Evaluation & characterization of isolated *lactobacillus spp*. from market dahi available in Bangladesh

A THESIS

BY

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Department of Dairy and Poultry Science

Faculty of Veterinary Medicine

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Chattogram-4225, Bangladesh

June, 2022

DEDICATED

To My

Beloved

Family and Honorable

Teachers

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Mahmuda Akter. Moni

June, 2022

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

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The Author
June, 2022

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List of Abbreviations and symbols

Abbreviations	Elaborations
CFU	Colony forming unit
ml	Milliliter
LAB	Lactic acid bacteria
pH	Potential of hydrogen
E. coli	Escherichia coli
HACCP	Hazard Analysis Critical Control Point
GI	Gastrointestinal
DDPS	Department of Dairy and Poultry Science
PRTC	Poultry Research and Training Centre
CVASU	Chattogram Veterinary and Animal Sciences University
MS	Master of Science
℃	Degree centigrade
N	Normal
NaOH	Sodium hydroxide
%	Percentage
Sec	Second
h	Hour
SPC	Standard Plate Count
MRS	De Mann Rogosa Sharpe
BSTI	Bangladesh Standards and Testing Institution
TVC	Total Viable Count
V/V	Volume per volume
NaCl	Sodium chloride
etc.	Et cetera
e.g	Example

Abstract

This study was carried out to evaluate and compare the quality of ten types of dahi available in the local markets of Chattogram metropolitan area, Bangladesh. These samples were collected randomly and analyzed for physicochemical and microbiological properties. Physicochemical analysis includes smell, taste, body and consistency, color, texture, and acidity. Microbial analysis was also performed to check the total viable count of bacteria of dahi. In addition, an attempt was made to isolate lactic acid bacteria *(Lactobacillus sp.)* from local Dahi through the phenotypic characterization method.

The organoleptic analysis showed that the highest mean value of total score was found in Sizzle brand (80±9.57) and the lowest mean value was found in Aarong brand (66.5±6.30). In most of the cases, Sizzle and Bonoful brands obtained the highest marks in score card but in the case of chemical test, the highest acidity percentage was obtained by Shakti plus (0.95±0.09), the lowest acidity was found in case of Sizzle brand dahi (0.61±0.02). The acidity percentage of Shakti plus exceed the standard level of acidity of dahi which might be due to uncontrolled incubation, postproduction handling, and prolonged storage. Microbial analysis of dahi showed that the highest total viable count of bacteria was found in case of Kutumbari restaurant dahi ($54\pm30.94\times10^3$) CFU/ml, and the lowest value was found in the case of Khulshi Mart dahi ($24\pm11.77\times10^3$) CFU/ml. In this study, *Lactobacillus spp*. were isolated and identified from these local market dahi. They can survive in high concentrations of salt and low concentrations of phenol. During the experiment period, Sizzle dahi had a good quality compared to other dahi tasted. Comprehensive research work is still required to set a standard for microbial quality to produce superior quality dahi in Bangladesh. In this study, *Lactobacillus spp*. were identified by phenotypic characterization method from local market dahi.

Key words: Dahi, *Lactobacillus spp*, isolate, organoleptic, microbial, quality evaluation, salt, phenol.

Chapter-I

Introduction

In recent days, dairy food is not considered based on taste only, but it must have immediate nutritional value and health benefits. The food businesses are engaged in intense competition to improve the nutritional content of the food products they promote. This increase in nutrition in today's concept is obtained by the consumption of food containing live bacteria. Dahi is a fermented dairy product by lactic acid fermentation of milk by using a starter culture of bacteria. It has been used for its nutritional and medicinal benefits from the beginning of time (Aneja et al., 2002). The starter culture used in dahi is not definite and that's why the quality of dahi varies according to the culture uses for the preparation of dahi. Dahi is mostly prepared by using a mixed culture of *Streptococcus lactis, Lactobacillus bulgaricus, Streptococcus thermophilus, Streptococcus citrophilus, Lactobacillus plantarum*, etc (Islam et al., 2017). But the lactic acid bacteria are the main microbial agent to produce this fermented milk product locally (Dewan and Tamang, 2007). The bacteria have probiotic properties which make the dahi more desirable to people. It improves appetite by stimulating B and T cells of macrophages by lactic acid bacteria (Meydani and Ha, 2000; Sinha and Sinha, 2000). It can control the growth of undesirable bacteria and incurs intestinal diseases like constipation, diarrhea, and dysentery (Shahani and Chandan. 1979). It also has an anti-carcinogenic effect (Shaham, 1980) and lowers the blood cholesterol level of human (Mann and Spoerry, 1974). Besides the therapeutic value of dahi, it is also unique in the case of nutritive value. It contains all the nutrients present in milk except for a little variation in lactose content. The lactose content of dahi is about 30% (percent) lower than milk because some portion of lactose is fermented for the formation of lactic acid (Akter et al., 2010). In Bangladesh, the popularity of dahi increasing day by day as a good item of sweet. Dahi is made using 4% of the entire amount of milk produced in Bangladesh (Mustafa, 1997). In India, about 7% of the total milk produced is converted to dahi whereas, it is 4% in Pakistan (Chakraborty, 1998). Mainly two types of dahi are available in local markets here, sweetened/misti dahi (sugar added) and sour dahi and both are prepared by a traditional method using previously made dahi (starter). Dahi is mainly prepared by two methods: traditional and standardized methods. In Bangladesh, household dahi is prepared by heating the milk at boiling temperature until the volume is reduced up to 15-20% and

8-10% sugar added (sweetened dahi), cooled down to body temperature, inoculated 2-3% starter and poured into earthenware and kept for curd formation overnight by wrapping woolen cloth or straw or jute bag to maintain warmth. In the shops, the method is more or less the same, and dahi is usually set in suitable containers (earthenware/ glass bottles/plastic cups) of the required capacity (Dey et al., 2011).

Over the past few decades, it has been abundantly evident that the human body coexists peacefully with an intricate ecosystem made up of more than 1000 different bacterial species that dwell in the skin, vagina, gastrointestinal (GI) tract, upper respiratory tract, and oral cavity. This collection known as the 'microbiota' is acquired soon after birth and persists throughout life. In the present day, it is important to seek natural bio-preservatives that can control both spoilage and pathogenic microorganisms and keep our food safe. Recent research has shown that having lactic acid bacteria species with antagonistic activity can enhance the quality and safety of meat, dairy products, and other food goods (Ibourahema et al., 2008). In this aspect, the contribution of lactic acid bacteria to the improvement of food safety and stability of fermented foods has long been known. LAB overgrows other microorganisms of the same ecological niche in food products mainly due to lowering the pH, competing for nutrients, and producing various antimicrobial substances such as organic acids, hydrogen peroxide, antibiotics, and bacteriocins.

To best of my knowledge very limited work has been conducted in our country particularly in Chattogram regarding the quality evaluation of market dahi and isolation of lactobacillus spp from the market dahi. Considering the above facts, the present study was conducted for the quality evaluation of market dahi and isolation of *lactobacillus spp.* from the market dahi.

Objectives:

Therefore, the present study was undertaken with the following objectives:

1. To evaluate and compare the quality of available market dahi in Bangladesh.
2. Isolation and characterization of *lactobacillus spp.* from available market dahi in Bangladesh.

Chapter-II

Review of Literature

Fermented dairy foods play an important role in the human diet. Dahi, which has an appearance similar to that of yoghurt, is a popular fermented dairy product in the Indian subcontinent. It is prepared by fermenting milk from cows, buffalos or goats with mesophilic lactic cultures, and its method of preparation and physico-chemical characteristics are well documented (Rati Rao et al. 2006). Dahi is easy to digest and has been found to reduce the risk of cardiovascular problems and cancerous tumors, besides strengthening general immunity (Sinha and Sinha 2000). Yoghurt, a western counterpart of dahi, besides its nutritive value, is believed to be effective in both the prevention and treatment of various illnesses, viz., gastrointestinal disorders, heart diseases and tumor development, in man as well as animals (Deeth and Tamime 1981). Several health benefits of dahi and yoghurt have been reported (Arvind et al. 2009; Pala et al. 2011; Yadav et al. 2008).

The prime objective of the present study was to evaluate the quality of market dahi and isolate the *lactobacillus spp*. from these dahi available in Bangladesh. 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.

2.1 Quality of market dahi

Use of poor quality milk, unhygienic practices associated with the process involved and the use of wild type strain of starter culture, storage dahi 1-2 day at room temperature give rise to poor grade dahi. This types of low grade dahi have only six-to-twelve-hour self-life (Younus *et al.*,2002), after that period, it becomes unhealthy for human consumption due to growth of undesirable bacteria occurs. So, it is necessary to use of quality milk, follow standardized method to increase the self-life of dahi. But most of the dahi manufacturing company doesn't follow the rules. So, there are found a wide variation among the physical, chemical and microbial qualities of dahi from region to region, shop to shop. In some case the dahi doesn't contain the proper amount of microorganism for probiotic action in human. But the most of the people take dahi as a probiotic food item for their beneficial activity in health. Some dahi prepared company

doesn't maintain the proper hygiene during preparation of dahi. Moreover, information is very scanty on the quality of dahi produced by small scale producers throughout the country as well as established renowned sweetmeat makers or large-scale dairy enterprises (Islam *et al*., 2017).

According to US public health milk and milk products should not contain more than 20,000 bacteria/gm and 10 coliform bacteria/gm (Bhowmick et al, 2006). That is why; a desirable standard for the manufacture of dahi should be established according to the average consumers of Bangladesh. Traditional method invariably involves production on a small scale, either in the consumer's household or in the sweetmeat-maker's shop in urban areas. In the house hold, milk is heated to boiling temperature until volume is reduced up to 15-20% and 8-10% sugar added (sweetened dahi), cooled down to body temperature, inoculated 2-3% starter and poured into earthenware and kept for curd formation overnight by wrapping woolen cloth or straw or jute bag to maintain warmth. In the shops, the method is more or less the same and dahi is usually set in suitable containers (earthenware/ glass bottles/plastic cups) of the required capacity (Dey et al., 2011)

Pazakova et al. (1999) studied on the sensorial evaluation of yoghurt produced from cow and goat milk. They reported that cow milk yoghurt is better than goat milk yoghurt.

Nergiz and Seckin (1998) manufactured Torba yoghurt using a traditional method (a strained yoghurt produced from different groups of milk). Milk was heated to 90° C for 10 min. cooled to 45°C inoculated with 3% starter culture and incubated at 43°C. The curd was cooled to 21°C and stored at 4°C for 12 hours.

Karthikeyan et.al (1998) evaluated in performance of 2 strains of yoghurt culture in different combinations in sweet cream buttermilk (SCBM) at various culture TS levels (9-24%) in terms of increasing acidity, rate of synerois, of curd and sensory quality of curd. They reported that acidity increased significantly with increase in TS content in the base mix up to 18%, the rate of synerosis of curd significantly and inversely decreased with increase in TS in SCBM was superior to that of control curd.

Bozanic et al. (1998) prepared yoghurt from goat milk and from cow milk. They reported that yoghurt samples prepared from milk had a softer consistency and lower viscosity than those prepared from standard cow milk; they also had a higher acidity

throughout the storage period. They also reported that sensory properties of samples of milk yoghurt were rated as being inferior to those of samples prepared from cow milk.

2.2 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	Uses in 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

2.3 Health benefits of lactic acid bacteria (LAB)

2.3.1 Biological Control of Food Borne Pathogens

Research has focused on the biological approach to the control and eradication of food borne pathogens. Scientists developed natural antimicrobial products for the biocontrol of pathogens and have exploited LAB for the competitive exclusion of pathogens and delivery of vaccines and bioactive compounds (Grasson, 2002).

2.3.2 LAB in competitive exclusion

The gastrointestinal tract of humans and animals contain a complex bacterial ecosystem. Commensal strains of LAB have a history of use with the intention of enhancing health in the form of probiotics and controlling human pathogens in farm animals. Research has demonstrated the capacity of *Lactobacillus spp.* to control arrange of human pathogens including *E. coli*, *Compylobacter jejuni* and *Clostridium perfringes* (Grasson, 2002).

2.3.3 LAB as vaccine delivery vehicles

Commensal LAB can be exploited to deliver vaccines and other biological active material to the gastrointestinal tract. Their use in vaccine delivery is of special value in stimulating mucosal immunity that is protective at the site of pathogen entry. The advantages of LAB delivery include: ease of administration; survival in stomach acid; inherent safety; particulate nature and size for uptake by cells; economic technology in that the bacterial manufacture the vaccine or therapeutic agent (Grasson, 2002).

2.3.4 LAB as beneficial microorganisms

The LAB are important commercially in the processing of meats, alcoholic beverages and vegetables. The products include sausages, cured hams, wines, beer, fortified spirits, pickles and sauerkraut (Collins and Lyne, 1976; Sharpe, 1981; Jay, 1986; Kandler and Weiss, 1986; Hastings and Holzapfel, 1987; Schillinger and Lucke, 1987; Jay, 1992). Although, LAB have beneficial effects in the food industry, they can sometimes be a nuisance as contaminants by producing off flavours (Kandler and Kunath, 1983; Aguirre and Collins, 1993; Cai *et al.,* 1998). *Lactobacillus* and *Streptococcus faecum* are beneficial microorganisms, which have been proven to replenish essential microflora and decrease the incidence of gastrointestinal disorders. Beneficial bacteria, especially *lactobacillus spp.* can produce anti- microbial substances, which have been observed to inhibit the growth of some pathogenic microorganisms. The addition of type O lactic culture may be an additional safeguard to established good manufacturing practices and Hazard Analysis and Critical Control Point (HACCP) programs in the control of growth of *E. coli* O157:H7 in minas cheese (Saad *et al.,* 2001; Yost and Nattress, 2002). These beneficial microorganisms are most effective during periods of disease or stress and following antibiotic treatment.

2.3.5 Importance of LAB and their effect on human health

Of interest is the role of LAB in the treatment of people suffering with tumors and immune compromised subjects. The evidence that LAB can stimulate the immune system is remarkable and fascinating in it and opens many questions about mechanisms and effective utilization. If this potential is supported in practice, then there are many components to conventional therapies. This may include cost effectively due to their ease of products derived from LAB seem to have relatively low toxicity compared to other treatments (Wood, 1992).

2.3.6 LAB and other effects on the immune system

The LAB are present in the intestine of most animals. The beneficial role played by this microorganism in humans and animals, including the effect on the immune system has been extensively reported (Perdigon et *al.,* 2001). The LAB are present in many foods and are frequently used as probiotics to improve some biological functions in the host. Through different mechanisms they send signals to active immune cells. Thus the

knowledge of the normal intestinal microflora, the contribution of LAB and their role in the numerous functions in the digestive tract as well as the functioning of the mucosal immune system. In the selection of LAB by their immune stimulatory capacity, it helps to know not only the effect which they have on the mucosal immune system, but the specific use to which these oral vaccine vectors are being put (Perdigon *et* al., 2001).

2.3.7 LAB as starter culture

Lactic Acid Bacteria (LAB) are the most important bacteria used in food fermentations. Apart from general demands for starter cultures from the view of safety, technological effectiveness and economics, numerous specific aspects have to be considered when selecting strains for the different food fermentations. Therefore, selection criteria for LAB depend on the type and the desired characteristics of the final product, the desired metabolic activities, the characteristics of the raw materials and the applied technology. A starter culture can be defined as a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process. The group of Lactic Acid Bacteria (LAB) occupies a central role in these processes and has a long and safe history of application and consumption in the production of fermented foods and beverages (Caplice and Fitzgerald, 1999; Ray, 1992; Wood, 1997; Wood and Holzapfel, 1995) (Table 1). They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid. Also, their production of acetic acid, ethanol.

From this section of review it is clear that numerous scientists around the globe have tried to know the quality of dahi or yoghurt prepared from different source of market milk under various condition. Very limited work has been conducted in our country particularly in Chattogram regarding the quality evaluation of market dahi and isolation of *lactobacillus spp*. from the market dahi. Considering the above facts, the present study was conducted for the quality evaluation of market dahi and isolation of *lactobacillus spp.* from the market dahi.

Chapter-III

Materials and Methods

3.1 Statement of the experiment

The experiment was carried out in the Dairy Science laboratory of the Department of Dairy and Poultry Science (DDPS) and Poultry Research & Training Centre (PRTC) of Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh, from July to November 2022.

3.2 Sample collection

Ten dahi samples were purchased from the local markets of Chattogram metropolitan city, Chattogram, Bangladesh. After collection, the samples were stored in a sterile plastic container and then preserved aseptically at a temperature of 4°C for the isolation of bacteria (*Lactobacillus* spp.).

3.3 Sensory/organoleptic quality evaluation

Organoleptic tests (smell and taste; body and consistency; color and texture) were done immediately after the preparation of each batch of Dahi. The organoleptic test was performed by a panel of expert judges (Nelson, 1948).

Figure 1. Sensory/organoleptic quality evaluation by judge panel

3.4 Chemical quality evaluation:

3.4.1 Acidity percentage: Acidity percentage was determined by the method described by Aggarwala & Sharma (1961) The titratable acidity was measured by titrating 9 gm of the diluted curd samples with 0.1 N sodium hydroxide (NaOH) until the substance reached a faint pink color corresponding to the end point of the phenolphthalein which is used 2-3 drops during titration as an indicator The amount of 0.1 N NaOH used was noted and then the titratable acidity was calculated by using formula.

Weighing of dahi

Adding distill water and phenolphthalein indicator

Titration of dahi sample

Faint pink end point

Figure 2. Determination of acidity percentage of dahi

3.5 Microbial quality evaluation

3.5.1 Total viable count: The total viable count of bacteria was determined by the standard plate count method by using the pour plate technique. For each sample, one gram of homogenized sample was dissolved in previously sterilized 9 ml of distilled water. The serial dilution was done from 10^{-1} to 10^{-10}.

One ml aliquot each of 10^{-3} dilution was placed on three Petri dishes and then pour on SPC (standard plate count) agar media on it and mixed, then allowed sometimes for solidification. After that, incubated for 48 hours at 32°C. The colonies were enumerated by using a colony counter (Model-STUART, SC-5) and counting the number of total viable bacterial colonies. and expressed as CFU /ml of sample.

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

Weighing of agar powder

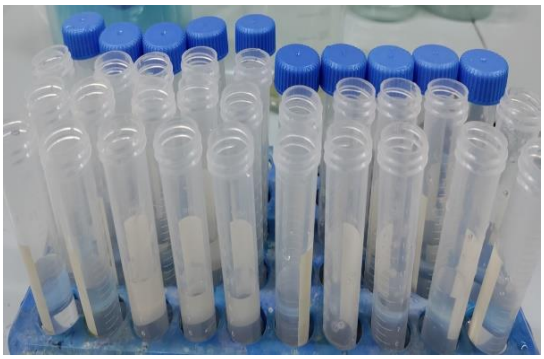
Mixing with distill water

Autoclaving

SPC agar

Figure 3. Preparation of agar media

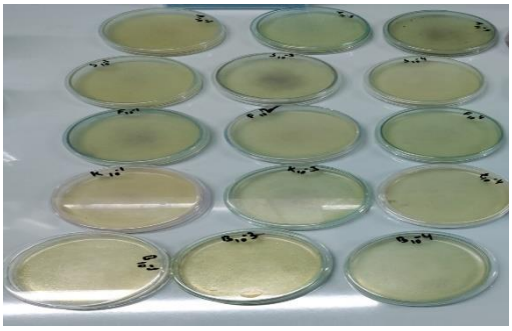
Taking inoculam of dahi sample



Dilution blank

Taking diluted sample into petridish

Pouring of agar



Waiting for solidification of agar

Incubation

Counting of TVC by colony counter

Figure 4. Microbial analysis of dahi

3.6 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 spp.*). MRS agar media was used to isolate *Lactobacillus spp.* from locally available Dahi (De Man et al., 1960).

Characterization of *Lactobacillus spp.*

Identification of the isolated bacteria as *Lactobacillus spp.* was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey s Manual of Systematic Bacteriology (Holt *et al.*, 1994; Williams and Wilkins, 1995). The tests carried out were Gram reaction, production of catalase, 0.4% phenol tolerance test and NaCl tolerance (4%) assay.

3.6.1 Procedure for the isolation and characterization of *Lactobacillus spp.*

The collected sample was mixed homogeneously and directly streaked onto MRS agar and incubated for 48-72 hours at 37°C. After incubation, bacterial growth was observed. Colonies differ in morphology, and pigmentation; shape, and size were subcultured. The growth of *Lactobacillus spp.* was suspected when off-white to cream color, shiny round shaped colonies yielded on an 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 and catalase-negative were considered *Lactobacillus spp.* (Salma *et al.*,2022).

3.6.2 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 the characterization of bacteria was recorded.

3.6.3 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 the surface of a clean, dry glass slide using a loop.

Then a drop of 3% H_2O_2 was placed onto 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.* is 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 of in the biohazard glass disposal container.

3.6.4 Determination of optimum growth of *Lactobacillus spp.*

The optimum growth of the isolate was determined by incubating 1% (v/v) fresh overnight culture into MRS broth at varying pH ranges from 3-9. For temperature assay, the culture was incubated at different temperatures (15-50°C) under anaerobic conditions. After 24 and 48 hr of incubation development of growth was measured by observing their turbidity (Hoque *et al.*, 2010).

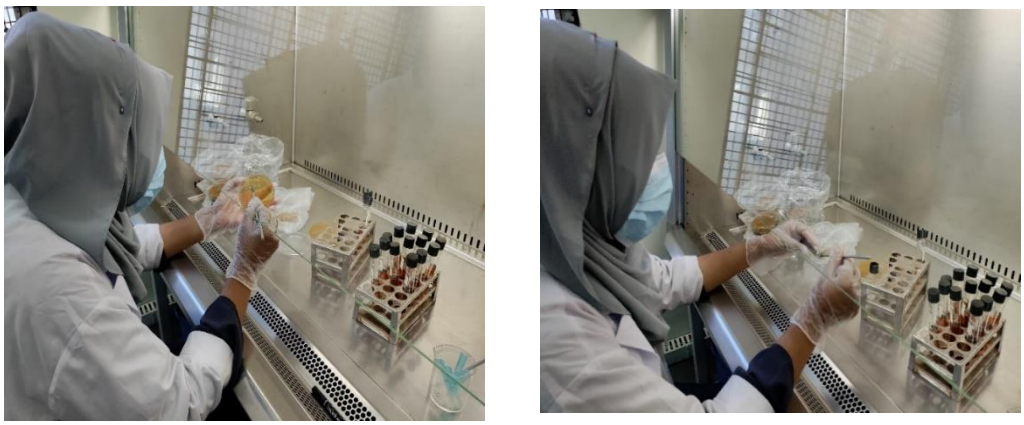


Figure 5. Culture inoculation on MRS broth

3.6.5 Assay for NaCl tolerance

For the determination of NaCl tolerance, 1% (v/v) fresh overnight culture of the isolate was incubated into MRS broth and adjusted with a NaCl concentration of 4%. After 24 and 48 hr of incubation growth of the isolate was determined by observing their turbidity.

3.6.6 Assay for phenol tolerance

For the determination of phenol tolerance, 1% (v/v) fresh overnight culture of the isolate was incubated into MRS broth adjusted with NaCl concentration of 4%. After 24 and 48 hr of incubation growth of the isolate was determined by observing their turbidity.

3.7 Statistical analysis:

The data obtained were imported, stored and coded according to recorded information in the data sheet using the Microsoft Excel – 2019 program for statistical analysis. A descriptive statistic was performed for chemical and microbial parameters according to different samples.

Chapter-IV

Results

4.1 Organoleptic quality of dahi samples collected from different sources: Organoleptic score was highest in Sizzle brand and lowest score in Aarong brand dahi during my experimental period. So Sizzle dahi was selected as the best dahi among these brands by the judge panel (**Table 2**).

Table 2 Sensory/ organoleptic score of dahi samples collected from different sources in Bangladesh

Parameter	Types of dahi									
	Si	Fu	Ar	Kh	Am	We	Ku	Sh	Mi	Bo
Smell and taste (50)	37.25 ± 6.61	35.5 ± 5.72	30.75 ± 3.27	33.25 ± 3.03	32± 2.45	36.5 ± 5.02	35± 6.36	34.25 ± 6.06	37.5± 2.5	37.75 ± 6.22
Body and consistency (30)	26.25 ± 1.20	22.25 ± 1.20	21.25 ± 2.16	25.25 ± 3.56	24.75 ± 1.09	22± 4.84	25.5 ± 0.5	20.25 ± 3.34	25.25 ± 3.56	25.5± 3.20
Color and texture (20)	16.5± 1.66	14.5 ± 1.80	14.5 ± 0.87	15.75 ± 1.20	16.25 ± 1.09	16± 2.55	17.25 ± 1.48	14.5± 1.5	15.5± 2.5	15.75 ± 3.49
Total score	80± 9.57	72.25 ± 8.82	66.5 ± 6.30	74.25 ± 7.89	73± 4.63	74.5 ± 12.42	77.75 ± 8.34	69± 10.90	78.25 ± 8.56	79± 12.91

- value expressed as mean value ± standard deviations

Si=Sizzle, Fu= Fulkoli, Ar= Aarong, Kh= Khulshi Mart, Am= Amman, We= Well food, Ku= Kutumbari restaurant, Sh= Shakti plus, Mi=Mithai, Bo= Bonoful

***Score card**

Excellent (91-100) %; Good (81-90) %

Fair (71-80) %; Poor < 70%

4.2 Chemical evaluation of dahi (Acidity Percentage):

The highest acidity found in Shakti plus and the lowest acidity found in Sizzle brand. So Sizzle brand dahi was good quality dahi and Shakti plus dahi was not good quality dahi during my experimental period **(Table 3).**

Table 3. Acidity Percentage of dahi samples collected from different sources in Bangladesh

Parameter	Types of dahi									
	Si	Fu	Ar	Kh	Am	We	Ku	Sh	Mi	Bo
Acidity %	0.61 ± 0.02	0.90 ± 0.04	0.83 ± 0.02	0.80 ± 0.07	0.90 ± 0.05	0.69 ± 0.03	0.80 ± 0.12	0.95 ± 0.09	0.66 ± 0.04	0.69 ± 0.06

- value expressed as mean value ± standard deviations

Si=Sizzle, Fu= Fulkoli, Ar= Aarong, Kh= Khulshi Mart, Am= Amman, We= Well food, Ku= Kutumbari restaurant, Sh= Shakti plus, Mi=Mithai, Bo= Bonoful

4.3 Microbial evaluation of dahi:

The highest TVC found in Kutumbari restaurant dahi and the lowest found in Khulshi Mart brand dahi. So Khulshi Mart dahi was good quality dahi and Kutumbari restaurant was not good quality dahi during my experimental period **(Table 4).**

Table 4. TVC of dahi samples collected from different sources in Bangladesh

Parameter	Types of dahi									
	Si	Fu	Ar	Kh	Am	We	Ku	Sh	Mi	Bo
TVC (CFU/ml) $\times 10^3$	30 ± 24.06	37 ± 19.51	36 ± 19.25	24 ± 11.77	48 ± 25.49	33 ± 18.06	54 ± 30.94	32 ± 15.33	35 ± 17.68	43 ± 26.28

- value expressed as mean value ± standard deviations

Si=Sizzle, Fu= Fulkoli, Ar= Aarong, Kh= Khulshi Mart, Am= Amman, We= Well food, Ku= Kutumbari restaurant, Sh= Shakti plus, Mi=Mithai, Bo= Bonoful

4.4 Isolation of *Lactobacillus spp.*

4.4.1 Morphological identification of *Lactobacillus spp.*

The characteristic growths of *Lactobacillus spp.* observed on MRS agar are displayed in **Figures 6.** 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 6. Growth of *Lactobacillus spp.* on MRS agar

4.4.2 Microscopic observation

All the presumptive isolates of *Lactobacillus spp.* **(Figure 7)** were purple colored, rod-shaped.

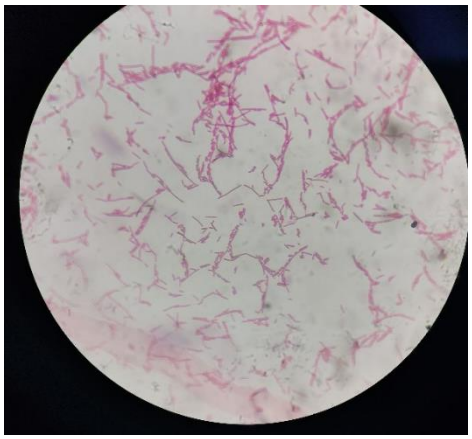


Figure 7: *Lactobacillus spp.* under microscope

4.4.3 Catalase test

No gas bubble was formed during the catalase reaction which means it was catalase negative. All the presumptive isolates were catalase negative **(Figure 8)**.

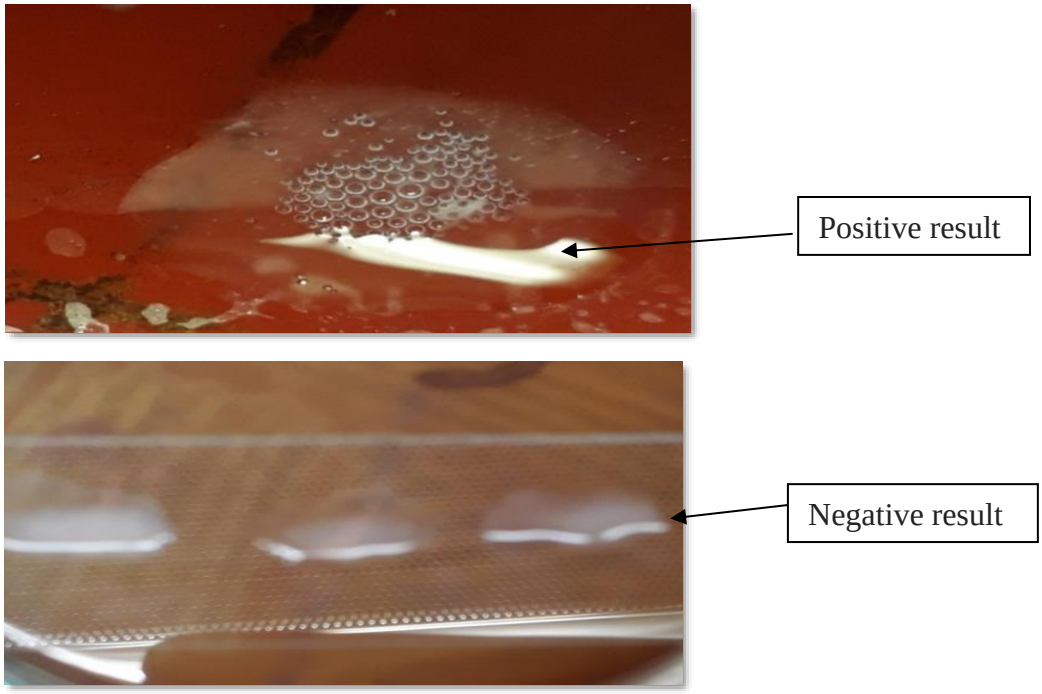


Figure 8. Catalase test

4.5 Determination of optimum growth of *Lactobacillus spp.*

A total of thirteen isolates were taken to determine the growth of *Lactobacillus spp.* It's observed after 24 and 48 hr. All most strains survived for 48 hr duration.
The values for growth of *Lactobacillus spp.* are shown in **Table 5 and 6-**

Table 5. Growth of *Lactobacillus spp.* after 24 hr

No. of inoculum	Growth rate after 24 hours
1	+
2	+
3	+
4	-
5	+
6	-
7	-

8	-
9	+
10	+
11	-
12	+
13	+

Legend: +, visible growth, -, no growth

Table 6. Growth of *Lactobacillus spp.* after 48 hr

No. of inoculum	Growth rate after 48 hours
1	+ +
2	+ +
3	+ +
4	+
5	+ +
6	+
7	+
8	+
9	+ +
10	+ +
11	+
12	+ +
13	+ +

Legend: ++, good growth, +, visible growth.

4.6 Tolerance to NaCl of *Lactobacillus spp.*

Total of thirteen isolates were taken for the NaCl test. It was observed after 24 and 48 hr. All the isolates were able to grow at 2% and 4% NaCl concentration but did not grow at 8% NaCl.

The values for growth in NaCl test are shown in **Table 7 and 8 -**

Table 7. Tolerance to NaCl of *Lactobacillus spp.* after 24 hr

No. of inoculum	Tolerance to NaCl after 24 hours		
	2%	4%	8%
1	+ +	+	-
2	+ +	+	-
3	+	-	-
4	+ +	+	+
5	+ +	+	+

6	-	-	-
7	+ +	-	-
8	+	+	+
9	+ +	+ +	-
10	+	+	-
11	+ +	+	-
12	+ +	+	-
13	+ +	+ +	-

Legend: ++, good growth, +, visible growth, -, no growth

Table 8. Tolerance to NaCl of *Lactobacillus spp.* after 48 hr

No. of inoculum	Tolerance to NaCl after 48 hours		
	2%	4%	8%
1	+ +	+	-
2	+ +	+	+
3	+ +	+	+
4	+ +	+	+
5	+ +	+	+
6	+	-	-
7	+ +	+	+
8	+	+	+
9	+ +	+ +	-
10	+ +	+	-
11	+ +	+	-
12	+ +	+	-
13	+ +	+ +	-

Legend: ++, good growth, +, visible growth, -, no growth

4.7 Tolerance to phenol of *Lactobacillus spp.*

A total of thirteen isolates were taken for the Phenol test. It's observed after 24 and 48 hr. All the isolates survived at 0.2% while 50% of isolates survived at 0.4% phenol concentrations.

The values phenol tolerance test are shown in **Table 9 and 10-**

Table 9. Tolerance to phenol of *Lactobacillus spp.* after 24 hr

No. of inoculum	Tolerance to phenol after 24 hours	
	0.2%	0.4%
1	+	+
2	+	+
3	+	-
4	+	-
5	+	-

6	+	-
7	+	-
8	+	+
9	+	-
10	-	+
11	+	-
12	-	+
13	+	+

Legend: +, visible growth, -, no growth

Table 10. Tolerance to phenol of *Lactobacillus spp.* after 48 hr

No. of inoculum	Tolerance to phenol after 48 hours	
	0.2%	0.4%
1	+	+
2	+	+
3	+	-
4	-	-
5	+	-
6	-	-
7	+	-
8	+	+
9	+	-
10	-	+
11	+	-
12	-	+
13	+	+

Legend: +, visible growth, -, no growth

Chapter-V

Discussion

5.1 Sensory/Organoleptic quality evaluation

It has been observed that the quality or integrity of a particular food sample can be determined by evaluating its sensory characteristic (USDA, 2001). Table 3 shows that the highest value of total organoleptic score was found in the Sizzle brand (80±9.57) and the lowest value was found in the Aarong brand (66.5±6.30). Starter culture, incubation temperature, processing conditions (e.g., heat treatment, homogenization), compositional properties of the milk base, and also many other factors affect the flavor, taste, and texture of dahi (Shaker et al., 2001).

5.1.1 Smell and taste

In the case of smell and taste the highest mean value was obtained by Bonoful dahi (37.75 ±6.22) and the lowest mean value was obtained by Arong (30.75 ±3.27). The mean value of others in case of smell and taste are Sizzle (37.25±6.61), Fulkoli (35.5±5.72), Khulshi Mart (33.25±3.03), Amman (32±2.45), Well food (36.5±5.02), Kutumbari restaurant (35±6.36), Shakti plus (34.25±6.06), Mithai (37.5±2.5). Ara et al, (2015) found that the addition of 10% jack fruit juice with dahi, smell and taste score was 41.44 ± 0.05, which agreed with the present findings of (Rangappa and Achaya, 1974) reported that milk stored too long before seeding often gives rise to broken curd of poor taste.

5.1.2 Body and consistency

In case of body and consistency scorecard showed that the highest mean value was obtained by Sizzle (26.25±1.20) and the lowest mean value was obtained by Shakti plus (20.25±3.34). The mean value of other brands for body and consistency are Fulkoi (22.25±1.20), Aarong (21.25±2.16), Khulshi Mart (25.25±3.56), Amman (24.75±1.09), Well food (22±4.84), Kutumbari restaurant (25.5±0.5), Mithai (25.25±3.56), Bonoful (25.5±3.20). Mangashetti et al. (2003) found that dahi produced from concentrated milk with 7.5% added sugar has smooth body and textural characteristics.

The variation in body and consistency score of dahi culture among different sources could be attributed to different starter cultures, total solids content, and manufacturing process employed. Kober et al. (2007).

5.1.3 Color and texture

In case of color and texture, the highest mean value was obtained by Kutumbari restaurant (17.25±1.48), and the lowest mean value was obtained by Aarong (14.5±0.87). The other brand score- Sizzle (16.5±1.66), Fulkoli (14.5±1.80), Khulshi Mart (15.75±1.20), Amman (16.25±1.09), Well food (16±2.55), Shakti plus (14.5±1.5), Mithai (15.5±2.5), Bonoful (15.75±3.49) in this case. Improving the textural quality of dahi such as firmness, viscosity, and creaminess, functional ingredients provide health benefits (Drake et al., 2000). These additional properties may affect consumer acceptability and preference (Fox, 2001).

However, it was also observed that some dahi brands were not significantly different from each other. Color, texture and thickness of dahi are important quality characteristics, but the flavor and taste of the product are generally considered the most critical and important indicators of consumer acceptance (Olugbuyiro and Oseh, 2011). As it had been earlier stated by (Olugbuyiro and Oseh, 2011) that low score in average overall acceptability is a function of flavor, taste and smell, the results obtained from this research conform to this statement.

The grading scores are excellent (91-100) %; good (81-90) %; fair (71-80) %; poor < 70%. According to score card the all collected dahi samples were fair quality during my experimental period.

5.2 Chemical quality evaluation (**Acidity**)

The acidity of different brands of dahi varies. In this case, the highest acidity was obtained by Shakti plus (0.95±0.09), and the lowest acidity was found in the case of Sizzle brand (0.61±0.02). The mean values of other are Fulkoli (0.90±0.04), Aarong (0.83±0.02), Khulshi Mart (0.08±0.07), Amman (0.90±0.05), Well food (0.69±0.03), Kutumbari restaurant (0.80±0.12), Mithai (0.66±0.04), Bonoful (0.69±0.06). The highest acidity of samples Bonoful and modhuban might be due to uncontrolled incubation, postproduction handling, and prolonged storage while sample Gousia brand dahi might be produced under controlled incubation and controlled storage temperature to controlled incubation & post-production handling & at 4°C. Alam (2014) found that

the acidity of dahi was 0.7% which agrees with the present findings. The result of the present findings (average acidity 0.7%) is nearly similar to the work of (Rashid and Miyamoto, 2005) who found that acidity of dahi was 0.6%.

5.3 Microbial quality evaluation

5.3.1 Total viable count

The highest total viable count of bacteria found in case of Kutumbari restaurant ($54\pm30.94\times10^3$) CFU/ml and lowest value found in case of Khulshi Mart ($24\pm11.77\times10^3$) CFU/ml. The variation in total viable count in different curd samples might be due to undefined starter culture in improper ratio and amount. It also contains a heterogeneous mixture of lactic acid bacteria. So as a result of Total Viable Count in dahi samples varies (Allai et al., 2015)

According to (Hasan et al., 2016) the dahi sample of Chittagong region contains 1.72×10^7 CFU/ml total viable count which supports the findings of this study. The highest total viable count/ml was recorded for Highway brand dahi sample which indicates that it contains more favorable condition for growth of microbes. Bacteria might have got more nutrients from commercial starch powder, and most probably less hygienic measure was taken during manufacturing.

5.4 Isolation of *Lactobacillus spp.*

In this study, the bacteria were identified presumptively by 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 edges. After gram staining, all the isolated microorganisms have been described as rod-shaped, convex, rough, smooth, glossy, irregular, circular, gram-positive, facultative anaerobic bacteria that indicate that they are *Lactobacillus spp.* (Fooks et al., 1999; Tharmaraj and Shah, 2003).

5.5 Growth of *Lactobacillus spp.*

A total of thirteen strains were isolated and formed turbidity on the MRS broth medium. This result was observed after 24 and 48 hr. All strains survived for 48 hr duration. This is in agreement with Forhad et al. (2015).

5.6 Tolerance to NaCl of *Lactobacilli spp.*

NaCl is an inhibitory substance that inhibited the growth of some bacteria (Hoque et al., 2010). NaCl tolerance test was performed at 2%, 4%, and 8% NaCl concentrations. All the isolates were able to grow at 2% and 4% NaCl concentration but did not grow at 8% NaCl. This is in agreement with Forhad et al. (2015) that *Lactobacillus spp.* were not able to grow in high NaCl concentrations.

5.7Tolerance to phenol of *Lactobacilli spp.*

Phenol is also an inhibitory compound produced in the deamination reaction of amino acids in intestine (Suskovic et al.,1997). Probiotic bacterial strains survive low concentrations of phenol. In the present study, a phenol tolerance test was performed in 0.2% and 0.4% of phenol concentrations. All the isolates survived at 0.2% while 50% of isolates survived at 0.4% phenol concentrations. A similar report was published by Hoque (2010), where most Lactobacillus strains survived up to 0.3% phenol.

Chapter-VI

Conclusion

In conclusion, this study showed that all the collected dahi samples of this study were fair quality. Among ten types of dahi Sizzle was comparatively good quality dahi during my experimental period. Products might be contaminated by poor quality milk, contaminated water, utensils; adulteration, and high temperature during the storage period. Comprehensive research work is still required to set a standard for the commercial production of dahi in Bangladesh to have uniformity and superiority in its organoleptic, chemical, and microbial quality. Also, Government should take proper steps with the help of BSTI to increase the quality of dahi all over Bangladesh. In this study, *Lactobacillus spp.* were isolated and identified from local market dahi and they can survive in high concentrations of salt and low concentrations of phenol. These findings revealed that the isolated Lactobacillus strains are suitable to survive in the environment of the human gastrointestinal tract. Though the phenol and NaCl tolerance values measured in this study varied from one to another, the level was acceptable, which is close to normal that could help to increase the shelf life of dairy food products. From this assessment, it could be concluded that the technique could help the new researchers to know the morphological characteristics, growth rate, phenol, and NaCl tolerance of *Lactobacillus spp.* It also helps to prepare better quality, highly nutritious, and palatable products.

Limitations and recommendations

Limitations:

1. Research fund was inadequate.
2. Lab facility should be more improved.
3. All tests of isolation and quality were not done.
4. Sample size of this investigation was not representative to the population due to short period of the study.

Suggestions for future research work:

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

1. Due to lack of time and others facilities I could not do all the isolation and quality tests.
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. Considerable further research study could be done to elucidate the present findings for the greater well-being of the consumers.

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Umme Salma Amin, AKM Humayun Kober, Nasima Akter, M.A. Hossain, Eaftekhar Ahmed Rana.,2022. Isolation and Identification of Lactic Acid Bacteria from Traditional Fermented Milk "Dahi" Towards Developing Probiotic Dahi in Bangladesh.

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Appendix

Score card for sensory evaluation of Dahi

Judge No:
Date of Judging:

Parameter	Types of dahi									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	Sizzle	Fulkoli	Aarong	Khulshi Mart	Amman	Well food	Kutumbari restaurant	Shakti plus	Mithai	Bonoful
Smell and taste (50)										
Body and consistency (30)										
Color and texture (20)										
Total score										

Signature of the evaluator

Brief biography of the student

The author of this paper, Mahmuda Akter. Moni was born in Patenga, Chattogram, Bangladesh in 1994. She is the elder daughter of MD.Golam Mowla and Kohinur Begum. She passed the Secondary School Certificate Examination from Patenga High School in 2010 and Higher Secondary Certificate Examination from Bangladesh Navy School & College, Chattogram in 2012. She completed her graduation degree on Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh in 2019. 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.