DEVELOPMENT OF PROBIOTIC FRUIT JUICE USING LACTIC ACID FORMING BACTERIA ISOLATED FROM 'DAHI' WITH SUPPLEMENTATION OF PREBIOTICS

Roll No. 0118/06

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Applied Human Nutrition and Dietetics

Department of Applied Food Science and Nutrition

Faculty of Food Science and Technology

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

December 2019

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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December 2019

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December, 2019

Dedicated
To
My Parents

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List of Abbreviations

Words	Abbreviations
MRS	deMan Rogosa and Sharpe agar
rDNA	Ribosomal DNA
GRAS	Generally Recognized as Safe
FAO	Food and Agriculture Organization
WHO	World Health Organization
LAB	Lactic Acid Bacteria
rRNA	Ribosomal RNA
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
BHI	Brain Heart Infusion
µl	Micro liter
mL	Milliliter
℃	Degree Celsius
%	Percent
rpm	Revolutions per minute
bp	Band size
NFW	Nuclease Free Water
TAE	Tri-acetate-EDTA

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Abstract

Usually probiotics are consumed in dairy based product such as dahi or yogurt. Dahi is a widely consumed fermented dairy product in Bangladesh. However, given the rise in various diet types, non-dairy alternatives have been developed such as inoculating fruit juices with probiotics. This study was carried out to develop a probiotic fruit juice using lactis isolates from dahi with prebiotic fortification. This study indicated an alternate effective way for those who are allergic or intolerant to dairy based products. Dahi samples were collected from different local shops of Chattogram and Bogura, Bangladesh. Lactic acid forming bacteria were isolated from the samples by using MRS (deMan, Rogosa and Sharpe) media. The presumptive isolates were identified on the basis of their morphological and biochemical characteristics. Gram's staining and Catalase test were done in this regard. Furthermore, these isolates were confirmed by Polymerase Chain Reaction (PCR) using specific primers sets designed for conserved 16S rDNA regions of *Lactobacillus* genus and *Lactococcus lactis sp. Lactis* isolates were confirmed and further used for the development of probiotic fruit juice. Prebiotic (asparagus & ginger powdered form) was added in fruit juice to support the growth and development of inoculates. Streaking plate technique was performed to check the growth of inoculated isolates. Growth of the bacterial colony on petri agar medium showed that asparagus exerted a positive effect on the survival of lactic acid bacteria in orange fruit juice.

Keywords: Lactic Acid Bacteria, Polymerase Chain Reaction (PCR), Probiotic Orange juice, Prebiotic.

Chapter 1: Introduction

Fermented dairy products supply an adequate amount of nutritious foods in an abundance of flavor, aroma and texture which are very significant to amplify the human diet **(**Sarkar and Misra, 2002). For the time being, consumers are increasingly demanding foods with special attributes, such as pleasant taste, low-calorie value or low fat content, and sound health effects. Functional dairy products are prominent as natural healthy products that contain crucial nourishing components of the balanced diet, along with health convenience that are compacted by the process of adding probiotics. Dahi or yogurt is a dairy product prepared by fermentation of milk with *Lactobacillus spp*. At present, helpful, prophylactic and nourishing properties of yogurt are altogether acknowledged (Boor, 2001). When administered in a sufficient amount, probiotics give health benefit to the host and improve microbial equalization (Fuller, 1989; Guarner et al., 2005). In this way, probiotics are live, non-pathogenic, beneficial microorganisms which play an effective role in the micro flora compartment of the host (Schrezenmeir and deVrese, 2001). Lactic acid bacteria (LAB) are the major group of probiotic microorganisms mainly *Lactobacillus* spp, *Bifidobacterium* spp, and *Enterococcus* spp. (Klein et al., 1998). Dahi or yogurt is a semisolid, custard-like food item which is consumed by people throughout Bangladesh for its nutrient value and eating it orderly as a part of the daily diet may enhance various health aspects such as minimizes the risk of heart disease, osteoporosis as well as aid in weight management (Sharma et al., 2006).

Lactic acid forming bacteria are assembly of Gram-positive, non-spore forming, cocci or rod shaped, catalase-negative and meticulous organisms often isolated from milk and dairy products. They are nonpathogenic to human and animals hence 'Generally Recognized as Safe' (GRAS) organisms (Mahantesh et al., 2010).The preparation of fermented foods by using lactic acid forming bacteria have acquired key consideration for their extensive usage (Farnwarth, 2005) which are specified by hygienic safety, better organoleptic properties and possibly the probiotic attributes **(**Savadogo et al., 2006). LABs have the capability to perform various antimicrobial properties such as organic acids, free fatty acids, diacetyl, H_2O_2 and bacteriocin, which have the receptivity to oppose the development of various food-borne spoilage and unwholesome organisms in a rapid manner (Jack et al., 1995).

The term ‘probiotic’ was used for the first time by Lilly and Stillwell (1965) to describe substances which develop the growth of other microorganisms. Probiotics are defined as 'live microorganisms which when managed in sufficient sums present a wellbeing benefit on the host'(FAO/WHO, 2002). LABs have been acknowledged as "probiotics" due to their innate capability to proclaim antagonistic activity against non-pathogenic and spoilage organisms (Gilliland et al., 1975). Moreover, in many cases, their effects are predominantly prophylactic in nature, rather than therapeutic, i.e. preventive rather than curative (Suskovic et al., 2001). *Lactobacillus* and *Bifidobacterium spp*. are leading members of the intestinal flora and are usually studied probiotics bacteria. They cause reduced lactose intolerance lessening of some diarrheas, inclined blood cholesterol, improved immune response and prohibition of cancer. The selection criteria for probiotic LAB include: safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelium ability to colonize the gastro intestinal tract, creation of antimicrobial substances, capacity to animate a host insusceptible reaction and the effectiveness to affect metabolic capacities, for example, nutrient generation, cholesterol absorption and lactose action (Savodago et al., 2006).

The word prebiotics was originated by Gibson and Roberfroid (1995). Prebiotics are non-digestible nourishment segments that fuel the development of bifidogenic and lactic acid bacteria in the gastro-intestinal tract. Generally, the prebiotics made of dietary fibers and oligosaccharides. Lactobacilli are members of the lactic acid bacteria whose primary fermentation end product is lactic acid. They are commercially significant bacteria with a wide diversity of application both in food and nonfood industries. Lactobacilli have been broadly studied for their molecular biology in order to improve their specific beneficial characteristics (Pouwel and Leer, 1993). They are acquired in a natural way or included intentionally, to spread a medical advantage for the customer and dahi or yogurt is one of the most popular indigenous fermented dairy items that contain probiotics (Oskar et al., 2004). From the wellbeing perspective, ingestion of live cells of specific species and strains the probiotic idea of lactobacilli in satisfactory sums should assign different beneficial physiological impacts on the host (Tannock, 2004), such as keeping a healthy and equilibrated intestinal micro biota and alleviating occurrence of intestinal infection (Gardiner et al., 2002).

The health benefits of certain foods had been explored for quite a while. Thus, development of foods that advance wellbeing and prosperity is one of the key research priorities of food industry (Klaenhammer and Kullen, 1999). Fruits and vegetables have been considered as perfect media for probiotic development since they contain essential supplements, they are elegant and have great taste (Luckow and Delahunty, 2004; Sheehan et al., 2007).There is an unsophisticated interest in the development of fruit juice with probiotics by including prebiotics in light of the fact that they have taste profiles that are engaging all age gatherings and because they are perceived as healthy and refreshing foods (Tuorila and Cardello, 2002; Yoon et al., 2004; Sheehan et al., 2007). In addition to take measures for advancing consumer health and well-being, functional foods such as probiotics in dairy products are a fascinating market sector, providing new economic opportunities. Method used for the detection of *Lactobacillus spp*. by the assurance of morphological and biochemical characteristics. Genomic DNA of bacterial isolates was extracted by using PCR (Polymerase Chain Reaction) performed with specific primers. This technique is utilized to distinguish microorganisms more precisely (Holzapfel et al., 1998; Charteris et al., 1997).

1.1 Objectives

- To develop a probiotic fruit juice using lactis isolates from dahi samples of Chattogram and Bogura city in Bangladesh.
- To conduct a molecular technique in order to verify phenotypic identification of *Lactobacillus & L. lactis sp. Lactis* which are isolated from dahi samples.
- To check the growth of isolated bacteria in fruit juice when fortified with prebiotics.

Chapter 2: Review of Literature

2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are widely used recently in food technology, microbiology and biotechnology and hygiene with respect to the production of fermented food. Lactic acid bacteria are used as industrial microorganisms in beverage, meat product, and sugar industry, souring of pickles, olives and dairy products. LAB (*Lactobacillus, Leoconostoc, Streptococcus* and *Pediococci*) are also responsible for fermentation of milk products. They alter flavor, texture, and appearance of foods, enhance nutritional values, retard spoilage, reduce contamination and are widely used in dairy processes because in addition to lactic acid production they also produce volatile compound such as acetaldehyde, diacetyl and alcohol. The large population of LAB in fermented milk products like yogurt competes strongly with microbial contaminants for available nutrient and thus enhances product safety. Some Lactic acid bacteria produce antimicrobial peptides, known as bacteriocin which may target certain pathogens (Havenaar and Huis int Veld, 1992).Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation products of the metabolism of carbohydrates described by Hayek and Ibrahim (2013).The taxonomic classification includes-

Phylum: *Firmicutes*

Class: *Bacilli*

Order: *Lactobacillales*

Family: *Lactobacllaceae*

Genus: *Lactobacillus*

2.2 Advantageous effects of lactic acid bacteria

There is a few potential wellbeing or wholesome advantages conceivable from certain types of lactic acid microorganisms. Among these are: improved dietary benefits of nourishment, control of intestinal diseases, improved absorption of lactose, control of certain sorts of malignant growth, and control of serum cholesterol levels. Some potential advantages may result from development and activity of the microbes during

the production of culture foods. Some may result from development and activity of specific types of the lactic acid microscopic organisms in the intestinal tract following ingestion of foods containing them (Gilliland, 1990).

2.2.1 Impacts on gastrointestinal tract

Microbial equalization is extremely fundamental for keeping up the intestinal homeostasis. Live lactic acid bacteria intake through dairy products have beneficial effects on gastrointestinal tract of human beings ranges from adjustment of lactose malabsorption, reduction of viral and drug induced diarrhea, post-operative pouchitis, irritable bowel syndrome, inflammatory bowel syndrome, antineoplastic effects on human cell line, maintenance of normal insulin level in blood and also helpful to enhance the absorption of fatty acids through intestine. produce these advantageous impacts by reclamation of normal intestinal flora, evacuation of intestinal pathogens, support of intestinal barrier capacity to foreign antigens, stimulation of nonspecific immunity such as phagocytosis, incitement of humeral insusceptibility and generation of anti-inflammatory products (Harish and Varghese, 2006; Heyman, 2000).

2.2.2 Consequences for lactose intolerance and malabsorption

Lactose intolerance is that the inability to digest lactose into its constituent's, i.e., glucose and galactose owing to low level of lactase enzyme within the brush border of duodenums. It generally happens in children. Side effects of lactase deficiency appear from half-hour to 2 hours after consumption of food that contain lactose in it. Symptoms include- bloating, cramping, flatulence, and loose stool. There are three clinical shorts of lactose intolerances, i.e., primary lactase deficiency occurs after weaning, secondary lactase deficiency due to diarrhea, inflammatory bowel disease and HIV infection and third type is congenital lactase deficiency which has genetic origin. Lactose malabsorption is that the condition wherein lactose is processed in to its constituents but since of lack of anatomical and cofactors these constituents are not appropriately consumed by the gastrointestinal system (Rusynyk and Still, 2001). It's been discovered that individuals with lactase insufficiency endure the lactose in yogurt superior to an identical measure of lactose in milk this likely due to the suspicion that either yogurt supply lactase protein or microscopic organisms which produce lactase catalyst (Fuller, 1991).From these discoveries it's derived that lactase deficiency issues are often decreased by routinely consuming the fermented dairy items due to the assembly of lactase compound by LAB present in them.

2.2.3 Impacts on diarrheal diseases

Diarrhea is that the frequent problem of both developed and developing countries however occurrences are progressively normal in developing countries because of their poor lifestyle and poor hygiene circumstances. Individuals at high danger of looseness of the bowels are little kids, older folks, people with intestinal contaminations and HIV transporters (Farthing, 2000). There are a couple of reasons for looseness of the bowels however among them is fecal contaminated food and water (WHO, 2000). There are numerous shorts of diarrhea like Rota virus induced diarrhea, antibiotic induced diarrhea, bacterial diarrhea travelers' diarrhea and fungal diarrhea .Lactobacillus GG strain has been to be viable against viral and idiopathic looseness of the bowels (Harish and Vargese, 2006). Because of these helpful impacts of lactic acid bacteria in Diarrheal ailment particularly in youngsters, utilization of LAB containing food, for example, yogurt and fermented milk ought to be advanced in children.

2.2.4 Effects of lactic acid bacteria on human immune system

Immunity is characterized because of the conflict of body against foreign invaders or any body abnormalities. Immunity is essentially of two types inborn and obtained resistances. Intrinsic insusceptibility incorporates mechanical boundaries, germ-free activities of body liquids, fiery reaction while obtained resistance comprises of lymphocytes, explicit shorts of proteins and antibodies to shield the body. These pathways are helpful to stay up the body functional. Antibodies are the significant segment of safe framework. Lactic acid bacteria are thought to possess a couple of probably valuable consequences for the safe capacity. There is proof to propose that they'll improve insusceptible capacity by expanding the number of IgA-creating plasma cells, expanding or improving phagocytosis, even as expanding the extent of T cells and natural killer cells (Ouwehand et al., 2002; Reid et al., 2003).

2.2.5 Utilization of lactic acid bacteria in food

LABs are considered because the most vital microorganisms with reference to as food industry care. Uses of live cultures can prompt better nourishment: improvement of insusceptibility and gut wellbeing with probiotics (Rodgers, 2008). LABs containing foods and supplements have also been found to balance inflammatory and hypersensitivity responses by due regulation of cytokine function. Clinical examinations recommend that they will forestall reoccurrences of inflammatory

bowel disease in grown-ups(Reid et al., 2003), even as improve milk hypersensitivities (Kirjavainen et al., 2003) and decline the danger of atopic skin inflammation in children (Kalliomaki et al., 2003).Realizing that people who have basic hypertension are at a high danger of making diabetes, another technique which will be utilized within the avoidance or deferral of diabetes and therefore the consequent decrease within the occurrence of hypertension might be the use of probiotics or probiotics based fermented foods (Aggarwal et al., 2013).

2.2.6 Role of lactic acid bacteria in preventing carcinoma

Colorectal disease is one among the foremost widely known sorts of threat in developed nations (Kolida and Gibson, 2011). The explanation for the use of probiotics to hinder malignant growth improvement within the colon would be the capacity of certain lactobacilli and bifido bacteria to bring down the degree of fecal chemicals embroiled in carcinogenesis, and their capacity to debase nitroso compounds (Mombelli and Gismondo, 2000). Change in micro flora arrangement is said with an expansion in fecal protein movement, β-glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase (Fuller, 1989). These proteins convert procarcinogens into cancer-causing agents and should therefore increase an expanded danger of colorectal malignant growth. Rather than past discoveries, investigations of Blanc et al. reflect very surprising outcomes. H_2O_2 engaged with the expanded expansion and spread of malignancy in colon, if this H_2O_2 level is diminished by any mean, it's conceivable to regulate or limit the movement and spread of disease inside the colon region. These H_2O_2 levels are often decreased by hostile to oxidant movement within the specific territory which thusly can be expanded by catalase action of the microscopic organisms. In the event that catalase creating microbes will colonize increasingly more within the colon territory it's expand cell reinforcement action which eventually decreases the danger of colon malignant growth. As *Lactococcus lactis* is that the strain with this novel movement thus it tends to be utilized as potential controlling operator of colon malignant growth (Blanc et al., 2008).*Lactococcus lactis* has anticolonic malignancy action on account of its capacity to expand the degree of anti-proliferative protein and diminishing the impacts of mutagenic protein more these organisms are often given orally (Kim et al., 2003).

2.3 History and definition of probiotics

The word ‘probiotic’ originated from Greek language ‘pro bios’ which signifies ‘for life’ against ‘antibiotics’ which suggests ‘against life’. The historical backdrop of probiotics started with the history of man by consuming fermented foods that's documented Greek and Romans consume considerably (Gismondo et al., 1999; Guarner et al., 2005).In 1908 a Russian scientist Ellie Metchnikoff, who features a novel prize, right off the bat proposed the helpful impacts of probiotic microorganisms on human wellbeing. Bulgarians are sound and seemingly a perpetual individual on account of the use of fermented milk items which comprises of rod shaped microscopic organisms (*Lactobacillus spp*.) was theorized by Metchnikoff (1908).During this manner, these microscopic organisms influence the gut micro flora decidedly and decline the microbial toxic activity (Gismondo et al., 1999;Chuayana et al., 2003).

The term probiotic, from Greek "for life," was utilized simply by Lily and Stillwell (1965) to characterize substances created by microorganisms which might develop the even period of various microorganisms. Afterward, (Parker, 1974) changed this definition to "living beings and substances that increase intestinal equalization." (Salminen et al., 1999) expressed that there are recorded wellbeing impacts of nonviable probiotics and even the cell divider segments on some probiotic microorganisms. This prompted the inspiration of the accompanying definition: "Probiotics are microbial cell arrangements or segments of microbial cells that beneficially affect the wellbeing and prosperity of the host." This definition introduces a second novelty with reference to the one proposed by Fuller (1991) and it’s the beneficial effect of probiotics on human health generally instead of specifically on intestinal health. The foremost acknowledged definition lately is that given by the earth Health Organization as “live microorganisms that, when administered in an adequate amount confers a health benefit to the host” (FAO/WHO, 2002).

2.4 Probiotic microorganisms

The microorganisms utilized as probiotics have an area with wide genera despite the very fact that the principal strains utilized have an area with the heterogeneous gathering of lactic acid bacteria (LAB), which contains *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* from which lactobacilli are most typically utilized during the formulation of probiotic items. LABs have a long and safe history of use within the detailing of fermented foods and drinks (Leroy and DeVuyst, 2004).

Table: 2.1: Commonly used probiotic microorganisms

Lactobacillus spp.	***Bifidobacterium spp.***	**Others**
L. acidophilus	*B. adolescentis*	*Lactococcus lactis sp. Cremoriss*
L. casei	*B. animalis*	*Lactococcus lactis sp. Lactis*
L. delbrueckii.sp. Bulgaricus	*B. breve*	*Pediococcus acidilactici*
L.lactis	*B. bifidum*	*VSL#3 (four strains of*
L. brevis	*B. infantis*	*Lactobacilli, three strains of*
L. fermentum	*B.lactis*	*bifido bacteria, one strain*
L. crispatus	*B. longum*	*Streptococcus salivarius sp.*
L. helveticus		*thermophilus),Saccharomyces*
L. gasseri		*boulardii(yeast), Saccharomyces*
		cerevisiae (yeast)
		Bacillus cereus
		E. coli

Source: (Heyman and Ménard, 2002; Ouwehand et al., 2002)

2.5 Concept of prebiotics

The prebiotics idea was presented for the first time by (Gibson and Roberfroid, 1995).Prebiotic may be a non-digestible substance of food origin which, when administered in adequate amounts, is useful to the buyer thanks to the selective promotion of growth and/or activity of one or more bacteria already present within the alimentary canal or taken alongside the prebiotic. (Hill et al., 2014).They're considered as functional foods. The foremost predominant shorts of prebiotics are healthfully classed as soluble fiber. Somewhat numerous shorts of dietary fiber show a point of prebiotic impact. Roberfroid offered a refined definition within the 2007

Journal of Nutrition expressing "A prebiotic may be a specifically fermented ingredient that permits explicit changes, both within the synthesis or potentially action within the gastro intestinal micro flora that presents benefits upon host well living and wellbeing "expounded the prebiotics idea by specific criteria viz. protection from gastric sharpness, hydrolysis by mammalian catalysts and gastrointestinal ingestion; aging by intestinal micro flora and particular incitement of the development, or potentially movement of intestinal microscopic organisms related with wellbeing and prosperity. Prebiotics are food constituents that animate specifically the event and action of explicit shorts of microscopic organisms within the gut, typically bifido bacteria and lactobacilli, with advantages to wellbeing (Gibson et al., 2004). By and by, they're short-chain carbohydrates (SCCs) that are non-digestible by human chemicals and that which are called safe SCCs. The expression "prebiotics" has increased tons of consideration within the ongoing years as clear within the logical writing and therefore the development of useful nourishments promoted with medical advantages related with its prebiotic properties. Balance of the micro flora synthesis by fermented foods with the target to improve the colonic condition may be a test. Prebiotics are useful within the capacity and by doing in order that help within the avoidance or the treatment of a wide range of maladies (Quigley et al., 1999).

2.6 Sources of prebiotics

Some prebiotics occur normally in several food items. Prebiotic carbohydrates are found normally in certain foods grown from the ground as crude Jerusalem artichoke, crude dandelion greens, crude garlic, crude leek, crude onion, crude ginger, tomatoes, asparagus, bananas, and berries. It's likewise found in numerous grains incorporate wheat, oats, grain, flour, whole grain foods such breads and oats and vegetables (lentils, kidney beans, chickpeas, white beans and dark beans) (Jackson, 2010; Moongngarm et al., 2011; Sharma et al., 2011).

2.7 Molecular identification

2.7.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) may be a scientific technique in biology to amplify one or a couple of copies of a portion of DNA across several orders of magnitude, generating thousands to many copies of a specific DNA sequence. Polymerase Chain Reaction was advanced in 1984 by the American biochemist, Kary Mullis. Mullis received the Nobel Prize and therefore the Japan Prize for developing PCR in 1993 (Bartlett and Stirling, 2003).

2.7.2 Basic principle of PCR

The basic PCR principle is straightforward. Because of the name implies, it's a sequence reaction: One DNA molecule is employed to supply two copies, then four, then eight and then forth. This continuous multiplying is performed by explicit proteins referenced as polymerases, enzymes that can to string together individual DNA building squares to shape long atomic strands. To try to their job polymerases require a supply of DNA building blocks, i.e. the nucleotides made up of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a little fragment of DNA, referred to as the primer, to which they attach the building blocks also as a extended DNA molecule to function a template for constructing the new strand. If these three elements are supplied, the enzymes will construct exact copies of the templates. PCR may be a method accustomed to acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a specific segment of DNA (Gibbs, 1990).

2.7.3 Steps of PCR

The key components of a PCR response are *Taq* polymerase, primers, template DNA, and nucleotides (DNA building blocks).The segments are collected during a tube, nearby cofactors required by the protein, and are gotten through rehashed patterns of warming and cooling that permit DNA to be combined.

The basic steps are-

i. **Denaturation** (94-98℃): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

ii. **Annealing** (55-65℃): Cool the reaction therefore the primers can bind to their complementary sequences on the single-stranded template DNA.

iii. **Elongation** (72℃): Raise the reaction temperatures so *Taq* polymerase extends the primers, synthesizing new strands of DNA.

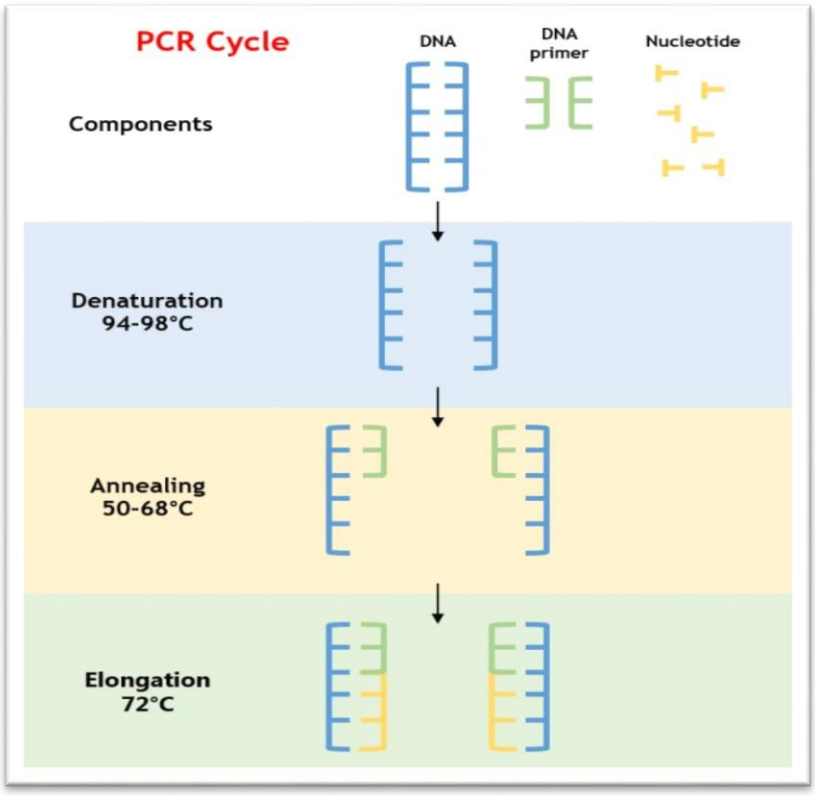

Figure: 2.1: Polymerase chain reaction (PCR) cycle phase

This cycle repeats 25-35 times during a regular PCR response, which for the foremost part takes 2-4 hours, contingent upon the length of the DNA locale being replicated. On the off chance that the response is proficient (functions admirably), the target area can go from only one or a couple of duplicates to billions. That's on the grounds that it's not simply the primary DNA that's utilized as a format whenever. Rather, the new DNA that's made in one round can fill in as a layout within the following round of DNA amalgamation. There are numerous duplicates of the preliminaries and numerous atoms of Taq polymerase drifting around in the response, therefore the quantity of DNA particles can generally twofold in each round of cycling (Gibbs, 1990; Arnheim and Erlich, 1992).

Chapter 3: Materials and Methods

3.1 Work place

The experiment was conducted in the laboratory of the Department of Microbiology and Veterinary Public Health, Applied Food Science and Nutrition, Applied Chemistry and Quality Assurance and Poultry Research and Training Centre (PRTC) of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram.

3.2 Study period

The study was carried out during the period of July to December, 2019.

3.3 Sample collection

Dahi samples were collected from various nearby superstores of Chattogram khulshi area and 'Mohoram Ali Dahi Ghor', Bogura in Bangladesh because of their wide acceptance among the consumers. Immediately after collection, the samples were stored in refrigeration temperature to protect from contamination and deterioration.

3.4 Media

The *Lactobacillus spp*. was isolated from dahi samples by using modified MRS (deMan, Rogosa and Sharpe) agar media.

3.4.1 Preparation of MRS media

MRS agar is employed for confinement, enumeration and cultivation of *Lactobacillus* species. It is confirmed by the authors to support affluent growth of all lactobacilli. All the ingredients required to organize MRS media were suspended in distilled water and heated to dissolve the media completely. The media was then sterilized in autoclave at 121℃ for 20 minutes and cooled at 50℃ (deMan et al., 1960).

3.5 Isolation and identification of bacteria

The lactic acid bacteria were isolated from dahi samples by using selective MRS agar media. At first, one loop of each fresh sample was streaking into the prepared MRS agar medium and then incubated at 37℃ for 2 days. After incubation, the presumptive isolates were identified on the basis of their cultural, morphological and biochemical characteristics (Gram staining, catalase reaction etc.).

3.5.1 Gram staining

The test was performed for all isolated strains according to the standard procedure. A smear of single colony was prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat fixed smear was flooded with crystal violet dye and after two minute, it was washed with water and flooded with mordant Gram's iodine solution (iodine & potassium iodide) for two minutes and washed with water. The smear was decolorized with 95% ethyl alcohol or acetone for 10 seconds and rinsed with water. Finally safranin was used as counter stain for 1 minute and washed with water, and examined under oil immersion (100X). The gram reaction of the isolates was observed by light microscopy after gram staining. LAB is known to be gram positive. It indicated that they give blue-purple color by gram staining.

3.5.2 Catalase test

Catalase is an enzyme that is produced by many microorganisms which breaks down the H_2O_2into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.

$$2H_2O_2 \rightarrow 2\,H_2O + O_2$$

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, a drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble indicated catalase-positive and no bubble indicated catalase negative. Lactic acid bacteria are known as catalase negative.

3.6 Long term preservation of isolates

Gram positive and catalase negative isolates were preserved into brain heart infusion (BHI) 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.

3.7 Molecular identification of bacteria by polymerase chain reaction (PCR)

3.7.1 DNA extraction from the isolates

Conventional boiling method was used for extraction of DNA from the obtained isolates. For this purpose, double boiling method was applied. Firstly, 200 µl deionized water was taken into a 2ml 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 99°C for15 minutes. Again, this procedure was repeated. Immediately after boiling, the suspensions were placed at -20°C for 5 minutes for cooling. Finally, the Eppendorf tubes along with the cell suspension were centrifuged at 10000 rpm for 5 minutes. About 100µl of supernatant containing bacterial DNA was collected in another sterile Eppendorf tube and preserved at -20°C until further testing.

3.7.2 Amplification of PCR products

The final identification of lactobacilli was carried out by PCR (Polymerase Chain Reaction) through the amplification of PCR product. The primer details used for detection and confirmation of LAB is given in table 3.1

Table: 3.1: Primers used for the detection and confirmation of LAB

Target organism	Primer	Primer sequence (5'-3')	Fragment size (bp)	Reference
Lactobacillus	LAC1F LAC2R	AGCAGTAGGGAATCTTCCA ATTTCACCGCTACACATG	340	Endo et al., 2009
L. lactis sp. Lactis	LacF LacreR	GTACTTGTACCGACTGGAT GGGATCATCTTTGAGTGAT	161	Pu et al., 2002

Table 3.2: Contents of each reaction mixture of PCR used to detect *Lactobacillus*

Content	Amount (µl)
Master mix	11.75
Forward primer	5
Reverse primer	5
Template DNA	2.5
Nuclease Free Water (NFW)	0.75
Total	**25**

Table 3.3: Contents of each reaction mixture of PCR used to detect *Lactococcus lactis sp. Lactis*

Content	Amount (µl)
Master mix	12.5
Forward primer	2
Reverse primer	2
Template DNA	4
Nuclease Free Water (NFW)	4.5
Total	**25**

Amplification was performed on a thermo cycler. All reactions were carried out in a final volume of 25 µl. The cycling conditions are shown in table 3.4 a total of 29 cycles and table 3.5 a total 35 cycles were run.

Table 3.4: Cycling conditions used for PCR to detect *Lactobacillus*

Serial No.	Step	Temp. and Time
1	Initial denaturation	94℃ for 3 minutes
2	Final denaturation	94℃ for 30 seconds
3	Annealing	55℃ for 30 seconds
4	Extension	72℃ for 3 minutes
5	Final elongation	72℃ for 10 minutes
6	Final holding	4℃

Source: (Negussie et al., 2016)

Table 3.5: Cycling conditions used for PCR to detect *Lactococcus lactis sp. Lactis*

Serial No.	Step	Temp. and Time
1	Initial Denaturation	94℃ for 5 minutes
2	Final Denaturation	94℃ for 40 seconds
3	Annealing	58℃ for 40 seconds
4	Extension	72℃ for 1 minute
5	Final elongation	72℃ for 10 minutes
6	Final holding	4℃

Source: (Pu et al., 2002)

3.7.3 Separation of amplified PCR products

3.7.3.1 Preparation of agarose gel

PCR products were separated by agarose gel electrophoresis. 0.75 g agarose was dissolved in 50 ml TAE buffer. Then the solution was boiled for 2 minutes. At that point it was cooled about to 50 °C and 5 µl ethidium bromide solutions were included. The prepared agarose gel was filled into the gel casting stand and the combs were placed. To solidify the gel, it was kept in room temperature for 20 minutes. At that point the gel was moved into an electrophoresis chamber loaded up with 1X TAE buffer. After having a rigid gel combs were taken to have wells for stacking.

3.7.3.2 Loading of agarose gel

5.5 µl of PCR products and 3 µl of loading dye for a gene were stacked into a gel-gap and 1 kb plus DNA marker was used for looking at the amplicon size of a gene product.

3.7.3.3 Electrophoresis of the PCR products

PCR products were electrophoresed at 90 volt, 120 Amp for 35 min. Amplification products were visualized by UV light.

3.8 Development of probiotic fruit juice

3.8.1 Materials required

Raw materials

- Orange
- Sugar
- Culture (isolated bacteria)
- Starch

Prebiotics

- Asparagus (powder form)
- Ginger (powder form)

3.8.2 Methodology

3.8.2.1 Preparation of fruit juice

The fruit (orange) was bought from the nearby market of Chattogram region and brought to the laboratory for collecting juice. Firstly, Orange (*Citrus sinensis L. Osbeck*) fruit was washed by flushing water and the skin was separated. Secondly, the white layer of the fruit was evacuated. Then the orange segments were squashed with the assistance of utilizing clean hand. After crushing the juice was filtered by using a juice strainer over a bowl or container. After that, strained the juice with a help of a spoon and continued squeezing and moving the spoon, so that the all the juice was strained well. At that point an adequate amount of sugar was added. They were kept in sterilized conical flask and stored at refrigeration temperature (5±1°C).

3.8.2.2 Probiotic culture and skim milk

Isolated culture was used as probiotic culture. Skim milk was gathered from the super shops, Khulshi, Chattogram.

3.8.2.3 Preparation of probiotic milk drinks

Milk was warmed until its weight lessens to around 20-25%. During heating, milk was mixed altogether with the assistance of a stirrer. After wanted warming milk container was taken out from the heater and permitted to cool. At that point when the temperature was about 40℃, after that milk was divided into two equivalent parts. Then one portion of milk was inoculated with common *Lactobacillus* and the other portion was inoculated with *Lactococcus lactis sp. Lactis* which was isolated from dahi samples.

3.8.2.4 Preparation of prebiotics paste

In this study, asparagus and ginger were used as prebiotic in power form. Starch was additionally used to set up the paste. At that point all ingredients were weighted and mixed approximately and were taken in a cup. After that, a sufficient measure of water was added into the cup to make a paste.

Table 3.6: Prepared paste with asparagus and ginger power per 200mL juice

Ingredients	Amount (gm)
Asparagus	0.5
Ginger	0.1
Starch	0.05
Total	**0.65**

3.8.2.5 Preparation of probiotic orange juice with prebiotics

Orange juice was prepared manually to include prebiotic which boost the development of probiotic bacteria. After addition of prebiotic paste, juice was pasteurized at 90℃ for 2 minutes. . At that point purified juice was permitted to cool. After that, when temperature was about 40℃, the fruit juice was incorporated with the probiotic milk. At that point, 200 mL of orange juice was divided into 7 bottles which are sterilized previously. One bottle contains only juice which is utilized as control. Another four bottles contain juice with isolated probiotic bacteria. What's more, the last two bottles contain juice, prebiotics and isolated probiotic bacteria. All prepared probiotic juice were then fermented for 24 hours at room temperature.

Table 3.7: Standardized parameter for the preparation of probiotic orange juice

Parameter	Standardization
Orange juice	200 mL
Prebiotics paste	0.65gm
Lactobacillus	5%
Lactococcus lactis sp. Lactis	5%
Fermentation time	24 hours

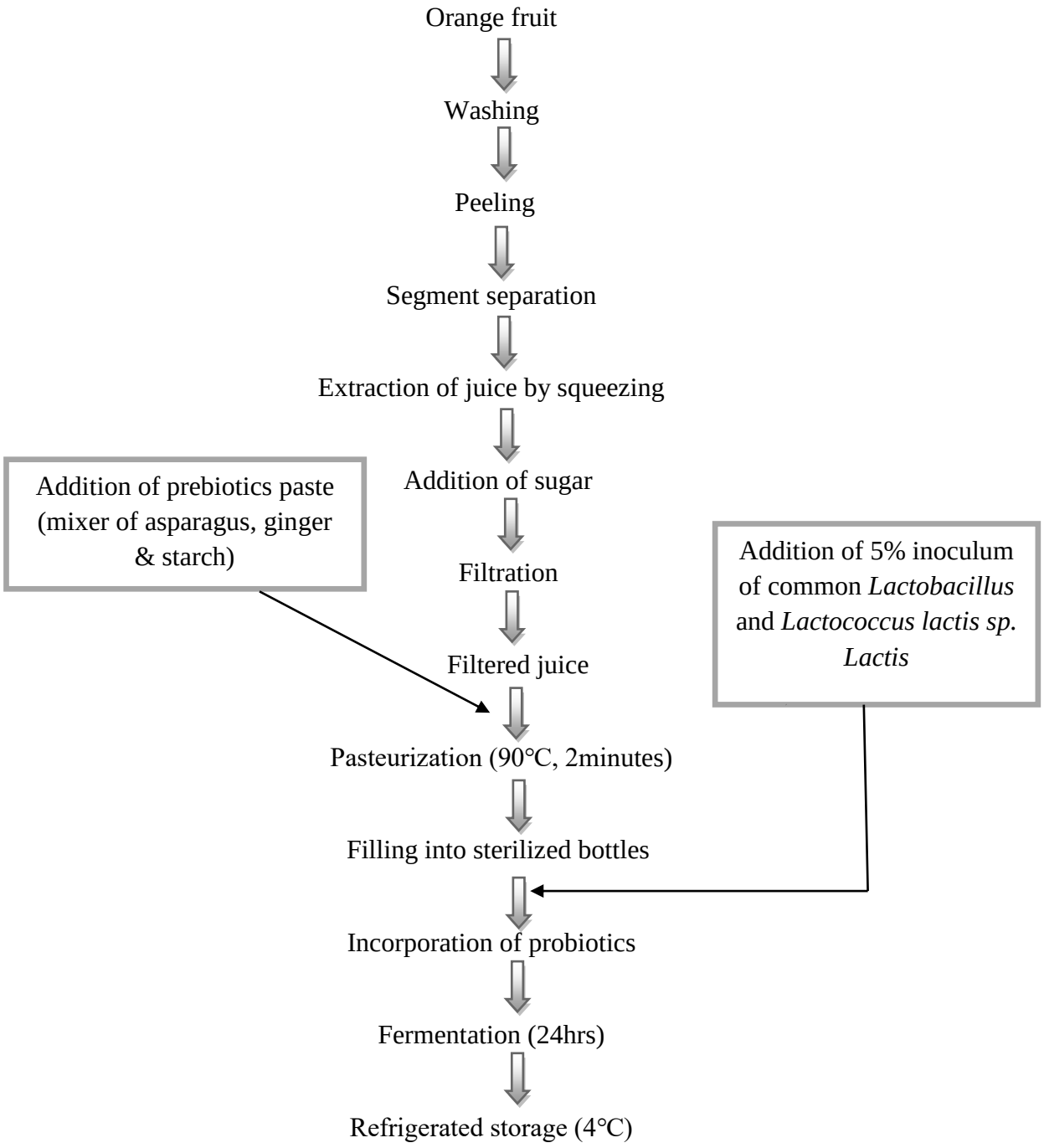

Figure 3.1: Preparation of probiotic fruit juice (orange) enriched with prebiotics

Figure: 3.2: Uninoculated Orange juice used as control

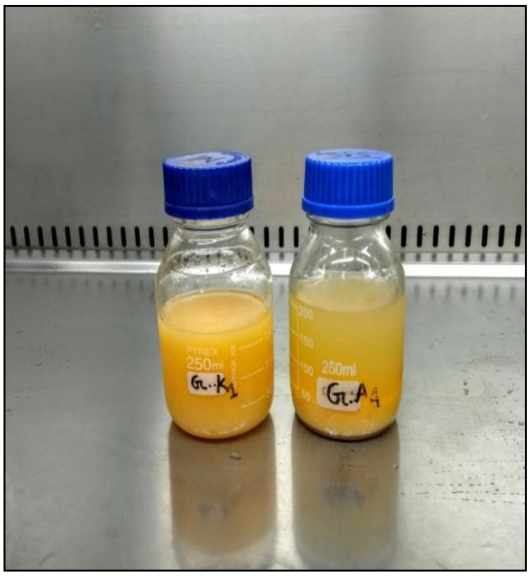

Figure: 3.3: Juice inoculated with isolated *Lactobacillus* genus

Figure: 3.4: Juice inoculated with isolated *Lactococcus lactis sp. Lactis*

Figure: 3.5: Juice inoculated with bacterial isolates and fortified with prebiotics

3.8.2.6 Streaking plate technique

After preparation of orange juice these are kept in room temperature for one day. At that point they were kept in refrigerated temperature at 4°C. Streaking plate technique was applied for each juice containing bottles aside from control to measure the growth of isolated bacteria. The sample is diluted by streaking it across the surface of the agar plate. These Petri plate were kept in incubator at 37°C for one day and afterward development of the growth of isolated bacteria were observed.

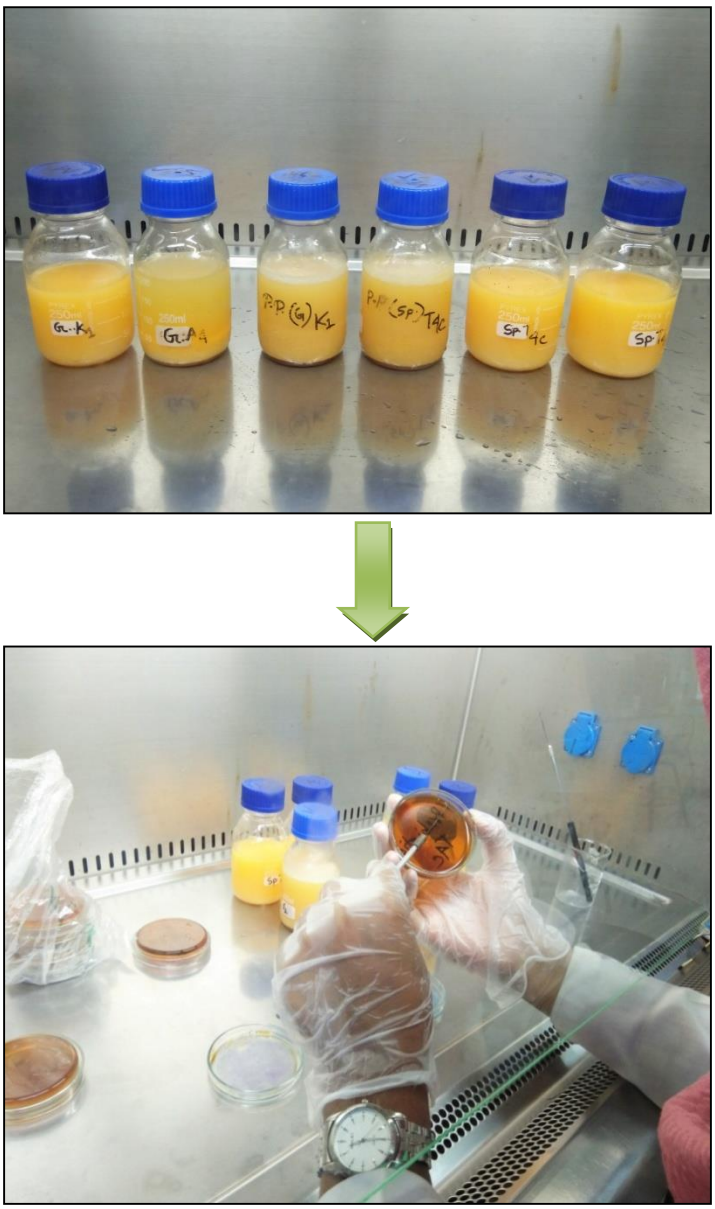

Figure: 3.6: Inoculation of isolated bacteria on MRS agar plate by streaking plate technique

Chapter 4: Results

4.1 Isolation and identification of bacteria

After overnight incubation at 37℃, the selected isolates were identified as *Lactobacillus* and *Lactococcus* spp. All the isolates produced cream colored, circular, convex, shiny and smooth texture. Microscopically the isolates were rod or cocci shaped. The results also showed that the isolate has no catalase activity.

Figure 4.1: Culture on MRS agar medium

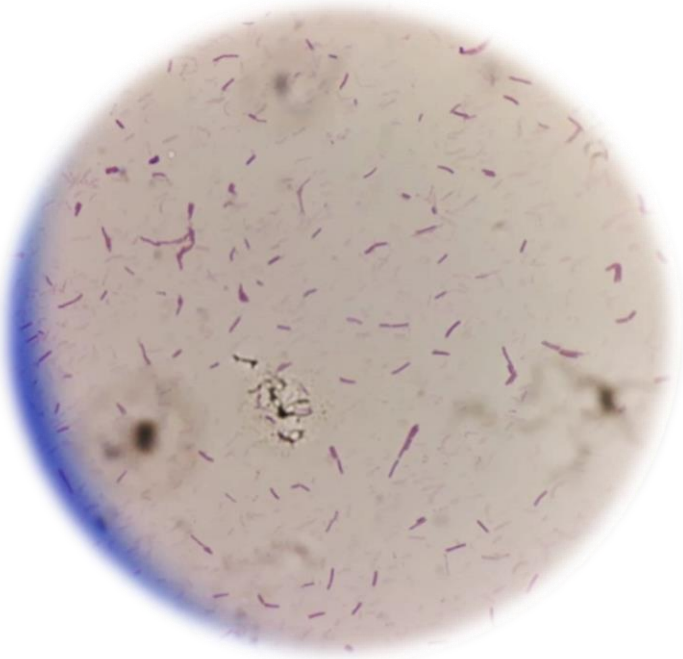

Figure 4.2: Microscopic features of the gram positive isolates

4.2 Molecular identification of the isolated bacteria

In this study, we built up a PCR (Polymerase Chain Reaction) based framework that permitted us to precisely recognize and identify lactic acid forming bacteria. Our outcome demonstrated that it is possible to distinguish the genera of *Lactobacillus* and *Lactococcus lactis sp. Lactis* and recognize these microorganisms from other dairy lactic acid organisms.

4.2.1 Amplification of 16S rDNA region

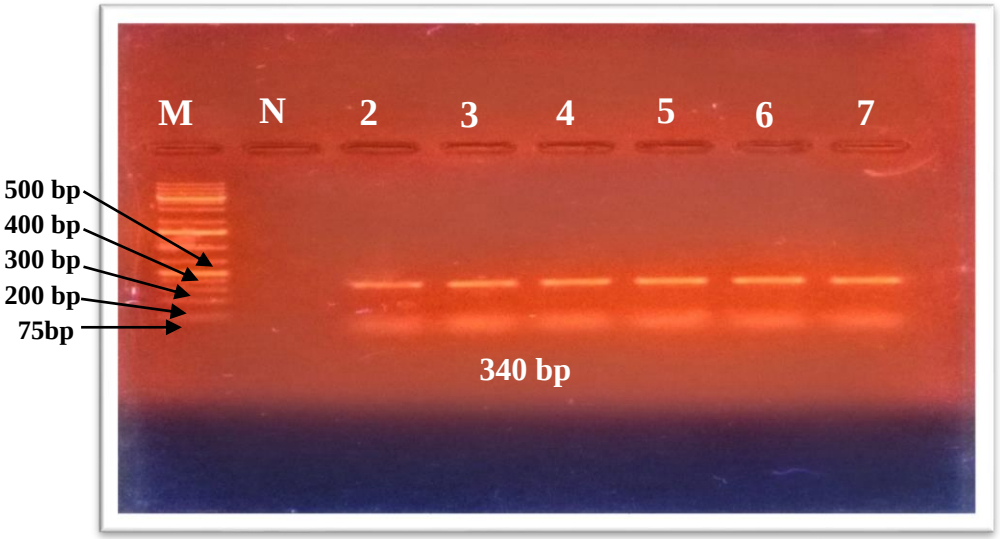

Figure 4.3: *Lactobacillus* genus specific PCR assay. This figure illustrates fragments specifically amplified by PCR by means of the primer *LAC1F* and *LAC2R*. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 2-7: PCR products showing the *LAC* gene-sized amplicon (340bp).

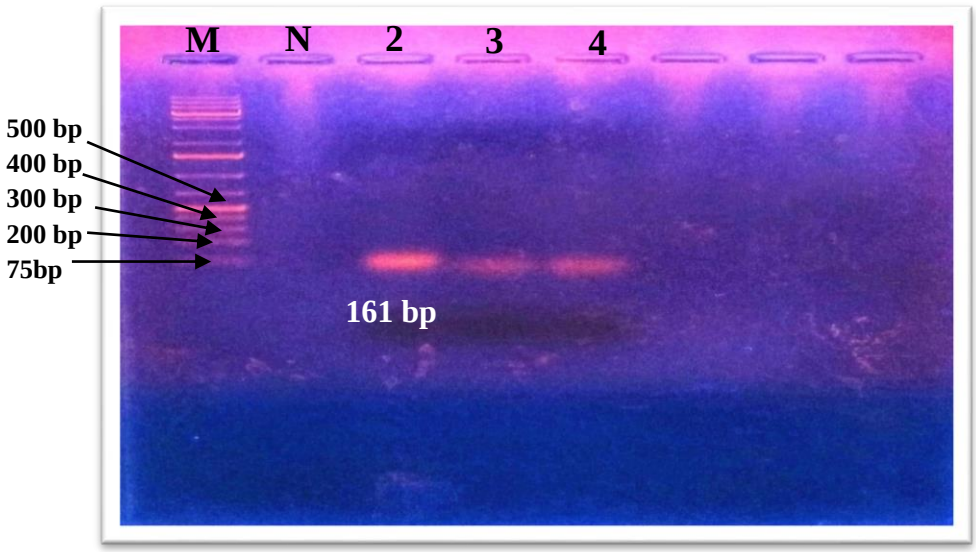

Figure 4.4: *Lactococcus lactis sp. Lactis* specific PCR assays. This figure illustrates fragments specifically amplified by PCR by means of the primer *LacF* and *LacreR*. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 2-4: PCR products; only Lane 2: PCR product showing the *Lac* gene-sized amplicon (161bp). Lanes 3-4: samples giving negative reaction in PCR.

4.3 Observation of the bacterial Growth in prepared fruit juice with prebiotics

In this examination asparagus were utilized to help the development of the segregated streaking plate strategy. After 24 hours fermentation, the expansion of bacterial growth was checked out. This outcome indicated the consequence of bacterial settlement develops on the Petri dish (figure 4.6) which implies that the prebiotic asparagus impact a positive effect on the survival of the growth of isolated bacteria in orange juice.

Figure 4.5: Observed the growth of isolated bacteria before adding prebiotics

Figure 4.6: Observed the growth of isolated bacteria after adding prebiotics

Chapter 5: Discussion

5.1 Identification of lactic acid bacteria

In the present study, bacteria isolated from different sources of dahi were identified as *Lactobacillus* and *Lactococcus* spp. based on their morphological and biochemical characteristics. The results of the study revealed that each one isolates produced white colored, circular, convex and smooth texture. After gram staining all the isolated microorganisms were identified as rod or cocci shaped, rough, irregular, circular and violet color gram positive bacterium. The isolates were also gave catalase negative reaction which was comparable with the study of (Hoque et al., 2010).In their study, the isolated Lactobacilli were detected as white, rough, irregular, shiny and smooth texture. Microscopically they were gram positive, rod shaped and catalase negative. However, another report showed that *Lactobacillus spp*. were identified as small, circular, irregular on the basis of characteristic morphology, catalase negative and gram positive rod shape (Mannan et al., 2017).

Assumedly selected colonies were further investigated by using molecular identification method to see and ensure their purity.

5.2 Molecular identification of the isolated bacteria

Genomic DNAs of bacteria were isolated using boiling method and then secluded DNAs were visualized by agarose gel electrophoresis under UV light. After DNA segregation the 16S rDNA area was intensified by PCR protocol. During this study, genomic DNA fragments of isolates were produced by PCR (Polymerase Chain Reaction) with 16S ribosomal DNA-targeted group-specific primers was utilized to acknowledge lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Lactococcus lactis* species. The findings of this study, PCR primers LAC1F and LAC2R (table 3.1) were determined for the amplification of 340 bp of the 16S rRNA gene (16S rDNA) of *Lactobacillus*. The result was agreed to the previous study, where the isolates identified as *Lactobacillus* at genus level by sequencing their 16S rRNA genes by using an equivalent PCR primer (Negussie et al., 2016).

On the opposite hand, the isolate which was detected as *Lactococcus* were then subjected to molecular identification at the subspecies level by using lactis specific primers. PCR primers LacF and LacreR (Table 3.1) were carried out for the

amplification of 161bp of the 16S rRNA gene (16S rDNA) of *Lactococcus lactis sp. Lactis.* This finding was correlated with the study conducted on isolation, phenotypic and molecular identification of *Lactococcus lactis* from traditionally produced village cheese by Sadik et al. (2010). Their study reported that they found the precise multi-copy PCR product regions of 161bp length for the isolate *Lactococcus lactic sp. Lactis* that on the brink of our study.

5.3 Development of probiotic fruit juice with prebiotics

In the present study we want to develop a functional food like probiotic fruit juice with isolated lactic acid bacteria by means of adding prebiotics. Initially juice was inoculated with different probiotic bacteria (*Lactobacillus and Lactococcus lactis sp. Lactis*) not including any prebiotics. Then the prepared probiotic juice was fortified with prebiotics (mixer of asparagus and ginger). During this study prebiotics were wont to support the expansion of probiotic bacteria. At that time, the bacterial growth was observed after 24 hours fermentation. The results of (figure 4.5) showed that the inoculated isolates were ready to grow in fruit juice with a very much low quantity without addition of prebiotics.

On the other hand, from (figure 4.6) we will see that the isolated bacteria were capable to grow immensely well in fruit juice with the supplementation of prebiotics. The outcome of this study indicated that the prebiotics has a significant effect on the survival of lactic acid bacteria in fruit juice beverage. However another study revealed that the influence of asparagus extract (used as prebiotic) exert a positive effect on the expansion of lactic acid bacteria in fruit juice (Majumder et al., 2017) that was close to our findings. Recently, another study investigated that pineapple juices were inoculated with *Lactobacillus* and *Bifidobacterium* strains were able to grow well within the pineapple juice. Supplementation with prebiotic fructo-oligosaccharides during the fermentation process increased the production of lactic acid by bifido bacteria and slightly improved the steadiness of fermented and probiotic cells (Nguyen et al., 2019). But in our present study, showed that the prebiotics have any capability to assist the development and growth of bacterial strains in orange juice and therefore the outcome gave a positive result suggested that the prebiotics have a remarkable impact on the growth of isolated bacteria in fruit juice.

Chapter 6: Conclusion

Fermented milk based dairy products are the foremost widely used vehicle for probiotics, but also drinks supported on fruit and vegetables are tested as conceivable medium for probiotics, representing a new technological challenge. Fruit juice when fortified with prebiotics and fermented with the isolated bacteria could be an acceptable alternative to dairy based probiotic products. This study revealed that the prebiotics have a significant impact on the growth of isolated bacteria in fruit juice. During this investigation, the vulnerability scope of lactic acid bacteria in natural fruit juice with prebiotic supplementation is indicated that prebiotics have a significant impact on the growth of isolated bacteria in fruit juice. This outcome makes fruit juice an appropriate carrier for those who can't consume probiotic dairy items because of hypersensitive responses. Since, fruits are naturally rich in essential macro and micro elements, incorporation of probiotics into fruit juices make them healthier.

Chapter 7: Recommendations and future perspectives

The current study was conducted to isolate and identify lactic acid forming bacteria from indigenous dairy product (dahi) and development of probiotic fruit juice using the isolated bacteria with prebiotics. The findings of this study suggest the isolated lactic acid bacteria from different source of dahi as potential source to supply functional foods. The market of functional foods is growing and this growth is fueled by an increased level of consumer attention to diet. The consumption of fruits is usually related to the thought of well-being, as these foodstuffs are rich in minerals and vitamins, antioxidants etc. During this research, development of fruit juice incorporated with the isolated lactic acid bacteria fortified with prebiotics are often successfully used as carriers of probiotics, maintaining good levels of growth capability of probiotic microorganisms. However, there have been some limitations during this study. On the idea of this investigation, the subsequent aspects need to be considered for further research work.

a) The present studies perhaps repeated for verification of the experimental findings.
b) The physiochemical characteristics of the juice should be investigated.
c) Survival of the probiotics and their effects on the sensory attributes must be determined.
d) Further assessment is required on progress of taste, storage stability, and packaging of such probiotic juice.

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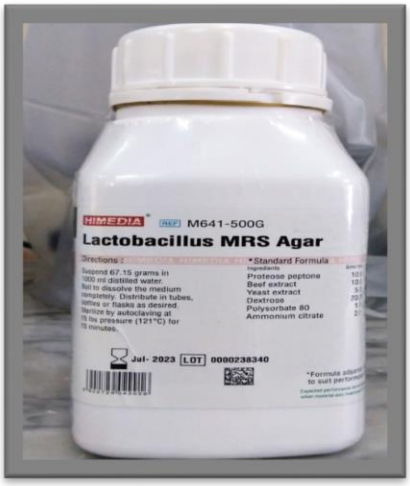
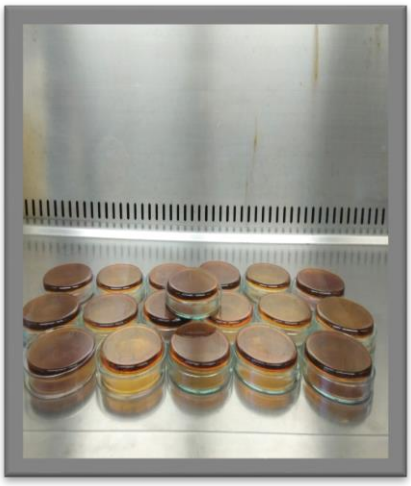
APPENDICES

Appendix A: MRS agar preparation



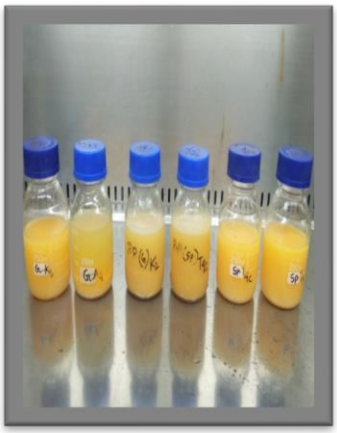
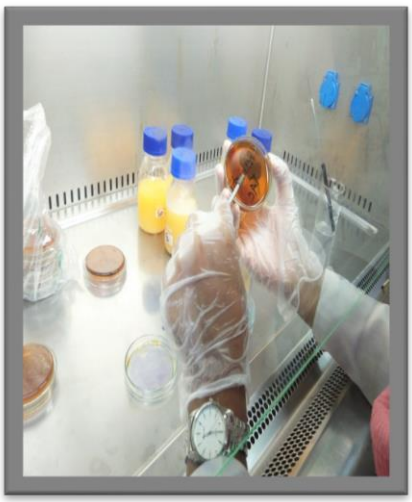
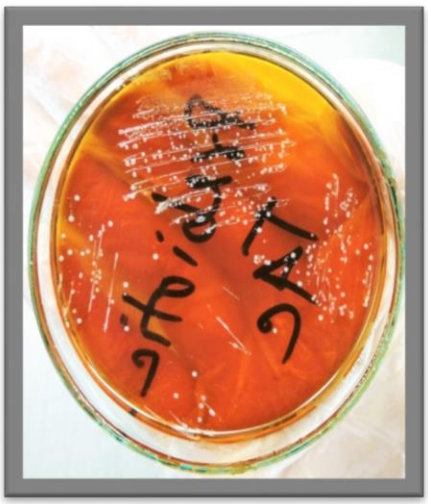


Appendix B: Genomic identification of bacteria using PCR method

Appendix C: Different steps of probiotic fruit juice preparation





Brief biography

Fahmida Akter completed B.Sc. (Hon's) in Food Science and Technology from the Faculty of Food Science and Technology of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh with CGPA 3.60 out of 4.00. Now, she is a candidate for the degree of MS in Applied Human Nutrition and Dietetics under the Department of Applied Food Science and Nutrition, CVASU. She has immense interest to work on the development of probiotic fruit juices with the prebiotic supplementation and the survival of the probiotics in juice and their effects on the sensory attributes.