fermentation. As a result, it causes syneresis on the surface of yogurts.

Studies showed that higher survival rates of lactic acid bacteria can be obtained at lower storage temperatures (Gilliland et al., 1988; Foschino et al., 1996). Preservation at low temperature not only hinders the excessive growth of the starter culture, but also gradually over acidification in general (Kneifel et al., 1993).

2.10.9 Packaging of Yogurt

Yogurt is packaged in such a way that is suited to individual groups as yogurt is targeted for a wide range of customers. It is usually packaged in plastic, glass or terra cotta cups; and also in a squeeze tube to make it child-friendly. Plastic is the regular medium of packaging for cup yogurt. Plastic lid as cover as optional can also be used.

Procedure of yogurt preparation

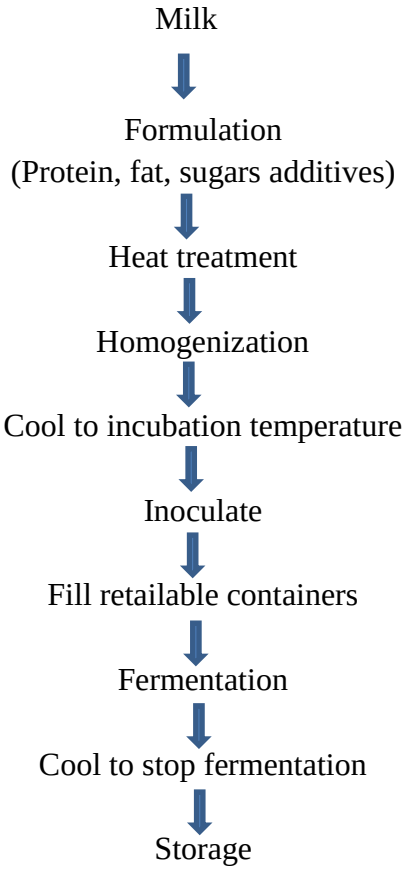

Figure 6. Process flow chart for the preparation of set yogurt

(Source: Duboc et al., 2001)

2.11 Viable count of fermented dairy products

Probiotics are defined as 'live micro-organisms that confer a health benefit on the host when administered in adequate numbers' (FAO/WHO, 2002). To achieve the therapeutic benefit, a minimum number of 10^6–10^7 colony forming units (CFU) of viable cells of probiotic cultures must be maintained in the products until the time of consumption (Boylston et al., 2004). The United States Food and Drug Administration (US FDA) has also suggested a minimum probiotic count in a probiotic food to be at least 10^6 CFU/ml (Bhadoria and Mahapatra, 2011). As stipulated by the Japanese fermented milk and lactic acid bacterial drinks association the minimum number of log10 10^6–10^7 microorganisms per gram or milliliter should be present in food products to meet the probiotic food requirements (Ishibashi and Shimanura, 1993).

2.12 Total coliform count of yogurt

Coliforms are bacteria which are commonly present in animals' digestive tracts, including human, and which can be found in their wastes. Total coliform counts give a general indication of a yogurt's sanitary condition. The total number of coliform bacteria in the food products received an indicator of the total number of coliform bacteria present in the food. As reported in Indian standard 9617 (1980), the coliform count per gram was limited to 10 cfu for dahi. Bakr et al., (2015) prepared bioyogurt and reported that coliform counts were not detected in all fresh and stored treatments that might be due to the effect of milk heat treatment and the role of yogurt bacteria in coliform control by their ability to produce a variety of antibacterial compounds.

2.13 The problem of whey separation

Wheying off 'is the appearance of whey on the surface of a gel' (Lucey, 2002). It is a common defect during gelation and subsequent storage of fermented milk products such as yogurt. 'Spontaneous syneresis is the contraction of a gel without the application of any external forces (e.g. centrifugation)' (Lucey, 2002). Excessive rearrangements of particles making up the casein gel network before and during gelation may be considered to be responsible for whey separation. Therefore, wheying off is related to the instability of the gel network and it has a strong tendency to undergo further rearrangement of network structure. As a result, it causes loss of the ability of the gel to entrap all the serum phase.

Whey separation could occur due to high incubation temperature (45°C), excessive pre-heat treatment (> 80°C for 30 min), and disturbances while the gel is still weak, low acid production (pH 4.9 instead of 4.6) and low total solids content (Lucey, 2002; Xu et al., 2008). It is said that, heat treatment increases the rigidity of yogurt gels (which is an important textural attribute), it is not very effective in preventing the whey separation that occurs in milk incubated at very high temperatures (Lucey et al., 1998). If there is an increase in loss tangent during gelation at higher frequencies in the acid gels made from heated milk and a reduction in fracture strain, both of which could assist in rearrangements and whey separation (Lucey, 2001). Thereby, the susceptibility of yogurt gels to whey separation varies markedly and is poorly understood.

2.14 Yogurt Starter Cultures

Yogurt starter cultures are active microorganisms which give desirable expected flavor, texture to milk product. Starter cultures are considered most crucial component in the manufacture of high-quality fermented milk products which regarded as harmless and food grade. Yogurt is usually inoculated with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* at the ratio of (Tamime et al., 1980). There is a symbiotic growth association between *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The acid production rate in mixed culture is greater than the rate of acid production using a single strain (Tamime et al., 1980). The combined culture produces an acidity of >10g/L within 4 hours, whereas 4g/L is produced by the isolated strain of *S. thermophilus* and 2g/L by *Lactobacillus bulgaricus* (Robinson, 2002). *Lactobacillus acidophilus*, *L. casei*, *L. paracasei* and *Bifidobacterium* species are the bacteria which are predominantly used in yogurt (Holzapfel et al., 2001). For the dairy industry, yogurt starter cultures are mass-produced under aseptic conditions. There are various methods of using starter cultures for large scale industrial production of yogurt. Cryoprotectants like glycerol, lactose, sucrose, ascorbate and glutamate for both frozen and lyophilized cultures may be used in order to maintain cell viability (Durso et al., 2003). Daily sub culturing of starter cultures can be done which have been propagated and prepared from the mother culture (Wigley et al., 1999).

Starter cultures should produce lactic acid very fast for yogurt fermentation. However, some major factors leading to inhibition of yogurt starters are as follows:

- Pathogenic organisms from the raw material may grow in the end-products at high pH, which can provide low acid production or complete inhibition in dairy fermentations. Problems are observed, especially when the starter cultures contain a single strain or when the same culture is reused over an extended period of time (Josephsen et al., 2004).
- Some environmental pollutants like insecticides have also a crucial effect on inhibition of yogurt starters (Teixeira, 1999).
- The favorable conditions for the growth and survival of starter cultures may be hampered by the presence of detergents and disinfectant residues like quaternary ammonium compounds, iodophors, hypochlorite and hydrogen peroxide used for cleaning of dairy equipment. It can also decrease the activity of starter cultures in yogurt (Surono et al., 2002).

Some solutions in order to overcome these problems are suggested as follows:

- It is very important to examine the milk for the presence of antibiotics as the antibiotics may slow down the fermentation process. *S. thermophilus* is sensitive to antibiotics like penicillin, ampicillin and streptomycin. These antibiotics may inhibit the fermentation process (Surono et al., 2002).
- Some points should be followed like as practicing good aseptic techniques, rotating the starter cultures, using phage resistant starter cultures or using multiple strain starters, each of which is resistant to different host-specific phages (Teixeira, 1999).
- Phage inhibitory media may be used for phage protection. The US Department of Agriculture introduced the idea of using substances to chelate Ca^{++} and Mg^{++} required for successful phage adsorption to the bacterial cell in 1956. For example, the phosphate salts have buffering capacity for fermentation (Durso et al., 2003).

2.15 The Role of Starter Culture on Yogurt

A starter culture is 'a microbial preparation of large number of cells of at least one microorganism to be added to a raw material to produce a fermented food by speeding up its fermentation process' (Leroy and De Vuyst, 2004).

- During fermentation, the yogurt bacteria, *S. thermophilus* and *L. bulgaricus* produce lactic acid from lactose. Acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes are also produced here. Consequently, they enhance shelf life and microbial safety, improve texture and contribute to the pleasant sensory profile of the yogurt (Leroy and De Vuyst, 2004).
- Distinct flavor of yogurt is produced by the proto cooperative action of lactobacilli and streptococci such as "acetaldehyde" at levels up to 40mg/kg is the major contributor to the flavor and it comes from lactobacilli (Teixeira, 1999). "Threonine aldolase" produced by the *S. thermophilus* showed almost linear relationship between the levels of "acetaldehyde" produced during milk fermentation and the activity of this particular enzyme in different *S. thermophilus* strains, turned out to be another potential flavor contributor (Chaves et al., 2002).
- Exopolysaccharides are responsible for important functional properties in cultured dairy products. For example, *S. thermophilus* which can produce EPS can increases the viscosity of the product by binding free water and preventing the gel fraction and whey syneresis (Hutkins et al., 2014).

The functional properties of LAB that contribute to the functionality of yogurt are listed in **Table7**.

Table 7. Contribution of starter culture (LAB) to functionality of fermented products

Functional property	Contribution to food functionality
Production of exopolysaccharides, amylase, aroma generation	Safety and/or organoleptic
Bacteriophage resistance, prevention of over acidification in yogurt	Technological
Production of bioactives, nutraceuticals, reduction of toxic compounds and anti-nutritional compounds	Nutritional and health

2.16 Role of Exopolysaccharides (EPS) in Texture of Yogurt

Water retention ability and firmness are the most important textural characteristics of yogurt that are related to the gel structure and can be influenced by the type of culture (Ruas-Madiedo et al., 2002). Smooth and creamy textured yogurt is a high consumer demand, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatine). The amounts of these ingredients required to achieve the total solids content similar to full-fat yogurt can lead to a powdery taste, excessive acid development from lactose fermentation, excessive firmness, higher whey expulsion, and grainy texture (Mistry and Hassan, 1992; Guzmán-González et al., 2000). LAB produces polysaccharides, which are considered as 'food-grade' additives. The polysaccharides provide a viable alternative for producing a creamy low-fat yogurt which, provides the consumer demand for products with low fat or sugar content and low level of additives, as well as cost factors (Jolly et al., 2002). Yogurt manufacture may be considered as the most important commercial application of EPS in the dairy industry. LAB that produce exopolysaccharides (EPS), which are generally recognized as safe, are widely used to improve the body and texture of yogurt (Faber et al., 2001; Broadbent et al., 2003). Yogurts which are made from EPS producing cultures have better water binding capacity, which decreases the product's susceptibility to syneresis (Hassan et al., 1995; Amatayakul et al., 2006). Among the conventional starters, *Streptococcus thermophilus* is a novel EPS producing LAB which is recently used for enhancing functionalities of yogurt and cheeses (Broadbent et al., 2001; De Vuyst et al., 2011; Vaningelgem et al., 2004).

2.17 EPS produced by lactic acid bacteria

EPS produced by LAB possess technological significance in the production of several fermented dairy products (Ruas-Madiedo et al., 2002). The amount of EPS produced in milk changes with the strain and species of the cultures used, and type of method of the EPS isolation. Different growth conditions (pH, temperature and incubation time) and composition of the medium (carbon, nitrogen sources and other nutrients) can alter the yield and the sugar composition of EPS produced.

The amount of EPS produced by lactic cultures, ranges from 50 to 350 mg/L for *S. thermophilus*, from 60 to 150 mg/L for *Lb. bulgaricus*, from 25-600 mg/L for *Lactococcus cremoris* and from 50 to 60 mg/L for *Lb. casei* (Cerning, 1995). The EPS concentration in milk cultures has been reported to go up to 3000 mg/L for *S. thermophilus*, to 2100 mg/L for *Lb. bulgaricus*, to 490 mg/L for *Lb. casei*, and to 600 mg/L for *L. lactis* ssp. *cremoris* by using modified medium and growth conditions (Duboc and Mollet, 2001). The non-EPS producing adjunct *S. thermophilus* can be used along with the EPS-producing *S. thermophilus* as a means of increasing the production of EPS (Zisu and Shah, 2003). The thermophilic strains among the LAB strains produce more EPS than mesophilic ones , but EPS yields are generally low (Mozzi et al., 2006). Ropiness or formation of capsular polysaccharide is strain dependent. It is reported that during the exponential growth phase of *Lb. acidophilus* 5e2 the increase in molecular mass of EPS secreted closely followed the increase in yield of EPS (Law et al., 2009). They found that the increase in yield during the growth, exponential and stationary phases was accounted for by an increase in chain length of the EPS secreted. The exopolysaccharides produced by LAB are tasteless, but their presence increases the time the milk product spends in the mouth, and hence imparts an enhanced perception of taste (Duboc and Mollet, 2001). Therefore, the incorporation of isolated EPS or EPS producing cultures may provide viscosity, stability, and water-binding functions (De Vuyst and Degeest, 1999) that may contribute emphatically to the mouth-feel, texture and taste perception of yogurt (Duboc and Mollet, 2001).

2.18 Beneficial effects of LAB

The metabolic activities of LAB are responsible for the production of lactic acid, the coagulation of milk proteins, and the production of various compounds that determine the organoleptic and textural characteristics of the final product (Van de Water and Naiyanetr, 2008). The LAB have proteolytic activity that could exert an effect on the formation and stability of the milk protein gels (Laws and Marshall, 2001). They produce antimicrobial agents, such as organic acids, bacteriocins, diacetyl and hydrogen peroxide which act as preservatives and also inhibit the growth of harmful putrefactive microorganisms (Noordiana et al., 2013). The lactic acid bacteria in yogurt are responsible for longevity of its consumers (Shah, 2007).

Probiotic bacteria must not only be viable and available in a high concentration, typically 10^6 CFU/g of a product, but also be able to survive passage through the harsh conditions of the GIT to reach their target site in live form (Mckinley, 2005; Shah, 2007). Studies have shown that administration of various strains of *Bifidobacterium*, *Lactobacillus* and *Lactococcus* can restore the normal intestinal permeability as a result improving intestinal processing of antigens ingested in the diet, reducing intestinal inflammation and IgE production by increasing the uptake of antigens by Peyer's patches (gut-associated lymphoid tissue), and potentiation of regulatory T cell cytokines, therefore decreasing symptoms of atopic dermatitis (Ji, 2009).

2.19 Health benefits of yogurt

Probiotic bacteria are supplements added to fermented milks such as yogurt. Most probiotic bacteria belong to the Lactobacillus genera which are common but non-dominant members of the human GIT's indigenous microbiota. Some of the potential health benefits of functional foods containing probiotic bacteria such as yogurt are enumerated below-

- Consumers get nutritional and health benefits as the probiotics are able to proliferate or even survive for a long period of time in human gastrointestinal tract (Gardini et al., 1999).
- Probiotic microorganisms are a good source of protein, calcium, phosphorus, potassium, vitamin B12, riboflavin (vitamin B2), thiamin (vitamin B1), folate, niacin, zinc and magnesium. They also make sure the calcium is efficiently absorbed into the bloodstream so the bones get an abundant supply of the calcium (McKinley, 2005).
- Yogurt has a similar composition to the milk from which it is made in terms of its nutritional profile, but will vary somewhat if other components are added such as fruit, cereal etc. It provides protein of high biological value. Moreover, the low-fat yogurts provide an array of important nutrients in relation to their energy and fat content, therefore making them a nutrient-dense food (McKinley, 2005).

- Potential function of probiotic bacteria also includes colonization in the gut, anticarcinogenic effect, hypocholesterolemic effect, immune modulation, reducing some forms of food allergies and prevention of inflammatory bowel disease (Gomes and Malcata, 1999; Vasiljevic and Shah, 2008).
- The essential health benefits of regular consumption of probiotic products include the improvement of the intestinal microbial balance; alleviating the symptoms of lactose intolerance through the production of lactase; reducing the risk of colon cancer in human studies and protection against breast cancer; lowering the blood cholesterol levels; suppression of blood pressure of hypertensive individuals; playing a crucial role in the prevention of diarrhoea; and inhibiting the growth of some pathogenic bacteria (Alhaj et al., 2007).
- Yogurt bacteria having microbial enzymes may degrade the protein which is a desirable process that improves milk digestibility and enhances nutritional quality. Though the Lactic acid bacteria are usually weakly proteolytic, they do cause a significant degree of proteolysis in yogurt (Abu-Tarboush, 1996).
- It is reported that a cell envelope-bound proteinase is rarely present in *S. thermophilus* and it possesses two additional peptidases, an oligopeptidase and an amino peptidase, which can be involved in bacterial growth by providing amino acids and in development of flavour in dairy products (Fernandez-Espla and Rul, 1999).
- Lactic acid bacteria have the proteolytic system that hydrolyses milk proteins to release ACE-inhibitory peptides (Yamamoto et al., 1993). ACE-inhibitory and antihypertensive peptides originating from milk usually contain up to 10 amino acids. The ACE-inhibitors derived from have moderate inhibitory potencies, usually within an IC50 range of 100-500 μmol/L (Hayes et al., 2007). Therefore, strain selection is one of the major factors that influence the release of ACE-inhibitors in dairy fermentations (Takano, 2002; Korhonen and Pihlanto, 2005).
- There are several proteolytic strains of the LAB species such as, *Lb. helveticus Lb. casei, Lb. plantarum, Lb. rhamnosus, Lb. acidophilus, L. lactis ssp. lactis and L. lactis ssp. cremoris,* as well as the two species used in traditional yogurt production *Lb. bulgaricus* and *S. thermophilus* which contain a particularly high

number of peptides including ACE-inhibitory and antihypertensive peptides (López-Fandiño et al., 2006). Addition of probiotics to yogurt has been shown to increase *in vitro* ACE-inhibitory activity due to improved proteolytic activity (Donkor et al., 2007).

- Various milk peptides may exert antihypertensive effect through several mechanisms such as inhibition of the release of endothelin-I by endothelial cells (Maes et al., 2004), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002), stimulation of bradykinin activity (Perpetuo et al., 2003), enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000) and a vascular relaxing mechanism (Miguel et al., 2007).
- A study reviewed the effects and mechanisms of action of fermented dairy products on serum cholesterol concentrations and recommended a moderate cholesterol-lowering action by fermented dairy products (St-Onge et al., 2000). Similarly, another study suggested moderate cholesterol-lowering action by dairy products fermented with appropriate strains of LAB and bifidobacteria (Pereira and Gibson, 2002).
- Feeding yogurt powder diet to normotensive rats reduced plasma TG, total cholesterol and HDL-cholesterol but did not influence their systolic BP (Yuan and Kitts, 1993).
- On the other hand, it was observed that a substantial increase in HDL-cholesterol level and decrease in TG and atherogenic index in the blood serum of rats fed milk fermented with *Lb. casei* EMC0409 and *S. thermophilus* TMC1543 along with a decrease in systolic BP of human volunteers consuming the fermented milk (Kawase et al., 2000).
- Long-term daily consumption of synbiotic yogurt, containing *S. thermophilus*, *L. lactis*, *Lb. acidophilus* 145, *B. longum* 913 and 1% oligofructose did not lower the total and LDL-cholesterol in healthy women but increased the serum concentration of HDL-cholesterol that lead to the desired improvement of LDL/HDL ratio (Kiessling et al., 2002).

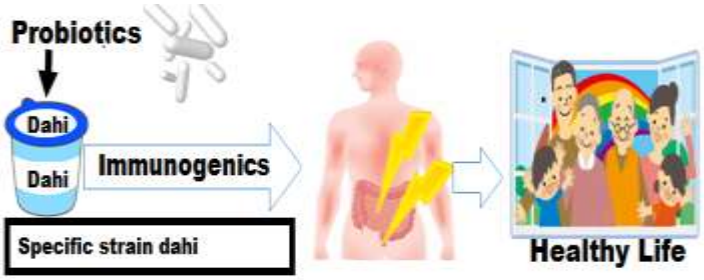

Figure 7. Health benefits of probiotic Dahi

2.20 Future Perspective in Probiotics

In the recent years, the scientific reports reveal that LAB has many positive health effects. Several recent studies have highlighted the critical role of intestinal microbes on health. Various studies including different probiotic strains have been performed in humans and animal models to investigate their beneficial effects (Lebeer et al., 2010; Bron et al., 2012). Probiotic bacteria are capable of controlling the colonization and eradication of pathogens in the intestine, including competition for scarce nutrients in the intestine, and mucosal immune modulation (Kamada et al., 2013). Moreover, it has been well established that probiotics are an important prophylactic or therapeutic strategy for many mucosal and non-mucosal immune-related conditions, such as inflammatory bowel diseases, celiac disease, metabolic syndrome, and diabetes (Ivanov and Honda, 2012). Recently, the use of genetically modified commensal and lactic acid bacteria to produce compounds of health concern has become increasingly important as an extesion of the probiotic principle (Martin et al., 2013).

2.21 Significance of the research work

In this study, efforts were made to isolate the *Lactobacillus* spp. and *Streptococcus thermophilus* from locally available Dahi. These isolates were identified by the PCR method and were used in this study as starters for production of dahi. This study will indicate the scope of using probiotic cultures in the preparation of commonly consumed fermented foods like yogurt to increase the probiotic quality of these products **(Figure 7)**. The findings from the present study are expected to encourage the people to consume more fermented dairy food, as it will provide some important knowledge of functional properties of probiotics which play a major role for beneficial health effects of consumers **(Figure 7)**. In addition, the study would assist to produce functional fermented dairy products which will have impacts on the national GDP, along with the improvement of the health status of the people of this country.

From this section of review, it is clear that various scientists around the globe have tried to find out the health effects of Dahi or yogurt prepared from different starter culture organisms. In our country, non-specific mixed culture is regularly used to make dahi. This study is one of the initiatives of developing specific strain dahi, which will support, encouraging people to consume more fermented dairy food.

Chapter-III

Materials and Methods

3.1 Statement of the experiment

The experiment was carried out at the Department of Dairy and Poultry Science (DDPS), Chattogram Veterinary and Animal Sciences University (CVASU) to develop Dahi using specific culture that is isolated from locally available Dahi. Analytical process or assay was performed at the PRTC and DDPS laboratories of CVASU during July to November, 2019.

3.2 Collection of raw materials or samples

The Dahi samples were collected by purchasing from the local market of Chattogram metropolitan city, Chattogram, Bangladesh. After collection, the sample was kept in sterile plastic container, and later the sample was stored aseptically in low temperature (4°C) for the isolation of bacteria (*Lactobacillus* spp. and *Streptococcus thermophilus*).

3.3 Isolation of *Lactobacillus* spp.

Conventional bacteriological methods were followed for primary isolation and identification of *Lactobacillus* spp. The collected samples were brought to the PRTC Laboratory, CVASU for isolation and identification of bacteria (*Lactobacillus*). MRS agar media was used to isolate *Lactobacillus* spp. from locally available Dahi (De Man et al., 1960).

3.3.1 Procedure for the isolation and identification of *Lactobacillus* spp.

The collected sample was mixed homogeneously and directly streaked onto MRS agar and incubated 48-72 hours at 37°C. After incubation the bacterial growth was observed. Colonies differ in morphology, pigmentation; shape and size were subcultured. The growth of *Lactobacillus* spp. was suspected when off-white to cream colour, shiny round shaped colonies yielded on a MRS agar plate. After several subcultures, finally pure cultures of *Lactobacillus* spp. were isolated. *Lactobacillus* spp. was further investigated by microscopic examination, gram-staining and catalase reactions. Only the gram-positive bacilli, catalase-negative were considered as *Lactobacillus* spp.

3.4 Isolation of *Streptococcus thermophilus*

Blood agar media was used to isolate *Streptococcus thermophilus* from locally available Dahi.

3.4.1 Procedure for the isolation and identification of *Streptococcus thermophilus*

The collected samples were brought to the PRTC Laboratory, CVASU for isolation and identification of *Streptococcus thermophilus*. For the isolation of *Streptococcus thermophilus*, sample was directly streaked onto blood agar. The agar plates then were incubated at 37^0C for 24 hours. Colonies differ in morphology, pigmentation; shape and size were subcultured. Small, dew-drop like colonies surrounded by a zone of hemolysis were suspected for *Streptococcus thermophilus.* After several subcultures, finally pure cultures were isolated. Cultures were further investigated by microscopic examination, gram-staining and catalase reaction.

3.5 Gram's staining

Gram's staining was performed to determine the size, shape and arrangement of bacteria. With a sterile cooled loop, a drop of sterile water or saline solution was placed on the slide. The loop was sterilized and cooled again and a bacterial colony was picked up and gently stirred into the drop of water/saline on the slide to create an emulsion. The smear was then heat fixed by quickly passing it two to three times through a flame. After fixation the Gram's staining was done as follows: Crystal violet (primary stain) was used for 2 minutes, Gram's iodine (mordent) for 2 minutes, Acetone (decolorizer) for 10 seconds and finally, Safranin (counter stain) for 1 minute. Gently rinsing was done with tape water after every step. Then the slide was blot dried with bibulous paper. The slide was then observed by microscope under 100X with immersion oil and characterization of bacteria was recorded.

3.6 Catalase test (Slide test)

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and oxygen production is observed by the generation of O_2 bubbles. The generation of gas bubbles indicates the presence of the enzyme, hence the catalase positive nature of the bacterium. A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop.

Then a drop of 3% H_2O_2 was placed on to the slide and mixed. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles. *Lactobacillus* spp. and *S. thermophilus* are both catalase negative and no O_2 production (gas bubbles) was observed when 3% H_2O_2 solution is dropped on top of the colonies grown overnight on agar medium. Then the slide was disposed in the biohazard glass disposal container.

3.7 Storage of isolated presumptive *Lactobacillus* spp. and *S. thermophilus*

All presumptive isolates were cultured into brain heart infusion (BHI) (Oxoid Ltd., UK) broth and incubated overnight at 37°C. For each isolate, 700 µl BHI broth culture was added to 300 µl 50% glycerol in 2 ml sterile eppendorf tube and stored at -80°C for further investigation.

3.8 Identification of *Lactobacillus* spp. and *S. thermophilus* by Polymerase Chain Reaction (PCR)

3.8.1 DNA extraction from the isolates

Conventional boiling method was used for extraction of DNA from the obtained isolates. Firstly, 200 µl deionized water was taken into a 2 ml eppendorf tube and a loop full of fresh colonies (about 5-6) was picked up from the agar plate, and transferred to the eppendorf tube. Then the tubes were vortexed for few seconds to make a homogenous cell suspension and boiled at 100°C for 15 minutes. Immediately after boiling, the suspensions were placed at -20°C for 5 minutes for cooling. After cooling, it was boiled again at 100°C for 15 minutes and then cooled at -20°C for 5 minutes. It is a slightly modified boiling method and it may be called a 'double boiling method' of DNA extraction. It was done as *Lactobacillus* spp. and *S. thermophilus* being gram-positive bacteria possess a thick cell wall. Finally, the Eppendorf tubes along with the cell suspension were centrifuged at 10000 rpm for 5 minutes. About 100 µl of supernatant containing bacterial DNA was collected in another sterile eppendorf tube and preserved at -20°C until further testing.

3.8.2 PCR of *Lactobacillus* spp.

The final identification of *Lactobacillus* spp. was carried out by PCR. The primer details used for detection and confirmation of *Lactobacillus* spp. is given in **Table 8**.

At first the stock solution (100 picomole concentration) of each primer was diluted with molecular grade water to make a 20 picomole concentration to be used for a PCR test. The specific primer sets used to amplify 16s rRNA gene of *Lactobacillus* were LAC1F (AGCAGTAGGGAATCTTCCA) and LAC2R (ATTTCACCGCTACACATG) (Endo et al., 2009). PCR reactions were conducted with a 25 µl reaction volume which was prepared with 2.5 µl of DNA, 11.75 µl of master mix (Thermo Scientific Dream Taq PCR Master Mix (2x) Ready to use), 5 µl of both primer sets (20 pmol), and 0.75 µl of nuclease-free water. The PCR program was carried out by the following program: Initial denaturation 94°C for 3 min, 29 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 3 min; and a final extension of 72°C for 10 min (Gebreselassie et al., 2016).

3.8.3 PCR of *Streptococcus thermophilus*

Primers used to identify *Streptococcus thermophilus* is enlisted in **Table 8**. At first the stock solution (100 picomole concentration) of each primer was diluted with molecular grade water to make a 20 picomole concentration to be used for a PCR test. The PCR reaction mixture (25 µl) was prepared from 4 µl of chromosomal DNA, 12.5 µl of master mix (Thermo Scientific Dream Taq PCR Master Mix (2x) Ready to use), 2 µl of specific primer sets (20 pmol) and 4.5 µl of nuclease-free water. The PCR program used for *Streptococcus thermophilus* had 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 52°C for 30 s and 72°C for 1 min, then 1 cycle of 72°C for 5 min (Vanatkova et al., 2009).

Table 8. Primers used for the detection and confirmation of *Lactobacillus* spp. *and Streptococcus thermophilus* for this study

Target organisms	Primer set	Primer sequence (5'-3')	References
Lactobacillus **spp.**	LAC1F LAC2R	AGCAGTAGGGAATCTTCCA ATTTCACCGCTACACATG	Endo et al., 2009
Streptococcus thermophilus	ThI ThII	ACGGAATGTACTTGAGTTTC TGGCCTTTCGACCTAAC	Tilsala-Timisjärvi et al., 1997

Amplification (PCR) was performed in a thermal cycler (Applied Biosystem®, 2720).

3.8.4 Visualization of the PCR products by agarose gel electrophoresis

PCR products were separated by agarose gel electrophoresis. A gel tray was accumulated in a proper set-up by setting comb in the tray. Then, 1.5% agarose solution (Seakem® LE agarose, Lonza) was made and kept in a water bath at 50°C for cooling, and 5µl ethidium bromide was added. Finally, the agarose was poured into the gel tray. To solidify the gel, it was kept in room temperature for 20 minutes. Then the gel was transferred into an electrophoresis chamber filled with 1X TAE buffer. 5.5 µl of each PCR product for a gene was loaded into a gel-hole and 3 µl ladder was used. Electrophoresis was run at 90 volts, 120 Amp for 35 minutes. Finally, DNA fragments were viewed by UV illumination and photographed using gel documentation system (BDA digital, biometra GmbH, Germany).

3.8.4.1 Procedure of agarose gel electrophoresis

1. For 1.5% agarose gel, 0.5 g of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask.
2. The mixture was heated in a microwave oven until agarose was completely dissolved.
3. The agarose-TAE buffer solution was then kept in a water bath at 50°C for cooling.
4. Gel casting tray was prepared by sealing ends of gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.
5. 5 µl of ethidium bromide was added to agarose-TAE buffer mixture, shaked well and poured into gel tray.
6. The gel was then allowed to be cooled (left for 15-30 minutes at room temperature).
7. The comb(s) were removed and the electrophoresis chamber was filled with 1x TAE buffer until the casted gel is drowned completely.
8. 5.5 µl of DNA and 3 µl of 1kb plus DNA marker (ladder) were loaded into gel.
9. The electrophoresis was run at 90 volts and 120 Amp for 35 minutes.
10. Then the gel was taken to the UV transilluminator for image acquisition and analysis.

3.9 Development of Dahi

3.9.1 Preparation of *Lactobacillus* spp. culture

Lactobacillus spp. cultures were reconstituted from frozen stock cultures (300 µl 50% glycerol in 700 µl BHI broth) by plating out in MRS agar. The plates were incubated 48-72 hours at 37°C. After incubation the bacterial growth was observed.

Thereafter a loopful colony was inoculated in 10 ml MRS broth. The inoculums were incubated at 37°C for 24 hours to get sample approximately close to the concentration level of 10^6 cfu susceptibility testing.

3.9.2 Preparation of *Streptococcus thermophilus* culture

Streptococcus thermophilus cultures were reconstituted from frozen stock cultures (300 µl 50% glycerol in 700 µl BHI broth) by plating out in blood agar. The plates were incubated 24 hours at 37°C. After incubation the bacterial growth was observed. Thereafter a loopful colony was inoculated in 10 ml BHI broth. The inoculums were incubated at 37°C for 24 hours to get sample approximately close to the concentration level of 10^6 cfu susceptibility testing.

3.9.3 Preparation of mother culture

The *Lactobacillus* spp. *and Streptococcus thermophilus* cultures grown in MRS and BHI broth respectively were centrifuged (4000 rpm for 5 minutes) to get bacterial pellets. Then the supernatant fluid was carefully discarded by pipetting. The bacterial pellets were washed twice with sterile PBS (phosphate buffered saline) to remove MRS broth. Finally the pellets were reconstituted in 2 ml of PBS. Out of this, a 200 µl aliquot containing 5 x 10^8 cfu/ml was added to 10 ml of the heat treated milk and incubated for 18 h at 37°C.

3.9.4 Preparation of Dahi with the isolated culture

Whole fresh cow milk was used for preparation of Dahi. After collection milk was filtrated by a strainer to remove any extraneous matter. Milk was brought to boil to destroy viable microorganisms. The volume was reduced to 25% by boiling, then 5% milk powder and 4% sugar were added to the milk. Powder milk and fine crystalline cane sugar of commercial grade was obtained from the local market was added during boiling milk and stirred for proper dissolution. The fat and protein content of the milk were 3.8% and 3.6% respectively after adding milk powder. The milk was stirred continuously until cool to 30-35°C. The milk was then seeded with 2% culture which was prepared previously. After filling in the containers, it was incubated at 37°C for 6-8 hours. Then the prepared Dahi was stored at 4°C. Dahi was prepared using isolated *Lactobacillus* spp. and *Streptococcus thermophilus* culture singly and or a combination process.

Finally, five probiotic Dahi samples were prepared (T_1, T_2, T_3, T_4 and T_5) from the prepared culture. Dahi (T_1, T_2, T_3) samples were made by adding *Lactobacillus* spp., whereas T_4 was prepared by using *Streptococcus thermophilus* culture and (T_5) Dahi sample was made with a combination of *Lactobacillus* spp. (T_3) and *Streptococcus thermophilus* (T_4) bacteria together. The whole procedure of probiotic Dahi preparation is presented below in **Figure 8**.

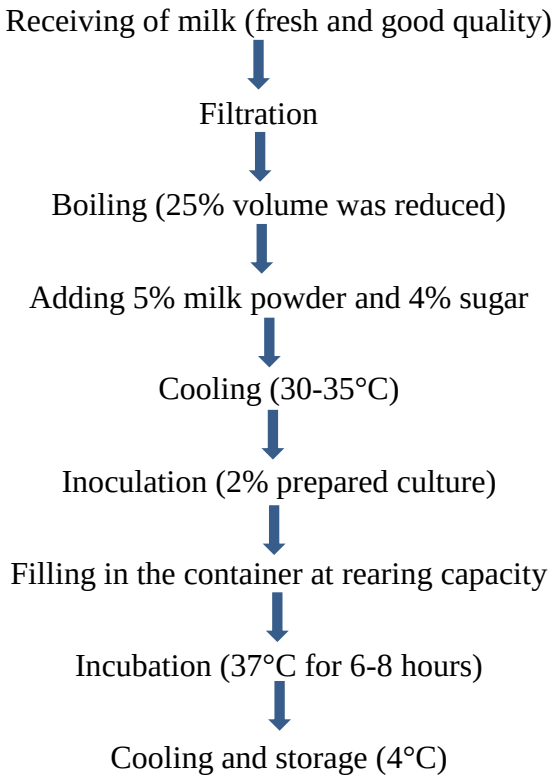

Figure 8. Development of Dahi using isolated LAB species

3.10 Physicochemical analysis of developed Dahi

The developed Dahi was analyzed for pH, titrable acidity, moisture, ash and protein. All the determinants were done in triplicate and the results were expressed as the average. The pH and titrable acidity were done on day 1 and 7.

3.10.1 Determination of titrable acidity

Acidity percentage was determined as per the method as followed by Aggarwala and Sharma., (1961).

Calculation:

$$\%\text{Titrable acidity} = \frac{\text{ml of alkali used} \times (\text{N})\text{of NaOH} \times 0.09}{\text{Total volume of the sample}} \times 100$$

3.10.2 Determination of pH

The pH of the preparations was measured using a digital microprocessor pH meter (pHepÒ3, Hanna Instruments, USA). The pH meter was standardized using reference pH 4.0 and 7.0 buffer solutions.

3.10.3 Determination of Moisture, Protein and Ash

The Dahi samples were tested for proximate analysis having dry matter (DM %), moisture %, crude Protein (CP %), and ash using standard laboratory procedures (AOAC, 2005). Dry matter estimation was done by oven dry method. Crude protein estimation was accomplished by Kjeldahl Method. Ash was measured by igniting the pre-ashing sample on a Muffle furnace at a temperature of 600°C for four to six hours.

3.11 Microbiological analysis

3.11.1 Viable count of starter culture

Pour plate technique was applied for enumeration and isolation of bacteria. Enumeration of bacteria was carried out by aseptically mixing yogurt sample (1 ml) with 9 ml of sterilized 0.9% physiological saline. The sample was thoroughly mixed and serial dilutions were performed using physiological saline as the diluents (Behrad et al., 2009). The sample was diluted up to 10^{-5}. Then 50 µl, 100 µl, 100 µl, 150 µl and 200 µl amount of sample from 1 to 5 dilution tubes were transferred respectively to the sterile petri-plates filled with melted, cooled MRS agar medium. Initially 15 ml of melted (45 °C) MRS agar medium was placed into a sterile petri dish followed by cooling of agar to temperature to allow solidification. The plates were rotated clockwise and anti-clockwise several times to spread the sample evenly on to the medium. All the petri-plates were labeled carefully, taken duplicates for each plate. The colonies formed were counted after 24-48 hours incubation at 37 °C in an inverted position (Benson, 2002). After incubation, the plates having well-spaced colonies were selected for counting.

The colonies were enumerated by using colony counter and counted the number of total viable bacterial colonies and expressed as cfu/ml of sample. The number of colonies or viable bacterial count per ml was calculated by multiplying the average number of colonies per plate by the reciprocal of the dilution.

cfu/ml =No. of colonies (Mean) x Dilution factor.

3.11.2 Total coliform count

Coliform count was determined by the methods described in the "Standard Methods for examination of Dairy Products" by APHA (1992) using VRB (Violet Red Bile agar). 1 gm of sample were measured and then dissolved it in 9 ml previously sterilized distill water and then serial dilution was done. Diluted sample was poured on petridishes and the pour the agar media on it and allowed it for solidification. Then it was incubated at 37°C for 24 hours. Then the plates were checked to observe the growth of coliform bacteria.

3.12 Sensory evaluation of prepared Dahi

Sensory characters such as color and appearance, aroma, taste, body and texture, and overall acceptability of the prepared Dahi samples were evaluated by a panel judges consisting of 5 members of the Dairy Science laboratory of CVASU, using hedonic scale developed for the purpose. The sensory characters of the prepared samples were measured by the panel expert following the method as described by Shekhar et al. (2013), the test was aimed at quality appraisal of the products. The sensory evaluation of prepared dahi was performed by the panel of judges based on “9-point hedonic scale” (1, 2, 3, 4, 5, 6, 7, 8 and 9 represent dislike extremely, Dislike very much, Dislike moderately, Dislike slightly, neither like nor dislike, Like slightly, Like moderately, Like very much and like extremely, respectively).

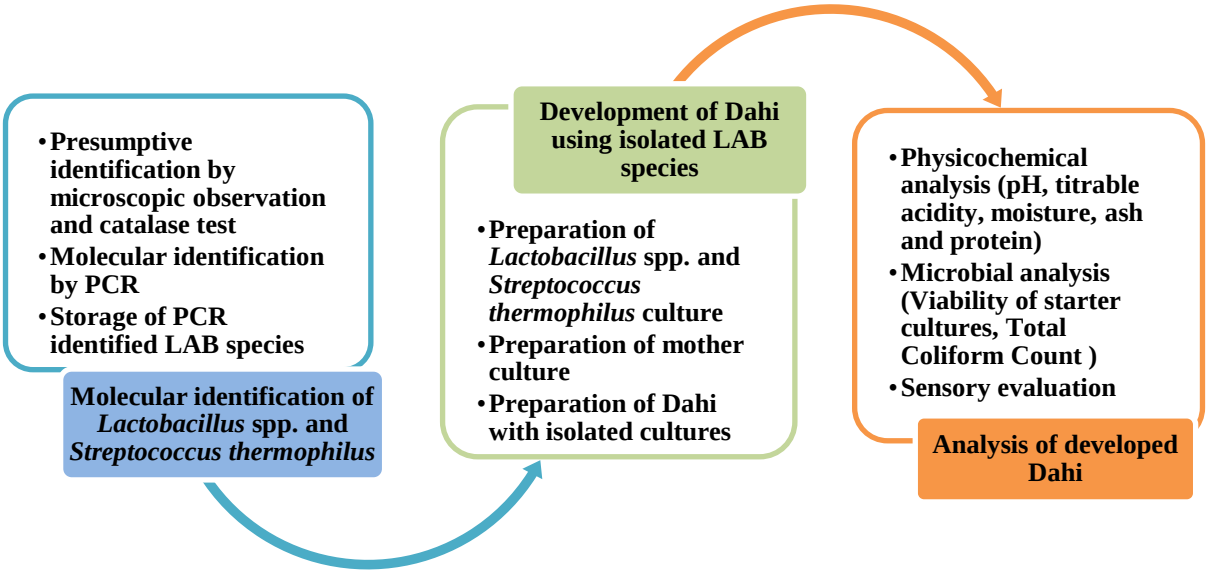

Figure 9. Experimental work flow chart

3.13 Statistical analysis

All data (proximate composition and sensory evaluation) were determined and stored in Microsoft Excel 2010 spread sheet to evaluate statistical analysis. All the collected data were subjected to statistical analyses by using one way ANOVA (Minitab version16, 2000). The significance of difference between means was determined by Fisher's least significant difference at $P \leq 0.05$.

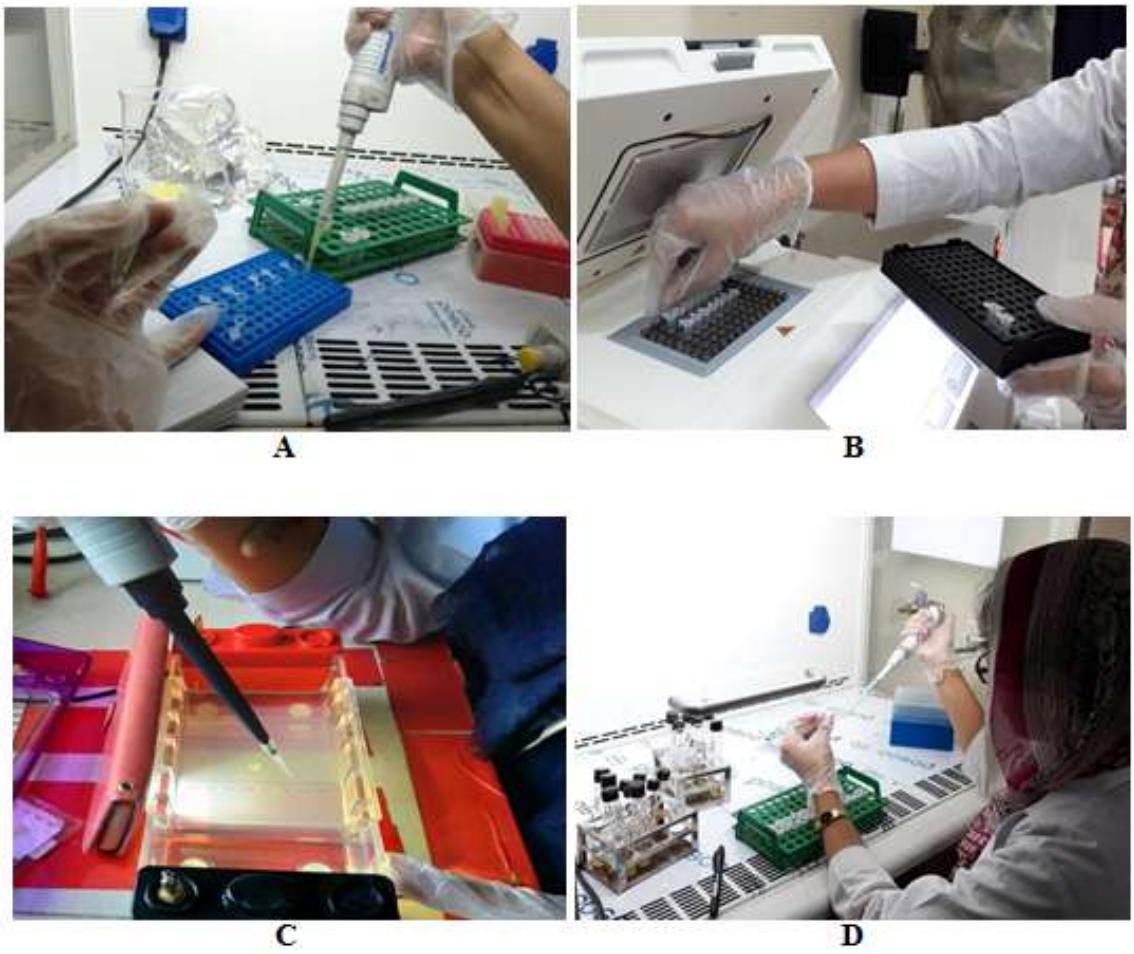

Figure 10. Different steps of PCR

(A) Preparation of PCR reaction mixture, (B) Kept PCR tube in thermal cycler, (C) Loading PCR product into gel-hole, (D) Storage of PCR identified culture.

Figure 11. Development of Dahi

(A) Culture in broth, (B) Preparation of mother culture, (C) Mother culture, (D) Measuring the culture, (E) Filling the container, (F) Kept in incubator.

Figure 12. Analysis of Dahi samples

(A) Determination of pH, (B) Determination of Acidity, (C) Counting of bacteria, (D, E, F) Sensory evaluation by judge panel.

Chapter-IV

Results

The results of isolated bacteria retrieved from locally available Dahi for uses as Dahi, obtained through the investigation are presented below through tables and graphs.

4.1 Isolation of *Lactobacillus* spp. and *Streptococcus thermophilus*

Lactobacillus spp. and *Streptococcus thermophilus* were isolated from the locally available Dahi. The bacteria were identified presumptively by the morphological characteristics, microscopic observation and catalase reaction.

4.1.1 Morphological characteristics of *Lactobacillus* spp. and *S. thermophilus*

The characteristic growths of *Lactobacillus* spp. and *S. thermophilus* observed on MRS agar and blood agar are displayed in **Figures 13** and **14**, respectively. Based on morphological characteristics, the isolates were detected as *Lactobacillus* spp. The isolates were grown in MRS agar at 37°C for 48-72 hours. All the isolates grew as cream colored, circular, convex, shiny, and watery with smooth edge. All the isolates were appeared to be morphologically similar to *Lactobacillus* spp.

Figure 13. Growth of *Lactobacillus* spp. on MRS agar

The isolates of presumptive *Streptococcus thermophilus* were selected on the basis of colony morphologies of the isolates; small, dew-drop like colony producing hemolysis on blood agar.

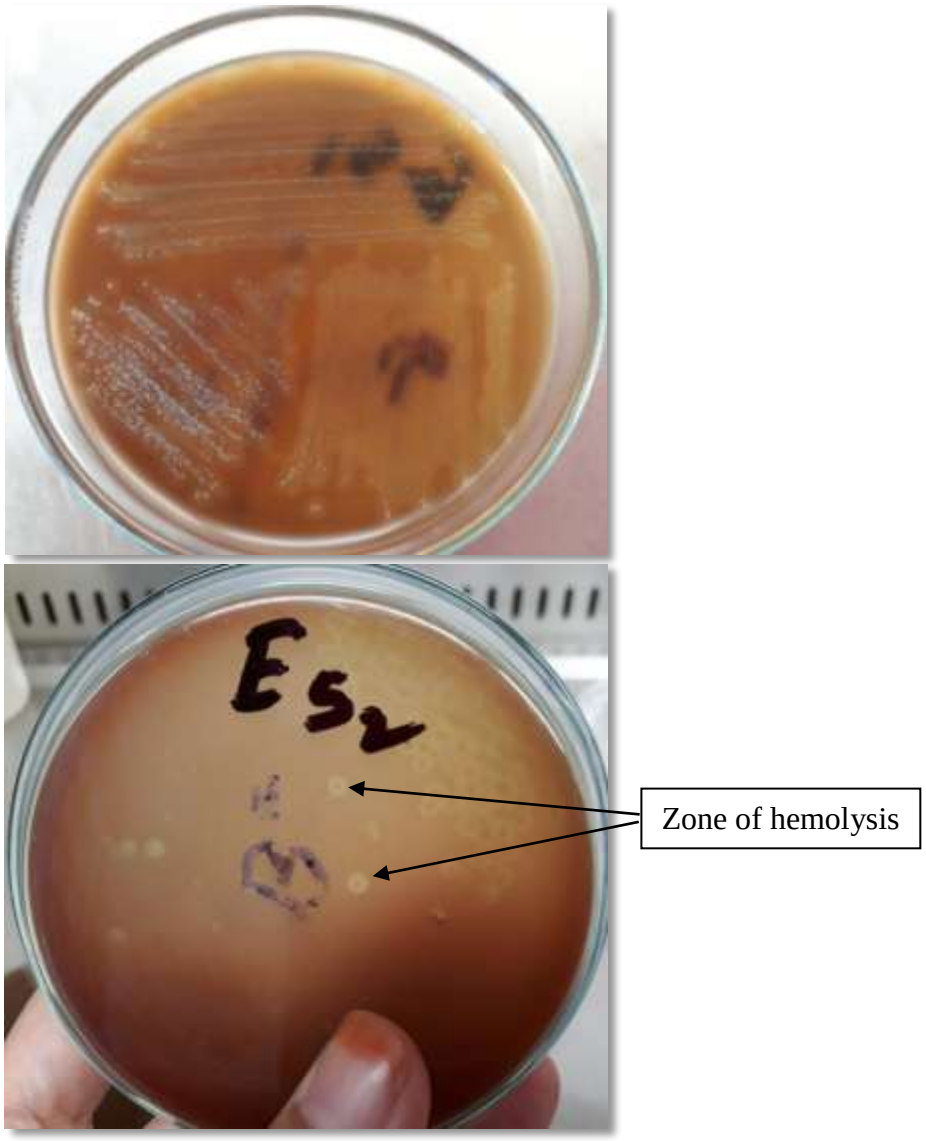

Figure 14. Growth of *Streptococcus thermophilus* on blood agar

4.1.2 Microscopic observation

All the presumptive isolates of *Lactobacillus* spp. **(Figure 15)** were purple colored, rod-shaped and the presumptive isolates of *Streptococcus thermophilus* **(Figure 16)** were gram-positive chain-forming cocci under light microscope.

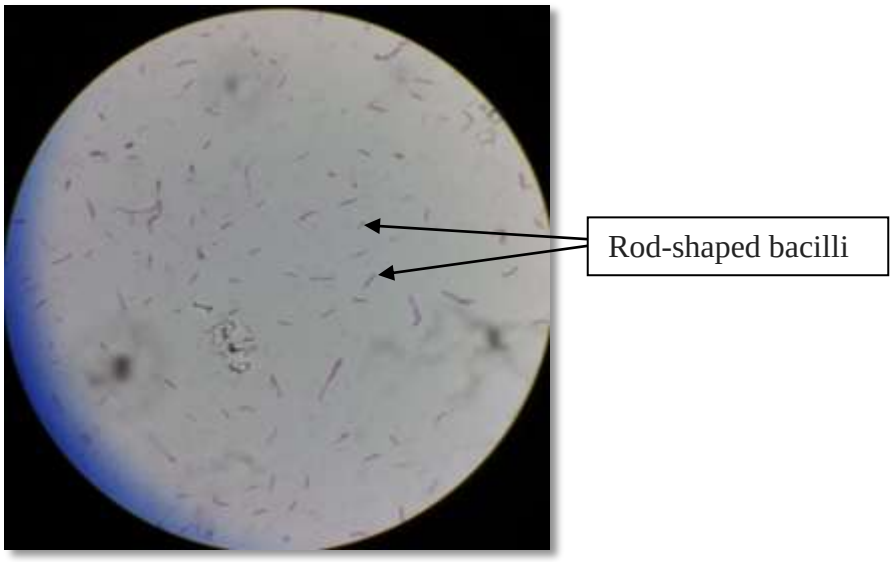

Figure 15. *Lactobacillus* spp. under microscope

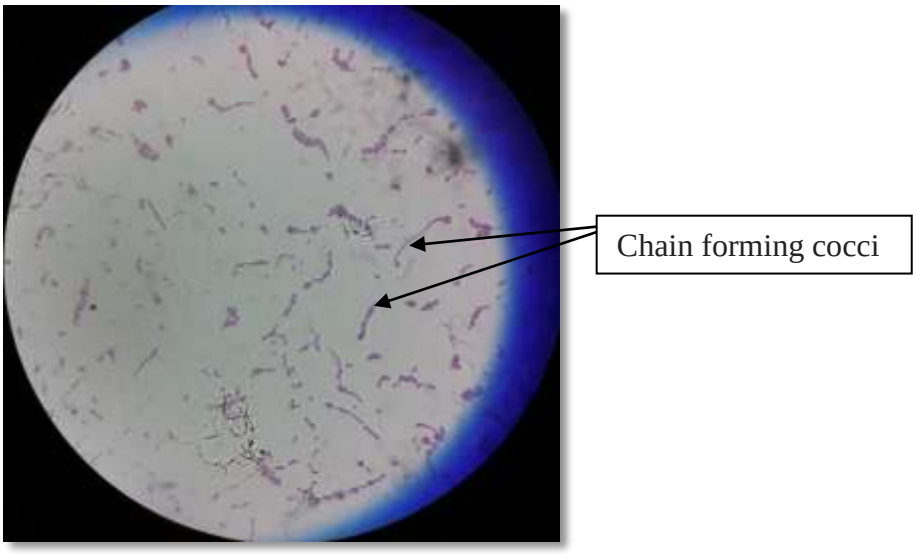

Figure 16. *Streptococcus thermophilus* under microscope

4.1.3. Catalase test

No gas bubble was formed during catalase reaction that means it was catalase negative. All the presumptive isolates were catalase negative **(Figure 17)**.

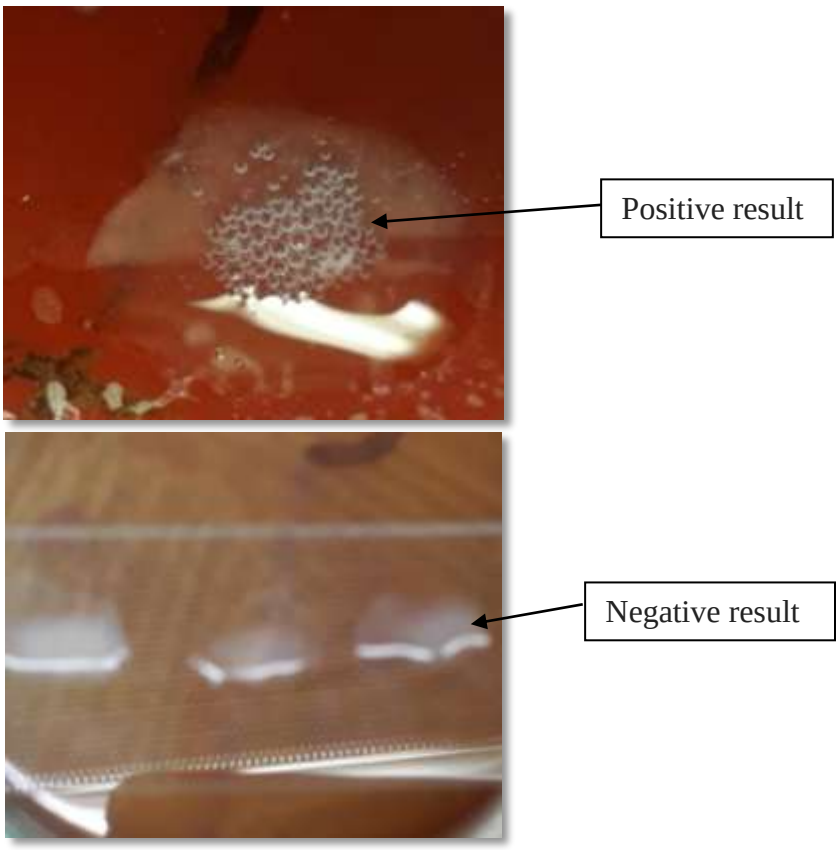

Figure 17. Catalase test

4.2 Molecular identification of *Lactobacillus* spp. and *Streptococcus thermophilus*

The presumptively identified isolates were then subjected to molecular identification by Polymerase Chain Reaction-Gel Electrophoresis method.

4.2.1 *Lactobacillus* spp.

Lactobacillus spp. was confirmed by PCR using primer set LAC1F/ LAC2R which amplified a 340bp fragment of the 16s rRNA gene **(Figure 18).**

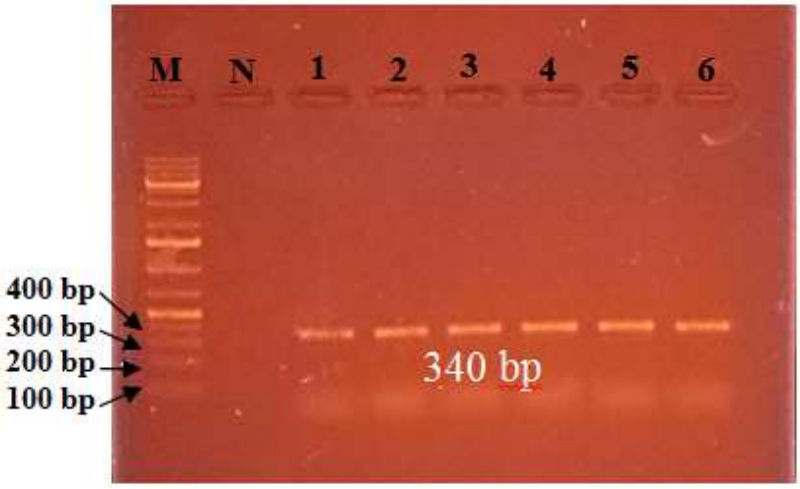

Figure 18. *Lactobacillus* spp. specific PCR assay

This figure illustrates fragments specifically amplified by PCR by means of the primer set LAC1F/ LAC2R. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 1-6: PCR products of amplified chromosomal DNA of *Lactobacillus* spp. (340bp).

4.2.2 *Streptococcus thermophilus*

Streptococcus thermophilus was confirmed by technique PCR using primer set ThI/ThII. Mentioned primers provided a PCR product with the expected size of 250bp **(Figure 19).**

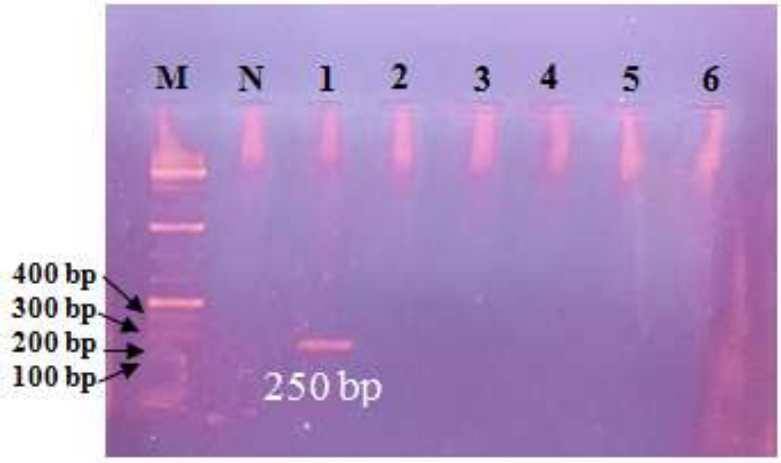

Figure 19. *Streptococcus thermophilus* specific PCR assay

This figure illustrates fragments specifically amplified by PCR by means of the primer ThI and ThII. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 1: PCR products of amplified chromosomal DNA of *S. Thermophilus* (250bp).

4.3 Development of Dahi using isolated LAB species

In this study, Dahi was developed using isolated *Lactobacillus* spp. and *Streptococcus thermophilus*. No artificial flavoring, coloring, thickening agents nor any chemical preservatives and stabilizer were added in any of the formulated dahi. The development of Dahi with these isolated cultures was successful as the preparation yielded a complete process. The developed Dahi can be a novel or potential delicious food item or popular dairy food products, as it has a better nutritive value with increased shelf life and greater acceptability, which could be introduced commercially for large scale dairy food production to meet the huge demand of the rising population across the globe.

4.4 Physicochemical analysis

The developed Dahi samples were analyzed for titrable acidity, pH, moisture, ash and protein. All the determinants were done in triplicate and the results were expressed as the average. The pH and titrable acidity were done on day 1 and 7.

4.4.1 Titrable acidity

Figure 20 shows the mean values of acidity of the different samples on day 1 and day 7. The acidity values for T_1, T_2, T_3, T_4 and T_5 on day 1 were 0.75, 0.97, 0.86, 0.74 and 0.76 respectively, whereas the values on day 7 were 1.12, 1.14, 1.06, 1.1 and 1.12 respectively. There was an increase in acidity with the progress of the storage time.

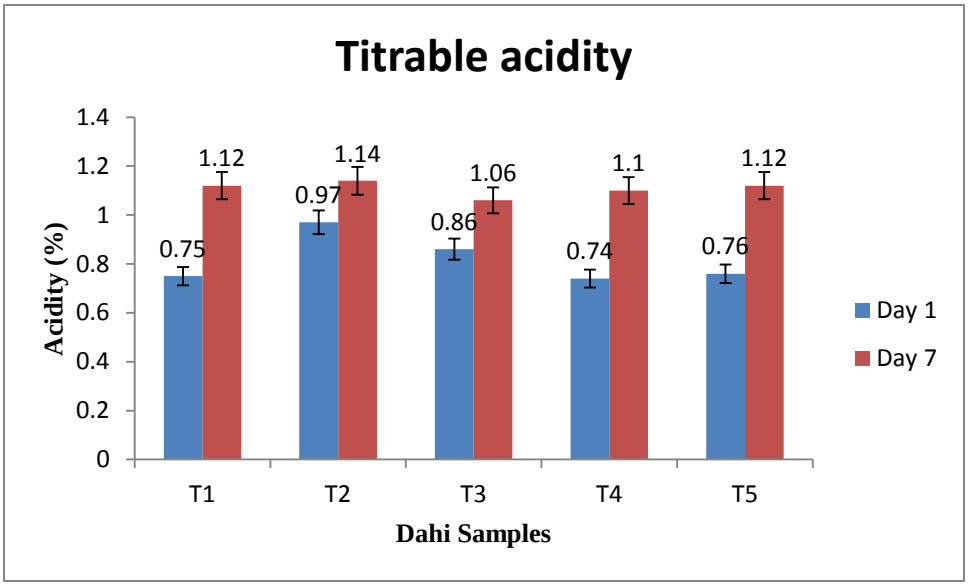

Figure 20. Titrable acidity of the Dahi samples on day 1 and day 7

(T_1, T_2, T_3 treatments refer to Dahi samples made from *Lactobacillus* spp., whereas T_4 means Dahi sample from *Streptococcus thermophilus* and T_5 denotes Dahi sample prepared by a combination of *Lactobacillus* spp. (T_3) and *Streptococcus thermophilus* (T_4) bacteria together).

4.4.2 pH

The pH values of the samples are presented in **Figure 21**. The figure indicates that mean values of pH level on day 1 were 4.52, 4.5, 4.49, 4.5 and 4.51, respectively, whereas 4.44, 4.4, 4.42, 4.38 and 4.43, respectively, were observed on day 7.

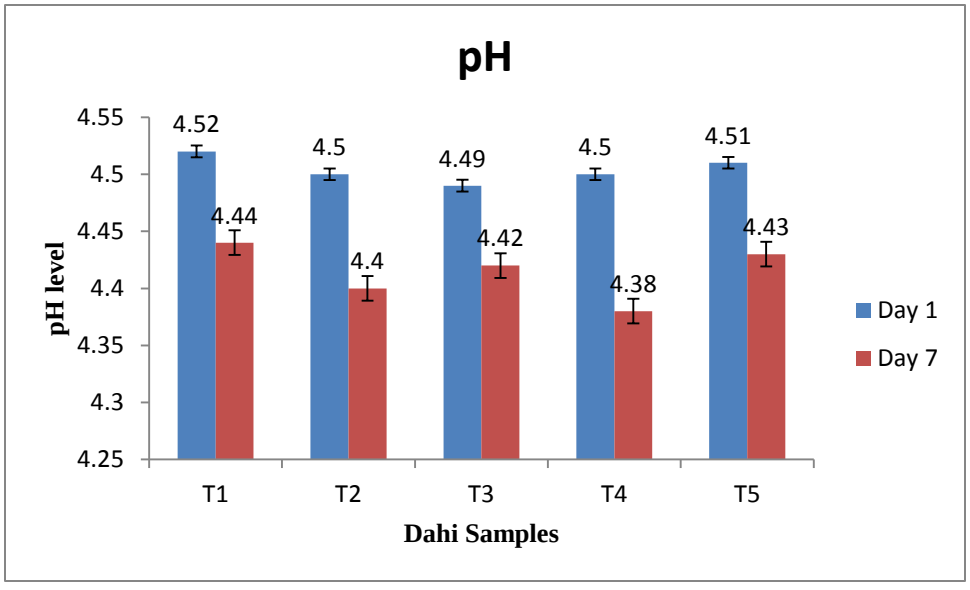

Figure 21. pH of the Dahi samples on day 1 and day 7

4.4.3 Chemical composition

The developed Dahi samples were analyzed to determine moisture, ash and protein content. The chemical composition was shown in **Table 9**. The data of moisture, protein and ash% of the samples showed significant difference between the treatments. T_5 sample had the highest moisture content 79.58% whereas T_2 being the lowest value 74.10%. The data showed that the moisture% of T_5 was significantly better than the other samples. The highest value of ash content 1.37% was found in the T_3 sample and the lowest value 1.09% was for T_4 sample. The result showed that the ash% of T_3 was significantly better than that of other samples. Protein content was higher 5.85% in T_3 whereas lower value 4.31% was found in T_4. The protein% of T_3 was significantly better than the other Dahi samples.

Table 9. Chemical composition (Moisture, Protein and Ash) of developed Dahi

Dahi samples	Moisture	Protein (Fresh basis)	Ash (Fresh basis)
T_1	77.63^{a}	5.15^{b}	1.31^{c}
T_2	74.10^{b}	5.64^{ab}	1.34^{b}
T_3	74.46^{b}	5.85^{a}	1.37^{a}
T_4	79.33^{a}	4.31^{c}	1.09^{d}
T_5	79.58^{a}	4.41^{c}	1.09^{d}
SEM	0.200	0.050	0.001
P- value	P<0.001	P<0.001	P<0.001

[Data refer to mean values consisting of three replicates; $^{a, b, c, d}$ Means bearing uncommon superscripts within a column is significantly different at the level mentioned in the above Table; SEM=Standard error of the means; T_1, T_2, T_3 treatments refer to Dahi samples made from *Lactobacillus* spp., whereas T_4 means Dahi sample from *Streptococcus thermophilus* and T_5 denotes dahi sample prepared by a combination of *Lactobacillus* spp. (T_3) and *Streptococcus thermophilus* (T_4) bacteria together].

4.5 Microbiological analysis

4.5.1 Viability of starter cultures

The values of the lactic acid bacterial counts (cfu/ml) of the Dahi samples are presented in **Figure 22**. The values of T_1, T_2, T_3, T_4 and T_5 were 1.6×10^7, 1.89×10^7, 1.9×10^7, 1.96×10^7 and 1.98×10^7 cfu/ml respectively. Numerically higher number of LAB were found in T_5 whereas T_1 being the lowest.

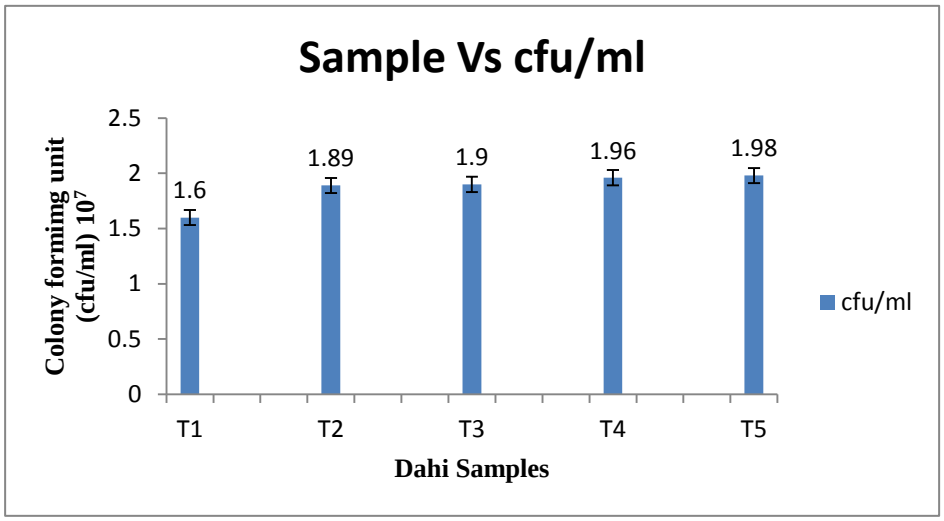

Figure 22. Viable count in the Dahi samples

4.5.2 Total Coliform Count (TCC)

The total coliform count of the dahi samples gave an indication of the total number of coliform bacteria present in the Dahi. TCC was nil in all Dahi samples.

4.6 Sensory evaluation of developed Dahi

The sensory evaluation scores of developed Dahi samples are presented in **Table 10**. The scores of aroma, taste and overall acceptability showed a significant difference between treatments. The maximum score of color and appearance was 9.00 in case of T_3 and T_4 samples. Similarly highest score of aroma (9.00) was recorded for these two samples. The highest score of taste was recorded for sample T_3 (9.00) while lowest score was (7.33) for T_2. The maximum score of body and texture was 8.66 for samples T_1, T_3 and T_4.

The maximum average overall acceptability score (9.00) was observed in T_4 followed by T_3 (8.66), T_5 (8.33) while minimum score of (8.00) was obtained by treatments T_1 and T_2. The color & appearance and body and texture scores obtained by different samples showed no significant difference between and within treatments. The highest sensory scores were recorded in the two samples (T_3, T_4).

Table 10. Sensory evaluation scores of developed Dahi

Dahi samples	Color & Appearance	Aroma	Taste	Body & texture	Overall acceptability
T_1	8.66	8.66^{ab}	8.33^{ab}	8.66	8.00^{b}
T_2	8.33	8.00^{b}	7.33^{b}	8.00	8.00^{b}
T_3	9.00	9.00^{a}	9.00^{a}	8.66	8.66^{ab}
T_4	9.00	9.00^{a}	8.33^{ab}	8.66	9.00^{a}
T_5	8.33	8.33^{ab}	8.00^{ab}	8.33	8.33^{ab}
SEM	0.115	0.094	0.115	0.133	0.094
P- value	0.233	**0.029**	**0.013**	0.452	**0.029**

[Data refer to mean values of three replicates; $^{a, b}$ Means bearing uncommon superscripts within a column is significantly different at the level mentioned made from in the Table; SEM=Standard error of the means; T_1, T_2, T_3 treatments refer to Dahi samples *Lactobacillus* spp., whereas T_4 means Dahi sample from *Streptococcus thermophilus* and T_5 denotes dahi sample prepared by a combination of *Lactobacillus* spp. (T_3) and *Streptococcus thermophilus* (T_4) bacteria together].

Figure 23. Appearance and texture of the sample T_4 (*Streptococcus thermophilus*)

Figure 24. Appearance and texture of the sample T_3 (*Lactobacillus* spp.)

Chapter-V

Discussion

5.1 Isolation of *Lactobacillus* spp. and *Streptococcus thermophilus*

In this study, the bacteria were identified presumptively by the morphological characteristics, microscopic observation and catalase reaction before molecular identification. All the isolates of *Lactobacillus* spp. grew as cream colored, circular, convex, shiny, and watery with smooth edge. After gram staining, all the isolated microorganisms have been described as rod-shaped, a convex, rough, smooth, glossy, irregular, circular, gram-positive, facultative anaerobic bacteria that indicates that they are *Lactobacillus* spp. (Fooks et al., 1999; Tharmaraj and Shah, 2003). *Streptococcus thermophilus* were chain-forming gram-positive cocci under microscope. Morphologically *S. thermophilus* is ovoid-spherical in shape with the range of diameter is approximately 0.7 to 0.9 μm and occurs in chains and in pairs (Hajira et al., 2017).

5.2 Molecular identification of *Lactobacillus* spp. and *Streptococcus thermophilus*

5.2.1 Molecular identification of *Lactobacillus* spp.

In this experiment, eighteen isolates of *Lactobacillus* spp. were identified by PCR using the primer set LAC1F/ LAC2R. Likewise, Gebreselassie et al., (2016) confirmed the *Lactobacillus* spp. by the method PCR-DGGE using the primer set LAC1F/ LAC2R. In their study, they obtained the *Lactobacillus* culture from naturally fermented butter milk. Also, Endo et al., (2009) identified the *Lactobacillus* spp. by PCR using this primer set. Walter et al., 2001 identified different *Lactobacillus* spp. by using the above mentioned primers which produced a PCR product of the intended size of 340bp.

5.2.2 Molecular identification of *Streptococcus thermophilus*

In our study, *Streptococcus thermophilus* was confirmed by PCR technique using primer set ThI/ThII. Vanatkova et al., (2009) revealed that, applied primer set ThI/ThII is suitable for the detection of *Streptococcus thermophilus*. In this study, the above mentioned primers produced a PCR product of the intended size of 250bp. The result is similar to that described by Vanatkova et al., (2009) and Brigidi et al., (2003).

They identified the PCR product size of primer set ThI/ThII specificity to target species in their study. It could be stated that the applied method are useful means for identification and detection of *Streptococcus thermophilus.*

5.3 Development of Dahi using isolated LAB species

In this study, heat treated whole milk was used to make our Dahi, which contains 5% milk powder, 4% sugar and 2% culture of PCR identified culture. The most commonly used heat treatment in the yogurt industry include 85°C for 30 minutes or 90-95ºC for 5 minutes (Tamime et al., 1999), whereas we used conventional boiling method for preparing milk to make dahi. The sensory and morphological quality of Dahi can be affected by heat treatment (Shekhar et al., 2012). However, among the other heat treatment, boiling treatment of milk resulted in least syneresis of whey in the dahi (Shekhar et al., 2012). In their study, dahi prepared from boiled milk, showed the highest value in cases of firmness, consistency and index of viscosity. High heat treatment of milk increases gel firmness and reduces syneresis in the final product (Lucey et al., 1998; Vasbinder et al., 2004).The acid production rate in mixed culture is greater than the rate of acid production using single strain (Tamime et al., 1980). In this study, artificial flavoring, coloring, thickening agents, chemical preservatives and stabilizer were not used to prepare our Dahi.

5.4 Physicochemical analysis

5.4.1 Acidity

In our study, it is observed that the acidity of Dahi varied from one to another samples. The reason behind this might be due to storage time, as the acidity of the experimental sample was tested between day 1 and day 7. Apart from this, the activity of LAB might be responsible for the variation of acidity including other factors. However, the result of titrable acidity of all our experimental samples are in agreement with the results reported by Vijayendra and Gupta, (2012). Aryana and Olson, (2017) reported that the most desirable yogurt resulted in a titrable acidity of 0.74 to 0.83%, when placing into cold storage and a acidity of 0.91 to 0.93% during cold storage. We know that bacteria produce lactic acid from lactose during fermentation, which could result in enhancing the shelf life and microbial safety, improve texture and contribute to the pleasant sensory profile of the yogurt (Leroy and De Vuyst, 2004).

5.4.2 pH

It is also clear from the data that the pH of Dahi varied from one to another samples. The reason behind this might be the different time of determining pH, as the pH of the samples was determined in different period *i.e.* day 1 and day 7, respectively. The increased pH might be due to higher alkalinity of the sample, and decreased pH indicates higher acidity. Samples become more acidic if the value of pH goes down than 7, whereas the more alkaline nature of the samples are considered when the value of pH goes above the level of 7 in any solution. The increased action of acid producing bacteria might contribute higher acidity, whereas low acidity could be due to lower acid production by the bacteria. The pH values of different yogurt samples were decreased with time.

This drop in pH is attributed to the absorption of lactose by microbial culture, which ultimately leads to the production of lactic acid, formic acid and small amounts of CO_2 (Panesar et al., 2011).

5.4.3 Chemical composition (Moisture, Protein and Ash) of developed Dahi

The chemical composition of different samples of our developed Dahi varied significantly as is observed from the data found in this study. Significant variation was observed in the values of moisture, protein and ash contents of the different samples of developed Dahi. It is obvious that the ash and protein% of T_3 sample were improved significantly compared to other samples. Low moisture content and ingredient composition might be reasons for enhanced nutrient contents of the T_3 Dahi.

5.5 Microbiological analysis

To achieve the therapeutic benefit, a minimum number of 10^6–10^7 colony forming units (CFU) of viable cells of probiotic cultures must be maintained in the products until the time of consumption (Boylston et al., 2004). The United States Food and Drug Administration (US FDA) has also suggested a minimum probiotic count in a probiotic food to be at least 10^6 CFU/ml (Bhadoria and Mahapatra, 2011). The viable count of Dahi cultures was well above the minimum recommended amount immediately after their production (zero hour).

The coliform count was nil in all Dahi samples. As reported in Indian standard 9617 (1980), the coliform count per gram is limited to 10 CFU for dahi. Bakr et al., (2015) prepared bioyogurt and reported that coliform counts were not detected in all fresh and stored treatments that might be due to the effect of milk heat treatment and the role of yogurt bacteria in coliform control by their ability to produce a variety of antibacterial compounds.

5.6 Sensory evaluation of developed Dahi

In our experimental Dahi, various sensorial characters such as color and appearance, taste, aroma, body and texture, overall acceptability etc. were evaluated by the panel expert following the method as described by Shekhar et al. (2012). It is obvious from the data that the aroma, taste and aceeptability of T_3 were improved significantly compared to others. The higher palatbility, increased nutrive value and the compatible action of using suitable organism might be responsible for enriching these characters of the Dahi (T_3). Higher fat content of the sample might increase palatability, taste or flavor nature of the Dahi, and which in turn, could enhance the consumer acceptability towards the products. The color & appearance and body & texture of the different samples were not influenced by the treatments. Dahi samples produced from *Streptococcus thermophilus* (T_4) and *Lactobacillus* spp. (T_3) were preferred by the panelists.

Chapter-VI

Conclusion

It is clear from the results that the developed Dahi with specific starter organism can be prepared successfully using culture obtained from the locally produced Dahi available in the market. The result could give us a new idea that PCR method can be used to identify particular organisms (*Lactobacillus* spp. and *Streptococcus thermophilus)*, which could be used effectively as starter culture for producing Dahi. This technology could further help us to prepare a single strain Dahi. The viable count of the Dahi denoted that the viability of starter bacteria was well enough as the standard limit ($>10^6$ CFU/ml) in all the developed Dahi samples. This way of preparing Dahi could also help us to manufacture other many fermented products say yogurt with acceptable quality, functional and sensory properties. Though the pH and acidity measured in this study varied from one to another, but the level was acceptable, which is close to normal that could help to increase the shelf life of the dairy food products. The nutrient contents particularly protein and ash of developed Dahi (T_3) were improved significantly that would enhance the nutritive value or quality of the product undoubtedly. The sensory characters such as taste, aroma and acceptability were improved in our developed Dahi (T_3) as well. From this assessment, it could be concluded that the technique could help the consumers or manufacturers to prepare Dahi following this method, which could assure them to prepare dairy food products with better quality, highly nutritious, palatable, safe, hygienic and higher acceptability and preserving quality. We can surmise from this findings that the Dahi prepared in this way would surely increase consumer attention and market demand in near future.

From all the above discussions it can be inferred that, *Lactobacillus* spp. and *Streptococcus thermophilus* could be used to make single strain dahi. *Streptococcus thermophilus* (T_4) and *Lactobacillus* spp. (T_3) showed the best performance among the other isolates with respect to nutritive value, sensory qualities and shelf life. The results suggest that the lactic acid bacteria identified in this study have the potential to contribute to Dahi production technology and product palatability.

Chapter-VII

Limitations and Recommendations

Limitations:

1. Research fund was inadequate.
2. Lab facility should be more improved.
3. Specific minerals present in the ash content were not determined for insufficient fund and facility.
4. Sample size of this investigation was not representative to the population due to short period of the study.
5. Monitoring systems or follow up system of every cases of research should be strictly maintained for fruitful outcome of the research.
6. Products preparation costs were not measured in this study.
7. No specific consuming guideline is given for diabetic and non-diabetic consumers.
8. Economy of the product preparation was not measured herein this study.

Suggestions for future research work:

From the present study, the following suggestions can be made for future work-

1. Other species of LAB which are commonly used for fermented dairy products (especially *Lactobacillus bulgaricus and Lactococcus lactis*) could be isolated.
2. Probiotic properties of the LAB species should be analyzed.
3. Storage study should be extended to evaluate the shelf life of the product for more days.
4. Both whole milk Dahi and low-fat Dahi may be developed with the specific starter culture in order to support the non-diabetic and diabetic consumer health.
5. The specific strain Dahi (Both whole milk Dahi and low-fat Dahi) is recommended for large scale production and commercialization.
6. Cost benefit analyses should be calculated for giving message to the consumer of economical dairy products production.
7. Considerable further research study could be done to elucidate the present findings for the greater well-being of the consumers.

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Appendix

Score card for sensory evaluation of Dahi

Name of the evaluator: Date:

- Please evaluate the following samples using the 9-point hedonic scale.
- Write the preferred score in the columns as per evaluation.

Sensory attributes	Sample number				
	1	2	3	4	5
Colour and appearance					
Aroma					
Taste					
Body and texture					
Overall acceptability					

Hedonic rating	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither Like or dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Suggestions and comments:

Signature of the evaluator

1. **Determination of titrable acidity**

Acidity percentage was determined as per (Aggarwala & Sharma., 1961) by titrating 9gm of the diluted Dahi samples against the standard N/10 NaOH solution until the substance reached a faint pink color corresponding to the end point of the phenolphthalein which was used 2-3 drops during titration as indicator. Then it was expressed in terms of % lactic acid.

i. 9gm of the diluted curd sample was taken in a porcelain beaker.
ii. 2-3 drops of phenolphthalein was added as an indicator.
iii. Titrated it against 0.1N NaOH.
iv. End point was indicated by appearance of a faint pink color which persisted for few seconds.

Calculation:

$$\%\text{Titrable acidity} = \frac{\text{ml of alkali used} \times (\text{N})\text{of NaOH} \times 0.09}{\text{Total volume of the sample}} \times 100$$

2. **Determination of moisture**

Moisture percentage was determined after determination of DM (dry matter).The enamel disc or crucible was dried in an oven regulated at 105°C which was cooled in a desiccator and weighted. 5gm of sample was weighted into the enamel disc and kept into the oven (105°C) for 24 hours. The enamel disc was removed from the oven with metal tong. After that it was cooled in desiccator and the final weight was taken after getting constant weight (AOAC, 2005).

$$\%DM = \frac{\text{Weight of Crucible with Dry Sample} - \text{Weight of Empty Crucible}}{\text{Weight of sample}} \times 100$$

% Moisture = 100 - % DM

3. Determination of Ash

The crucible was cleaned & dried in hot air oven. Than it was cooled in desiccator and weighted. 5 grams of sample was placed there and the sample was burned up to no smoke in heater. The crucible with sample was cooled and transferred to the muffle furnace. Then the sample was ignited at 550-600°C for 6-8 hours until white ash. The furnace was cooled at 150°C & the sample was transferred to desiccators and weighted (AOAC, 2005).

$$\%Ash = \frac{\text{Weight of Crucible and Ash } - \text{Weight of Crucible}}{\text{Weight of sample}} \times 100$$

4. Determination of protein content

0.5 gram sample was weighted and one spoonful catalyzer mixture (KOH, NaOH, and Se) was added there. 10ml Conc. H_2SO_4 was added and the digestion flask was placed in Kjeldahl Digestion Set. After that heat was increased gradually & continued up to clear residue (45 min-1hr). The Flask was then removed & cooled. 10ml 2% Boric Acid solution and 2 drops mixed indicator was taken in a conical flask. The conical flask was fitted in the collection arm of distillation set. 50ml distilled H_2O was added in the digestion tube and fitted in the distillation flask. 40ml of 40% NaOH was added there & the distillation was continued up to 100ml of distillate. The Distillate was titrated against 0.1N HCl. Titration was continued until the color changed into pink. Then the titration volume was calculated (AOAC, 2005).

$$\text{Protein}\% = \frac{\text{Titration value} \times \text{Normality of HCl } (0.1) \times 0.014 \times 6.25}{\text{Sample weight}(0.5\text{g})} \times 100$$

Chemical composition (Moisture, Protein and Ash) of developed Dahi

Dahi samples	Dry matter (%)	Moisture (%)	Ash (%) (DM basis)	Ash (%) (Fresh basis)	Protein (%) (DM basis)	Protein (%) (Fresh basis)
T_1	20.83	79.17	5.92	1.32	23.62	5.28
T_1	23.78	76.22	5.87	1.31	23.97	5.36
T_1	22.5	77.5	5.88	1.32	21.52	4.81
T_2	26.63	73.37	5.2	1.35	21	5.44
T_2	25.45	74.55	5.15	1.33	21.87	5.59
T_2	25.6	74.4	5.17	1.34	22.75	5.89
T_3	26.1	73.9	5.37	1.37	22.4	5.72
T_3	25.02	74.98	5.36	1.37	23.27	5.94
T_3	25.5	74.5	5.38	1.37	23.1	5.9
T_4	21.05	78.95	5.26	1.09	21.35	4.41
T_4	20.45	79.55	5.3	1.09	20.3	4.19
T_4	20.5	79.5	5.28	1.09	21	4.34
T_5	20.5	79.5	5.36	1.09	21.87	4.46
T_5	20.34	79.66	5.37	1.1	20.83	4.25
T_5	20.4	79.6	5.36	1.09	22.22	4.54

Brief biography of the student

The author of this paper, Umme Salma Amin was born in kulgaon, Chattogram, Bangladesh in 1993. She is the elder daughter of Nurul Amin and Nur Jahan. She passed the Secondary School Certificate Examination from Kulgaon City Corporation High School & College in 2009 with GPA 5.00 followed by Higher Secondary Certificate Examination from Chittagong Public School & College in 2011 with GPA 5.00. She completed her graduation degree on Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh in 2017 with CGPA 3.92. During her graduation, she received clinical training from Madras Veterinary College and Veterinary College & Research Institute, Namakkal, Tamilnadu, India and from Tufts Cummings School of Veterinary Medicine, USA. She has great interest to work in Dairy microbiology sector. Now, she is a candidate for the degree of MS in Dairy Science, Dept. of Dairy and Poultry Science, Faculty of Veterinary Medicine, CVASU.