PROBIOTIC POTENTIALITY OF LACTIC ACID BACTERIA ISOLATED FROM *THEKI DAHI* AROUND VARIOUS PLACES OF POKHARA VALLEY

by

Pawan Khanal

Central Department of Food Technology

Institute of Science and Technology

Tribhuvan University, Nepal

2022

Probiotic Potentiality of Lactic Acid Bacteria Isolated from *Theki Dahi* Around Various Places of Pokhara Valley

A dissertation submitted to Central Department of Food Technology, Institute of Science and Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of M.Tech in Food Technology

by

Pawan Khanal

Central Department of Food Technology, Dharan

Institute of Science and Technology

Tribhuvan University, Nepal

March, 2022

Tribhuvan University

Institute of Science and Technology

Central Department of Food Technology, Dharan

Approval Letter

This ***dissertation*** entitled ***Probiotic Potentiality of Lactic Acid Bacteria Isolated from Theki Dahi around Various Places of Pokhara Valley*** presented by **Pawan Khanal** has been accepted as partial fulfillment of requirement for the **M. Tech. degree in Food Technology**

Dissertation Committee

1. **Head of Department:** ……..……..
   (Mr. Basanta Kumar Rai, Prof.)

2. **External Examiner:** ……..……..
   (Mr. Uttam Kumar Bhattarai)

3. **Supervisor:** ……
   (Mr. Dambar Bdr Khadka, Asst. Prof.)

4. **Internal Examiner:** …….
   (Mr. Bunty Maskey, Asst. Prof)

March, 2022

Acknowledgements

I am very delighted in the submission of my dissertation entitled Probiotic Potentiality of Lactic Acid Bacteria Isolated from *Theki Dahi* around Various Places of Pokhara Valley prepared for the fulfilling the partial requirement for the M. Tech Degree in Food Technology. This dissertation is presented in attempt to give a synopsis of traditional fermented dairy product: *theki dahi*.

First of all, I would like to express my immense gratitude to my supervisor, Mr. Dambar Bahadur Khadka for his continual support, expertise, guidance and encouragement throughout my research work. I am really grateful to my internal examiner Mr. Bunty Maskey for his valuable insights.

I would like to acknowledge Prof. Basanta Kumar Rai, Department Head of Central Department of Food Technology, Dharan for his valuable comments and advices. I would also like to thank Dr. Keshav Singh, Sr. GM Sujal Dairy Pvt. Lt., Mr. Hari Pd. Khanal, Campus Chief, Pokhara Bigyan tatha Prabidhi Campus for his generous support in completing my project. I would also like to thank Mr. Naresh Khanal as well as Mr. Krishna Gurung for helping me throughout my research pursuit. Also, I am thankful to Miss Dikshya Shilpakar for her helping hands.

Lastly, I would like to thank all our Lecturers for their supervision and valuable suggestions throughout the work.

Date of Submission: March 2022

_____

(Pawan Khanal)

Abstract

This research was undertaken to study the probiotic potentiality of lactic acid bacteria (LAB) isolated from *theki dahi* around various places of Pokhara valley. A total of 48 samples were collected from different locations, pooled together and finally examined accordingly. Based on the morphological, biochemical and physiological characteristics, three types of Lactobacilli (LAB A, B and C), two types of *Leuconostoc* (LEU A and B), two types of *Bifidobacterium* (BIF A and B), one type of streptococci and three types of Lactococci (LCC A, B and C) were identified. Also, probiotic strain of *Lactobacillus casei* sub sp. Shirota was isolated from yakult sample. All the isolated microbes were then subjected to further examination for probiotic potentiality test and compared to *Lactobacillus casei* sub sp. Shirota.

All the bacteria were found to be Gram positive, catalase-oxidase negative, non-motile bacteria, growing optimally at 30°C (expect *Streptococcus thermophilus*) and at 0% NaCl. In terms of acid resistance, all of them were found to be resistant to low pH 2 and 4 for 2h with a high resistance of LAB A and BIF B. Similarly, all of them were resistant to bile salt (0.3% and 0.4%) and able to hydrolyze it. Among them, LAB A and BIF B showed high resistance. With regard to antibiotic resistance, all of them were sensitive to penicillin G, ampicillin and amoxicillin but only Lactobacilli and *Bifidobacterium* showed resistance to ciprofloxacin. Finally, all the isolates were able to adhere to hexane. And, LAB A, BIF A and BIF B showed high hydrophobicity. This study implies that *theki dahi* comprises variety of LAB with probiotic properties. Statistically, in this study, LAB B and BIF A demonstrated significantly similar probiotic properties ($p<0.5$) to the control organism. Thus, they can be considered as potential candidate for probiotic organism in *theki dahi*. This research ultimately helps to document as well as commercialize *theki dahi* as a potential probiotic food of Nepal.

Contents

List of Table

List of Figure

List of Plates

List of Abbreviations

Abbreviation	Full form
AMP	Ampicillin
AMX	Amoxicillin
BIS	Bureau of Indian Standards
CIP	Ciprofloxacin
EPS	Exo polysaccharide
FDA	Food and Drug Administration
GIT	Gastro intestinal tract
GRAS	Generally regarded as safe
LAB	Lactic acid bacteria
LcS	*Lactobacillus casei* strain Shirota
MRS agar	De Man, Rogosa and Sharpe agar
MSNF	Milk solids-not-fat
NABL	National Accreditation Board for Testing and Calibration Laboratories
NSLAB	Non-starter lactic acid bacteria
PBS	Phosphatase buffer solution
PEN-G	Penicillin G
TDM	Total dry matter
TMTC	Too many to count
ISO	International Organization for Standardization
FAO	Food and Agriculture Organization

Part I

Introduction

1.1 General introduction

Probiotics are usually defined as microbial food supplements with beneficial effects on the consumers. Most probiotics fall into the group of organisms' known as lactic acid producing bacteria and are normally consumed in the form of yogurt, fermented milks or other fermented foods (Parvez *et al.,* 2006). Nowadays, the term refers to viable, nonpathogenic microorganisms (bacteria or yeasts) that, when ingested, are able to reach the intestines in sufficient numbers to confer health benefits to the host. Commonly used bacterial probiotics include various species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, as well as *Lactococcus lactis*, *Leuconostoc* and some *Enterococcus* species (Hawaz, 2014).

Out of the many indigenous fermented food around the world, *dahi* or curd is one of the popular fermented dairy products from South Asia. It is prepared from boiled milk, fermented in a traditional way by natural microflora (Bhattarai *et al.*, 2016). *Dahi* is popular throughout the Indian subcontinent. Indigenous *dahi* is prepared in *theki* or earthen ware pots at home and as well as at local level for sell. The most flavorful *dahi* is prepared in theki (Bhattarai and Das, 2013). *Theki* is a close-necked wooden vessel carved out of wood like *daar* (*Boehmeria rugulosa*). *Boehmeria rugulosa* Wedd. (*Urticaceae*) is an evergreen tree, distributed in sub montane to montane Himalaya, Himachal to Bhutan, and Myanmar (Semwal *et al.,* 2009). The main purpose of using *daar theki* is to give flavorful *dahi* and to serve as natural microflora reservoir (Bhattarai *et al.*, 2016).

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, which excrete lactic acid as a main fermentation product into the medium. This biochemical definition associates lactic acid bacteria of different phylogenetic branches of bacterial evolution: the "low GC" taxa e.g. *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leucononstoc*, *Pediococcus* and *Streptoococcus* and the "high GC" genus *Bifidobacterium*. Some LAB strains from animal and human intestinal micro flora have been adopted as 'probiotic' food supplements including *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus casei* subsp. *Rhamnosus* and several *Bifidobacterium* and *Propionibacterium* species (Mathur and Singh, 2005). Many studies have reported that the best matrices to deliver

probiotic lactic acid bacteria are dairy fermented products such as yogurt, cheese and other fermented milk products. Their unique presence in intestinal epithelium and human gastrointestinal tract, and their traditional use in fermented foods and dairy products without remarkable problems prove their safety (Hawaz, 2014).

Proper microbial characterization of the fermented food and beverages leads to better understanding and management of food fermentation process. This helps to improve the quality of fermented food products. Also there have been several methods for isolation and identification of the microorganisms involved in food fermentation (Tamang *et al.*, 2015).

The probiotic potentiality of microorganisms can be evaluated from five *in-vitro* tests namely acid resistance, bile salt resistance, bile salt hydrolysis, cell surface hydrophobicity and antibiotic resistance tests. Probiotic microorganism shows 100% resistance to acid (HCl, pH 2.0) and bile salt (0.3% w/v), completely hydrolyses 0.5% w/v bile salt and shows no zone of inhibitions for test antibiotics (Hawaz, 2014). In this study, on the basis of the results obtained from above *in-vitro* tests, the probiotic potentiality of lactic acid bacteria isolated from traditional dairy product *dahi* fermented in *theki* will be evaluated.

1.2 Statement of problem

Recently, probiotics have been gaining popularity due to its numerous health benefits and thus, they are incorporated in many food products, mainly fermented dairy foods (Kechagia *et al.*, 2013). However, the commercialized probiotic foods are not easily associable and can't be afforded by majority of the Nepalese society.

Dahi is consumed as a part of daily diet in Nepal. It is believed to have many health benefits (Khanal and Koirala, 2019). Moreover, it is also believed to demonstrate probiotic properties. However, enough research and documentation regarding this is still lacking (Balamurugan *et al.*, 2014). Additionally, *theki dahi* is one of the most loved delicacies of Nepal. Yet, proper investigation and documentation regarding its microbial and health benefits is still overshadowed (Bhattarai and Das, 2013). This has endangered one of the famous and potential probiotic food of Nepal; *theki dahi*. Henceforth, this study is undertaken to evaluate the probiotic potentiality of *theki dahi* so that probiotic food will become easily accessible to Nepalese society.

On the other hand, proper documentation of technology of *dahi* and its nutritive value is lacking. This is required not only to authenticate its origin, preserve its culture but also to improve and standardize its technology and commercialize this product. Moreover, the knowledge of the biochemical and microbiological properties of these products enhances its production and consumption on a larger scale. On that account, this thesis is also aimed at scientific documentation of *theki dahi*, isolation and identification of microbes associated with it and subsequently examining the probiotic potential of isolated LAB which will ultimately lead to preservation and commercialization of the product.

1.3 Objectives

1.3.1 General objective

The general objective of this research work was to examine the probiotic potentiality of lactic acid bacteria isolated from *theki dahi.*

1.3.2 Specific objectives

- To isolate and characterize *Lactobacillus, Streptococcus, Leuconostocs, Bifidobacterium, Lactococcus* species from *dahi* fermented in *theki.*
- To study probiotic potentiality of the isolated organisms by Acid and Bile resistant test, Bile hydrolysis test, Antibiotic resistant test and Cell surface hydrophobicity test.

1.4 Significance of study

Probiotic bacteria are largely associated with numerous health benefits to human. Thus, they are highly incorporated in many food products, mainly fermented dairy products (Daly and Davis, 1998). *Theki dahi* is one of the most popular and loved dairy product of Nepal. It is prepared and consumed by maximum group of Nepalese society (Bhattarai *et al*., 2016).

This research is mainly undertaken to evaluate the probiotic potentiality of *theki dahi* so that probiotic food will become easily accessible to Nepalese society. The outcome of this research will be used to examine the probiotic potentiality of the isolated and characterized microbes associated with *theki dahi*. Consequently, this will help to commercialize *theki dahi* as a probiotic food which is easily accessible to many Nepalese society.

Additionally, this research is also aimed at the scientific documentation of traditionally fermented dairy product, *theki dahi*. The microbial characterization of *theki dahi* will be used for better understanding of *dahi* in general. This will thereby help to develop starter culture for the product. Moreover, this research will be helpful to dairy entrepreneurs from Pokhara region to develop and launch *theki dahi* on a commercial level.

1.5 Limitation of the work

- Only phenotypic characterization of isolate microbes was carried out.
- Only in-vitro study of the isolated organisms was studied.
- Genotypic characterization of the organisms was not performed
- In vivo test for probiotic potentiality was not studied.

Part II

Literature review

2.1 Introduction to probiotics

The expression “probiotic” was probably first defined by Kollath in 1953, when he suggested the term to denote all organic and inorganic food complexes as “probiotics”, in contrast to harmful antibiotics, for the purpose of upgrading such food complexes as supplements. In recent years, the gastrointestinal micro flora has been featured strongly in scientific, veterinary and medical research. As a result, it has become obvious that the gut micro flora is an essential component of the healthy animal. Not only is it involved in digestion of food, but also it is essential for the optimal resistance to disease (Schmid *et al.*, 2006).

Great attention is currently drawn to probiotics, prebiotics or their combined use as symbiotic, to improve human health via natural sources. Probiotics are defined by the FAO/WHO as “live microorganisms that, when administered in adequate amounts, confer health benefits on the host”. Probiotics have become a major focus of lactic acid bacteria (LAB) research over the past 10 years, with most attention drawn to the genera *Lactobacillus* and *Bifidobacterium*. These organisms have been widely reported to exert many beneficial effects, such as activation of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity and presentation of an antagonistic environment for pathogens. There has been an increase of interest regarding the commercial utilization of *Lactobacillus* strains isolated from traditional and naturally fermented dairy products, which possess health-promoting effects. Research on lactobacilli isolated from such traditional and naturally fermented dairy products reveals a long history of safe use (Saarela *et al.*, 2000).

Most probiotic microorganisms belong to LAB, such as *Lactobacillus* sp, *Bifidobacterium* sp and *Enterococcus* sp. Most scientists agree that probiotic strains shall be able to survive transit through the gastric acid environment as well as exposure to bile and pancreatic juice in the upper small intestine to be able to exert beneficial effects in the lower small intestine and the colon, although there are convincing data on beneficial immunological effects also from dead cells (Ljungh and Wadström, 2006).

2.2 Probiotic microorganism

Lactic acid bacteria have contributed in the increased volume of fermented foods worldwide especially in foods containing probiotics or health promoting bacteria. Microorganisms of genera *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Pediococcus* are involved in these fermentations. In addition, *Lactobacillus spp*. and species of *Bifidobacterium* which is not LAB in nature are part of normal human intestinal micro flora and they exert a positive effect on human health (Daly and Davis, 1998). The probiotic bacteria used in commercial products today are mainly members of the genera *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* species from which probiotic strains have been isolated (Heller, 2001).

2.2.1 *Lactobacillus casei* sub sp. Shirota

The first commercially used *Lactobacillus casei* strain is *Lactobacillus casei* sub sp. Shirota. It was used as a starter culture in manufacturing the dairy probiotic fermented drink; LcS has also been documented as GRAS by the FDA (Das *et al.*, 2016). *Lactobacillus casei* sub sp. Shirota has been used in fermented milk products for more than 80 years and is one of the most intensively studied probiotics (Wang *et al.*, 2015). LcS has been suggested to provide health benefits through balancing the gut microbiota, improving gastrointestinal dysfunction, preventing infection and cancer, and modulating inflammatory and immune responses. It is also said that LcS improves mood disturbance in the elderly and decreases anxiety symptoms in patients with chronic fatigue syndrome, and suppressed the onset of physical symptoms in healthy students exposed to academic stress in a pilot trial. However, it has not been examined fully whether LcS relieves psychological stress-induced responses in association with the microbiota–gut–brain (Kato-Kataoka *et al.*, 2016).

There are many suggested mechanisms of the probiotic action of LcS in the intestine, but aside from immune modulation, the production of lactic acid (resulting in a reduction of local pH) and the competitive adhesion or displacement of pathogenic bacteria have been quoted most often (Sutula *et al.*, 2013). *Lactobacilllus casei* sub sp. Shirota has been proven to have high tolerance to acid and bile, and had the ability to adhere to intestinal surfaces. Also, these strains supported higher acid-production activity and proteolytic activity in the fermented milk (Guo *et al.*, 2009). LcS has also been reported to colonize in gut strongly, reduce plasma-cholestrol levels and exert antagonistic activity against methicillin-resistant *Staphylococcus aures* (Das *et al.*, 2016).

2.2.2 Genus *Lactobacillus*

Lactobacillus is the largest genus of the family *Lactobacillaceae*. Lactobacilli are gram-positive, rod-shaped, facultative anaerobic or microaerophilic, non-spore-forming, acid-tolerant, and catalase-negative bacteria (Huang *et al.*, 2018). The optimum growth temperature is 45°C (minimum at 22°C and maximum growth at 50-55°C). They are able to ferment lactose, fructose and glucose but do not utilize arginine. They grow well under anaerobic conditions but may grow in microaerophilic as well as aerobic conditions. They exhibit optimum growth at slightly lower acidic condition (pH 5.5 – 6.0) while growth is often restricted at neutral or somewhat alkaline condition (pH above 7.0 to 7.5) (Khalil and Anwar, 2016). *Lactobacillus delbrueckii* subsp. *bulgaricus* is considered as the dominant Lactobacilli in *dahi* products (Koirala *et al.*, 2014). These organisms can survive in the adverse conditions by modifying the rate of synthesis of certain protein. They are capable of withstanding high temperature too (Haddaji *et al.*, 2015a).

Major flavor compounds of yoghurt are produced by the joint action of streptococci and Lactobacilli. *Lb. delbrueckii* is mainly responsible for the production of acetaldehyde in yoghurt (Cheng, 2010). The proteolytic activity of *Lb. delbrueckii* ssp. *bulgaricus* is important for acetaldehyde production, because threonine enzyme which is necessary for the conversion of aldehyde to glycine and acetaldehyde mainly results from the activity of the peptidases of Lactobacilli (Erkus, 2007).

Lactobacilli is one of the major probiotic bacteria found in yoghurt (Khanal and Koirala, 2019). Of all the genera of LAB, *Lactobacillus* is the most economically important for human nutrition and for its probiotic properties (Shao *et al.*, 2015). They are considered as health promoting bacteria. Certain strains of Lactobacilli are believed to show modulatory activity, anti-hypertensive, calcium binding activity and anti-cancer activity (Rakhmanova *et al.*, 2018). Moreover, *Lactobacillus delbrueckii* ssp. *bulgaricus* has a preservative effect on the product not only because of the production of lactic acid and hydrogen peroxide, but also by the help of the antimicrobial compound (bacteriocin) it produces (Erkus, 2007).

Lactobacillus isolates from yoghurt shows the potential to be used as a probiotic. They are able to survive in a favorable environment of gastrointestinal tract such as high salt, low pH and high bile salt concentration. The benefits of *Lactobacillus* spp ranges from reducing allergies, preventing antibiotic-associated diarrhea, removing cholesterol, prevent urinary tract

infection, stimulation and development of immune system of the host (Khanal and Koirala, 2019). Additionally, they are 'generally regarded as safe' as they have long been used in the manufacture of dairy foods and are desirable members of the intestinal microflora (Das *et al.*, 2019).

2.2.3 Genus *Streptococcus*

Streptococcus thermophilus is a gram-positive, non-pathogenic, facultative anaerobic lactic acid bacterium. They have the ability to metabolize lactose into exopolysaccharides (EPS), vitamins, and several flavor compounds including carboxylic acids, aldehydes, ketones, alcohols, and esters. Among the many flavor compounds in yoghurt, acetic acid has a significant effect on the final flavor of yoghurt. They are responsible for increasing the acidic flavor in yoghurt (Dan *et al.*, 2018). However, similar to *Lactobacilli*, they may also produce off flavor in yoghurt by producing off flavor compounds from proteolysis of amino acid, excessive lipid oxidation or lipolysis (Cheng, 2010).

Streptococcus thermophilus can produce EPS which increases the viscosity of product by binding free water and preventing the gel fraction and whey synerysis. This organism also enhances lactose digestion of lactose intolerant people. The viable cells of *Streptococcus thermophilus* survive in the stomach and are lysed in the gastrointestinal tract. The intracellular β-galactosidases are released and hydrolyze lactose. Lactose does not reach the large intestine and the symptoms of lactose intolerance does not occur (Erkus, 2007).

2.2.4 Genus *Bifidobacterium*

Bifidobacterium sp. are anaerobic, non-motile, non-acid-fast, gram-positive, short, irregular rods. They appear as bifid or irregular V or Y-shaped rods with rudimentary branching. They constitute the normal flora of the gastrointestinal tract and the oral cavity and are not frequently isolated from human clinical specimens. (Brook and Frazier, 1993; Lee and O'Sullivan, 2010). *Bifidobacterium* have been recognized as bacteria possessing probiotic, nutritive and therapeutic properties (Awasti *et al.*, 2016). It is also believed to help in prevention of enteric infection, reduction of serum cholesterol levels, immuno-stimulation, anti-carcinogenic properties, alleviation of constipation and treatment and lactose intolerance (Cronin *et al.*, 2011). Moreover, Bifidobacteria are generally considered to be food-grade organisms that do not impose health risks on the consumer or the environment (Masco *et al.*, 2006).

Thus, these strains of *Bifidobacterium sp*. are currently added to variety of fermented food to attain probiotic health benefits (Ruiz *et al.*, 2011; Waddington *et al.*, 2010). Bifidobacteria display a set of mechanisms in order to overcome the adverse situations encountered through the human GIT and transiently colonize this competitive environment (Ruiz *et al.*, 2011). Moreover, they are ingested to improve the intestinal microflora, which in turn improves the intestinal environment and contributes to the health of the intestine thereby preventing intestinal tract diseases or infection (Mishra *et al.*, 2012). They participate in the defense against pathogen by preventing their adhesion to the epithelium in the human gastrointestinal tract (Liu *et al.*, 2020).

2.2.5 Genus *Lactococcus*

Lactoccocci are Gram–positive, non-motile, catalase negative cocci belonging to lactic acid bacteria group. With the optimum growth temperature of 30^{o}C, they can survive at 10^{o}C but not able to grow at 45^{o}C. Among many lactococci species, *L. lactis* sp. *lactis* and *L. lactis* sp. *cremoris* are most frequently used as starter cultures in dairy industries (Buyukyoruk *et al.*, 2010).

Lactococcus lactis is non-pathogenic, mesophilic, coccus bacterium of 0.5 – 1 µm diameter. They are fermentative anaerobe, Gram-positive bacteria (Faudzi *et al.*, 2018). They have a complex proteolytic system that together with other proteolytic enzymes convert casein into peptides and amino acids. These amino acids are the key precursors of volatile flavor compounds as they are metabolized to aldehydes, ketones, amides, alcohols, and sulphur compounds (Gutierrez-Mendez *et al.*, 2008). Additionally, proteolytic enzymes are responsible for the hydrolysis bitter peptides responsible for bitterness in the product (Samaržija *et al.*, 2001). Moreover, excessive lipid oxidation or lipolysis can cause off flavor in the product (Cheng, 2010).

Some strains of the *Lactococcus lactis* species produce exopolysaccharides that modify the texture of the product by increasing its viscosity. Moreover, few of them have the ability to produce a range of bacteriocins which help to control certain pathogens and spoilage bacteria in fermented milk products. They also help in improving keeping quality and health of consumer by reducing the symptom of lactose intolerance and balancing the intestinal microflora (Samaržija *et al.*, 2001).The *L. lactis* sp. *lactis* can be discriminated from the *L. lactis* sp. *cremoris* by arginine hydrolysis and differences in their growth characteristics at pH 9.2, 40^{o}C and at 4% saline concentration in the medium (Buyukyoruk *et al.*, 2010).

Lactococcus lactis are mostly isolated from dairy products including raw milk and kefir grains. They adapt easily to environments including milk or containing milk or dairy products and hence quickly and easily metabolizes lactose. Moreover, In vitro and in vivo studies on the probiotic properties of *L. lactis* have suggested its use as a potential probiotic strain to improving nutritional value of foods (Yerlikaya, 2019). They could be used in functional foods. *L. lactis* has properties such that it can maintain its viability in the presence of pancreatin, pepsin and bile salts, and in the extremes of pH (Ozdogan *et al.*, 2014). Furthermore, *Lactococcus lactis* is also recognized as generally regarded as safe (GRAS) by United States Food and Drug Administration (USFDA) (Akbar *et al.*, 2019).

2.2.6 Genus *Leuconostoc*

In the genus *Leuconostoc*, the organisms that are associated with dairy starter cultures are *Leuconostoc mesenteroides* subsp. *cremoris* (previously known as *L. cremoris* or *L. citrovorum*), *L. mesenteroides* subsp. *dextrunicum*, and in some instances *L. luctis* (Tamime and Robinson, 2002). *Leuconotsoc* are Gram-positive, non-motile, facultative anaerobe, non-spore forming, vancomycin-resistant, and they usually have a spherical shape and occur usually in pairs or chains (Rodriguez, 2017; Harun-ur-Rashid *et al.*, 2006). They do not possess arginine dehydrolase and catalase, are hetero fermentative thus produces CO_2 from glucose metabolism beside d-lactate and ethanol or acetate. They are facultative anaerobic bacteria, which shows mesophilic characteristics and grow at 10^oC. They are often present as dairy starter culture and even in the dairy environment and thus can be considered as non-starter lactic acid bacteria (NSLAB). (Hemme and Foucaud-Scheunemann, 2004).

To increase the flavor of yogurt, strains belonging to the genera *Leuconostoc* and *Lactococcus* are often incorporated as adjunct cultures. The major compounds related to the utilization of *Leuconostoc* are diacetyl, acetic acid and ethanol (Cheng, 2010). Carbohydrate degradation and citrate metabolism by *Leuconostoc* inhibits the growth of spoilage as well as pathogenic bacteria. Moreover, they produce bacteriocins which can be used for food preservation. Citrate metabolism is also important for aroma and gas production in fermented dairy products (Hemme and Foucaud-Scheunemann, 2004). *Leuconostoc mesenteroides* has a great economic importance in the dairy industry due to its capability of producing CO_2 from carbohydrates, flavor compounds (diacetyl, acetate and ethanol) in many cultured dairy product (Cardamone *et al.*, 2011).

However, this microorganism may be involved in the deterioration of some products and, in rare cases, it may also be involved in diseases in immunocompromised patients. In contrast, *Leuconostoc mesenteroides* have been evaluated as a potential probiotic culture. Moreover, this microorganism has important technological properties, such as production of dextran, acetaldehyde, diacetyl and acetoin, lipolytic and proteolytic enzymes, low production of acid, and ability to grow under stress conditions (acid, high salt content, and elevated temperature) (de Paula *et al.*, 2014). Presence of these organisms in food is also associated with improving food preservation, flavors, nutrition and human health (Kwon and Son, 2016).

2.3 Identification of microbes

2.3.1 Gram staining

Gram staining is the fundament of phenotypic characterization of bacteria. It is a differential staining technique which differentiates bacteria on the basis of their cell wall structure. Bacteria with thick layer of peptidoglycan (20 to 80 nm) traps the primary color of stain (crystal violet) and gives purple color are designated as gram positive bacteria. While those with thing layer of peptidoglycan (1 to 3 nm) do not retain the color of primary stain upon alcohol treatment, rather are counterstained with safranin and are labelled a Gram negative bacteria (Moyes *et al.*, 2009).

2.3.2 Catalase test

Catalase test assists the detection of the enzyme catalase in bacteria. This enzyme facilitates cellular detoxification. Catalase accelerates the breakdown of hydrogen peroxide into water and oxygen. Thus, it neutralizes the bactericidal effects of hydrogen peroxide as its concentration in bacteria has been correlated with pathogenicity. The catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme (Reiner, 2010).

2.3.3 Citrate utilization test

In this test, citrate agar containing citrate as carbon source and inorganic ammonium salts ammonium di-hydrogen phosphate ($NH_4H_2PO_4$) is used to determine bacterial ability to utilize citrate as a source of energy. Bacteria that can grow on this medium produces an enzyme citrate permease capable of converting citrate to pyruvate. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns

the bromothymol blue indicator in the medium from green to blue above the pH of 7.6 (Aryal, 2019a).

2.3.4 Growth at different temperature

Microorganisms grow over a wide variety of temperature. Each microorganism has a minimum, optimum, and maximum growth temperature. Microorganism shows decreased growth rate above or below the optimum temperature due to denaturation of enzymes and protein, and due to breakdown of the cytoplasmic membrane. Bacteria can be classified as psychrophiles, mesophiles, and thermophiles according to the influence of temperature on their growth (Erkmen and Bozoglu, 2016).

Thermophiles grow at a relatively high temperature with optimum from 55 to 60°C and ranging between 45 and 70°C. Mesophiles grow at ambient temperature with optimum between 30 to 40°C, and ranging from 10 to 45°C. Most of the foodborne pathogens are in this group. Psychrophiles grow at cold temperature with optimum between 12 and 15°C and ranging from 10 to 20°C, and cannot grow above 25°C (Erkmen and Bozoglu, 2016).

2.3.5 Growth at different pH

The acidity or alkalinity of an environment has an effect on the activity and stability of macromolecules, such as enzyme functions and metabolism and cellular activities which ultimately affects the growth of microbes. Every microorganism has an optimum pH and a pH range for its growth. Yeasts and molds have higher tolerance to acidic pH. Yeasts grow best in the pH ranging from 4.0 to 6.0. Molds grow best at the pH ranging from 3.5 to 5.0. Lactic and acetic acid bacteria grow well in the pH ranging from 5.0 to 6.0 (Erkmen and Bozoglu, 2016).

2.3.6 Growth at different salt concentration

Salt concentration affects the growth of microbes in various ways. Some organisms are obligate halophiles, which means they require salt to survive and will die if the salt level drops to a low level. Other halophilic organisms are halotolerant, which means they don't need salt to survive but can tolerate moderately salty environments. Slight halophiles thrive in environment that contain 1 to 6% salt. Moderate halophiles prefer 6 to 15% salt. Extreme halophiles enjoy the saltiest environment of all at 15 to 30% salt. While for non- halophiles salt can be deadly (Mayer, 2018).

2.3.7 Arginine dihydrolase test

The ability of the microbe to hydrolyze arginine can be determined by incubating the bacterial culture in arginine broth for about 24-48 h with few drops of Nessler reagent added. Appearance of brown color indicates hydrolysis of arginine and presence and presence of arginine dihydrolase in bacteria. While for arginine breakdown of Lactobacilli, MRS broth was used in which the ammonium citrate was replaced with 0.3% of arginine hydrolysis (Mehmood *et al.*, 2009).

2.3.8 Sugar fermentation test

Bacterial ability to metabolize certain carbohydrate and its related compounds is used for the identification of bacteria. This test is based on the principle that the action of organism on a carbohydrate substrate result in the acidification of the medium which is detected by the pH indicator dye (bromocresol purple). The fermentation medium consists of a basal medium of single carbohydrate (glucose, lactose, sucrose, mannitol etc.) for fermentation and pH indicator (bromocresol purple) to detect acid production by fermentation. The medium also contains Durham tube to capture gas produced by carbohydrate metabolism. The indicator in the media changes from purple to yellow upon carbohydrate metabolism (Aryal, 2019b).

2.4 Characteristics of probiotics

There are certain desirable properties which are expected to exert by potential probiotic strain to justify its beneficial effects. Some of them which can be determined by in-vitro tests are: (Amraii *et al.*, 2014; Hawaz, 2014; Kechagia *et al.*, 2013).

i) The organism must be fully identified: genus, species and strain
ii) It must be safe for consumption:
    - Not pathogenic or carrying antibiotic resistance genes
    - Not degrading to intestinal mucosa or conjugating for bile acids
iii) It must survive intestinal transit: acid and bile tolerant
iv) It must adhere to mucosal surface and colonize the intestine (at least briefly)
v) It must possess documented health effects:
    - Produce antimicrobial substances and antagonize pathogenic bacteria
    - At least one phase 2 study documenting benefit
vi) It must be stable during processing and storage

2.4.1 General characteristic

2.4.1.1 Source

Application of probiotics greatly determines the source of probiotics. It should be originated from targeted animal micro flora. The probiotic source may be food like raw milk or fermented food, animal origin, human origin like human large intestine, small intestine or breast milk. For micro flora to be safer for human purpose, probiotic strains should be isolated from human micro floras which are more likely to adhere to human intestinal wall than others. It has also been reported that the bacterial composition of breast milk reflects the infant micro flora. Therefore, the natural microbiota of human milk could be proposed as a source for the isolation of novel probiotic bacteria. The strain should be properly isolated and identified before use (Shewale *et al.*, 2014).

2.4.1.2 Strain identification

According to Erkus (2007), most commonly used characteristics for the identification of bacteria are morphology, staining reactions, nutritional requirements, cell wall chemistry, ability to use different energy sources, fermentation byproducts, gas requirements, temperature and pH tolerance, antibiotic sensitivity, pathogenicity, immunological characteristics and habitat.

Phenotypic characteristics include colony appearance, cell morphology, Gram staining, growth at different temperatures (8–65°C), pH (3.9–9.6), and salt tolerance (4.0–18%). Biochemical tests are based on the metabolic activities of microorganisms such as carbon and nitrogen sources, energy sources, sugar fermentation, secondary metabolite formation, and enzyme production (Tamang *et al.*, 2015).

2.4.1.3 Biosafety of microbial strain

European Food Safety Authority (EFSA) listed character of *Lactobacillus* species, *Bifidobacterium* species and *Streptococus* (*Enterococcus*), *Lactococcus lactic* and *Leuconostoc sp* species which should be following Qualified Presumptions of Safety (QPS). Other than these selected strains should be nonpathogenic and nontoxic. Before selecting other probiotics, toxicological studies must be performed. Soil based organisms and spores claimed as probiotics. Generally the Lactic acid bacteria have good record in safety (Kosin and Rakshit, 2006).

2.4.2 Functional characteristic

2.4.2.1 Resistant to gastric condition

Gastric juice is a crucial barrier to most pathogens; it displays large variability in characteristics under different conditions, for example during the fed and fasted states. Under fasted conditions, the pH in a healthy human stomach is acidic, generally ranging between 1 and 3. After food, the stomach environment varies considerably over the course of gastric residence of the meal (Mudie *et al.*, 2010; Sahadeva *et al.*, 2011).

The survival of ingested probiotics in different parts of gastrointestinal tract varies with the strain. Some strains are rapidly killed in the stomach while others, such as strains of *Bifidobacteria* or *L. acidophilus*, can pass through the whole gut at very high concentration. Strains of *Lactococcus lactics*, *Strptococcus thermophillus* and *Leeuconostoc sp* are more acid tolerant in the food career matrix (Marteau *et al.*, 1997; Mater *et al.*, 2005; Silva *et al.*, 2017; Watson *et al.*, 2008) On the other hand, food matrix determines their pH tolerance. Survival of these organisms in the acidic environment has also been enhanced in presence of the metabolized sugar that allow the cell membrane proton pumps to operate and prevent the lowering of intracellular pH. The *bifidobacteria* however proved less acid resistance than the lactobacilli, particularly when exposed to the human gastric juice (Kalantzi *et al.*, 2006).

2.4.2.2 Resistance to the bile acid

Apart from its normal physiological function, bile is highly toxic for those microorganisms un-adapted to the intestinal conditions. Therefore, enteric bacteria, including *Lactobacilli* and *Bifidobacteria*, *Streptococcus thermophillus* must have evolved specific defense mechanisms to resist the deleterious action caused by these compounds (Mater *et al.*, 2005). The strong lipophilic nature of the steroid ring makes the cell membrane the main target of these molecules, in which they disturb the lipid packaging and disrupt the proton motive force, causing cell death (Burns *et al.*, 2010). Although intrinsic bile tolerance appear to be strain-dependent, both *Lactococcus* and *leuconostoc* can progressively adapt to the presence of bile salts, and resistant derivatives has been obtained from sensitive wild type strains by sub culturing in gradually increasing concentrations of bile (Kumar *et al.*, 2006; Ruiz *et al.*, 2011; Silva *et al.*, 2017).

Overall, bile response is a multifactorial phenomenon, implicating a variety of processes addressed toward detoxification of bile counteracting the deleterious effect on bacterial

structures. Active efflux of bile acids/salts, bile salt hydrolysis, and changes in the architecture/composition of cell membrane and cell wall appear to be the most prevalent bile-specific mechanisms mediating resistance in both genera (Pfeiler and Klaenhammer, 2009). In addition, general stress response, protection against oxidative damages, as well as global glycolytic reorganizations are other common consequences of bile exposure, that might be employed to counteract some of the cellular damage caused by these compounds (Hamon *et al.*, 2011; Ruiz *et al.*, 2011).

2.4.2.3 Adherence and colonization of intestinal epithelium/ tissues

Adhesion ability to the host is a classical selection criterion for potential probiotic bacteria that could result in a transient colonization that would help to promote immunomodulatory effects, as well as stimulate gut barrier and metabolic functions (Haddaji *et al.*, 2015b). Since the GIT is a dynamic environment, the flow of the gut digesta may wash out any bacterium not attached to the intestinal mucosa. Thus, probiotic strains with adherent ability are more likely to have an increased opportunity to colonize the GIT (Shokryazdan *et al.*, 2014).

Bacterial adhesion to intestinal surfaces could be driven initially by non-specific physical binding as hydrophobic interactions followed by a second stage of adhesion by specific cell wall components (Haddaji *et al.*, 2015b). Some researchers have reported a correlation between hydrophobicity and adhesion (Pan *et al.*, 2006). In this regard, the presence of some surface proteins such as cell wall–anchored proteinases have been shown to enhance hydrophobicity and adhesion in some lactic acid bacteria (Zhang *et al.*, 2013). It was reported that LAB strains possessing hydrophobic cell surface and aggregation capacity were more capable to adhere to the intestinal cells to perform beneficial effects (Guan *et al.*, 2020).

2.4.2.4 Antimicrobial property

Antimicrobial properties against potentially pathogenic bacteria and capability of producing antimicrobial substances are the most important in developing probiotic supplement and probiotic rich foods. Several metabolic compounds (including organic acids, fatty acids, hydrogen peroxide and diacetyl) produced by lactic acid bacteria have antimicrobial activity. Production of lactic acid also inhibits the growth of other microorganisms and allow them to be established in the intestinal tract (Shewale *et al.*, 2014).

Lactobacillus lactis has actively shown production of bacteriocin which is also approved for use in product intended to human (Nehal *et al.*, 2019). *Stereptococcus salivarius* has also been proven to have antimicrobial activity against bacteria involved in halitosis (Masdea *et al.*, 2012). Strains of *Lactococcus lactis* produce exopolysaccharides when produced from camel milk. Also, *Leuconostoc gelidum* was evaluated for its ability to inhibit a wide spectrum of lactic acid bacteria including meat spoilage bacteria. Lactic acid bacterium was inhibitory to most of lactobacilli, all the Leuconostocs and their strains of *Listeria monocytonenes* (Harding and Shaw, 1990). Apart from nacin, other probiotic strains also produce a various kind of bacteriocins which has an adverse effect on a various pathogenic organisms for eg: Lactoccin, Salivaricin, Acidocin 8992, Lantaricin, Lacticin A (Pisano *et al.*, 2015).

2.4.2.5 Resistant to antibiotic

Resistance to antibiotics is one of the selection criteria for probiotic microorganisms (Hawaz, 2014; Niazi Amraii *et al.*, 2014). The determination of antimicrobial susceptibility of a bacterial strain is an important prerequisite for its approval as a probiotic. Some authors claim that in cases of co-administration with antibiotics to prevent and treat intestinal disorders, probiotics should be resistant to certain antibiotics in order to survive in the gastrointestinal tract. However, this opinion is controversial. Probiotics containing resistance traits may have negative consequences to human health. Risks relating to potential transfer of antibiotic resistance from probiotic strains to intestinal pathogens are a concern (Moubareck *et al.*, 2005). For this reason, the presence of acquired antimicrobial resistances is one of the first safety criteria to be checked during the selection process of a potentially probiotic strain (Masco *et al.*, 2006).

Bacteria used as probiotics or in starter cultures may serve as hosts of antibiotic resistance genes, which can be transferred to pathogenic bacteria. Before launching a starter culture or a probiotic product into the market, it is therefore important to verify that the single bacterial isolates (strains) do not contain transferable resistance genes (Danielsen and Wind, 2003)

2.4.3 Basic health characteristic of probiotic

The selection of the probiotic organisms depends upon a health claim. Probiotic must be able to exert their benefits on the host through the growth and /or activity in the human body. Most proven probiotics strains are human origin, a strong case can be made that they are normal commensals and, therefore, safe to use. To achieve the health benefits, probiotic bacteria must

be viable and available at high concentration, typically 10^5 to 10^7 CFU/g of product (Shewale *et al.*, 2014). There is a need for refinement in vitro tests to predict the ability of probiotics to give health benefit to human being. They should provide benefit against gastroenteritis, irritable bowel syndrome, and Inflammatory Bowel Disease (IBD; Crohn's disease and ulcerative colitis), diarrhea, cancer, depressed immune function, inadequate lactose digestion, infant allergies, failure-to-thrive, hyperlipidaemia, hepatic diseases and helicobacter pylori infections (Orel and Kamhi Trop, 2014).

2.4.4 Production characteristic

2.4.4.1 Acid production

The rate of acid development is a critical criterion for the selection of the probiotics in milk-fermented products. A rapid acid production in the raw material not only helps to prevent the growth of unwanted microorganisms, but is also essential for the aroma, texture, and flavor of the end product, which has positive influence on its overall acceptability in the form of pH, texture, flavor, and aroma of the product (Hussain *et al*., 2008).

2.4.4.2 Proteolysis

Proteolysis (Casein hydrolysis) contributes to the texture, flavor and body development in the end product like different varieties of chesses and yogurts. Hence, this criterion is an important consideration in selection of the above products. The ability to produce cell wall bound Extra Cellular Proteinases (ECP) is very important feature of LAB in the hydrolysis of milk proteins (casein) which supply amino acids to the cells that are essential for growth of LAB. Protein degradation is mainly associated with *Lactobacillus bulgaricus* and *Streptococcus thermophillus* (Kosin and Rakshit, 2006).

2.4.5 Probiotic stability and viability

Probiotic must have the capabilities for its survival in the food, feed and dietary supplements. Manufacturer has given a great attention to probiotic stability. More importantly, the probiotics strain should be stable enough to withstand a conventional industrial production process. Stability is also a strain specific (Salminen and Wright, 1998).

2.5 Indigenous methods of milk fermentation

Traditional and indigenous foods are the integral components of traditional and indigenous foods system of the country. These foods are densely nutritious and have a long history of supporting health and wellness (Durust and Bayasgalanbat, 2014). Traditionally fermented foods are mostly specific to certain geographic regions and also to particular communities. The major fermented traditional foods and beverages of Nepal include *Masyaura*, *Fulaura*, *Jilebi*, *Selroti*, *Kinema*, *Gundruk*, *Sinki*, *Khalpi*, *Mesu*, *Chhurpi*, *Dahi*, *Mahi*, *Ghiu*, *Jandh*, and *Rakshi* (Dahal *et al.*, 2005).

Fermented foods are prevalent in Southeast Asia to balance the fluctuation in food availability in the area during the stage of monsoonal circulation (Law *et al.*, 2011). The fermentation techniques may vary from place to place but a key element influencing the quality of the fermented product is the fermentation vessel. These vessels are usually simple and are made from locally available materials such as woven grass, wood fiber, earthenware pots, wood or animal skin bags (FAO, 1990).

In ancient days, human beings realized that fermentation occurred, without, however, knowing their cause. Originally milk fermented spontaneously, and the reuse of fermentation vessels and tools contributed to a certain repeatability and stability in the fermentation process. This led to the use of specific microorganisms for the manufacture of more or less refined products. Different countries or even different parts of the same country developed their own fermented milks (Anagnostopoulos *et al.*, 2019). The quality and safety of traditional indigenous fermented foods can be improved by selecting microbial strains with various functional properties (Mathara *et al.*, 2004).

2.6 *Dahi*

Curd, also known as commercially prepared yoghurt or homemade *Dahi*, is formed during the slow lactic fermentation of lactose from milk by thermophilic LAB (Mahato and Shahani, 2019). It is prepared from boiled milk, fermented in a traditional way by natural microflora (Bhattarai *et al.*, 2016). *Dahi* has been reported to contain a mixture of LAB in addition to *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mostly used cultures for yoghurt making (Maqsood *et al.*, 2013). Use of standard cultures is still not in practice. Instead, people use the "back-slopping" technique i.e.; using previous fermented product as source of the inoculum (Dewan and Tamang, 2007). The term *dahi* might have been derived as originated in

the different languages spoken in the country as depicted as *argha* in Sanskrit and as *dahi* in Nepali (Bhattarai and Das, 2013).

Dahi is a fermented dairy product popular throughout South Asia where it constituents a significant part of the daily diet (Balamurugan *et al.*, 2014). However, the flavor and body of *dahi* varies widely in different parts of the subcontinent. It may be made from cow milk or buffalo milk or their combination. Buffalo milk, being high in solids content, yields a very firm product, while cow-milk *dahi* is a relatively softer product. In terms of flavor, in certain regions a mildly acidic, yeasty-sweet product is desired. In others, a more acidic *dahi* is preferred. During the production of *dahi* in households and the cottage industry, "back-slopping" is practiced. It refers to the practice of using a small remnant of the previous day product to inoculate a fresh batch (Vedamuthu, 2013).

According to PFA rules (2006), *dahi* can be defined as: "It is the product obtained from pasteurized or boiled milk by souring, natural or otherwise, by harmless lactic acid or other bacterial culture. *Dahi* may contain added cane sugar. *Dahi* shall have the same minimum percent of milk fat and MSNF as the milk from which it is prepared. Where *dahi* is sold or offered for sale without any indication of the class of milk, the standards prescribed for *dahi* prepared from buffalo milk shall apply. Milk solids may also be used in the preparation of this product".

2.7 *Theki dahi*

Theki is a close-necked wooden vessel carved out of wood like *daar* (*Boehmeria rugulosa*). *Boehmeria rugulosa* Wedd. (Urticaceae) is an evergreen tree, distributed in sub montane to montane Himalaya, Himachal to Bhutan, and Myanmar (Semwal *et al.*, 2009). Mostly people from the Himalayan region of Nepal traditionally use *theki* to prepare *dahi* (Rai *et al*., 2016). The main purpose of using *daar theki* is to give flavorful *dahi* and to serve as natural microflora reservoir (Bhattarai *et al.*, 2016).

There is no strict definition of *dahi* as there is for yoghurt. *Dahi* contains various strains of LAB. For *dahi* fermentation, a small portion of product containing microbes of a previous fermentation (back slopping) is generally added to milk (Mitra *et al*., 2010). One night fermented *dahi* in *theki* kept near fireplace or covered with cloth gives best quality *dahi.* It does not require to previous day culture to carry out fermentation (Bhattarai and Das, 2013; Yadav *et al.*, 2006).

1. If *theki* is new, 5-8 g of pervious day *dahi* must be added.
2. If *theki* is new, *dahi* is allowed to go waste for at least 4-5 times until natural and good microflora is obtained

2.7.1 *Theki*

It is a close-necked wooden vessel carved out of wood like *daar* (*Boehmeria rugulosa*). *Boehmeria rugulosa* Wedd. (*Urticaceae*) is an evergreen tree, distributed in sub montane to montane Himalaya, Himachal to Bhutan, and Myanmar (Semwal *et al.*, 2009). They have sack shaped body constricted neck and wide rim. It can be called *theki* (for wooden pots) and *theko* (small wodden pots) (Pradhan, 2017). In 1975 by Judy Birmingham, *theki* is cited as very useful utensil for variety of uses in milk, he also suggested they might have black patches which are the result of the burning the wood during the course of the preparation of the vessel. A representative image of *theki daar* is shown in Fig. 2.1.

Theki is widely used to ferment *dahi*. But, it has also been as a storage container for grains like finger millet or oilseed like mustard or sesame (Sthapit *et al.*, 2001). Mostly, people from mountains and hills use *theki* for fermentation. One-night fermented *dahi* in *theki* kept near fireplace or covered with cloth gives best quality *dahi*. It does not require to previous day culture to carry out fermentation (Bhattarai and Das, 2013). The main purpose of using *daar theki* is to give flavorful *dahi* and to serve as natural microflora reservoir (Bhattarai *et al.*, 2016).

Fig. 2.1 *Theki* made of daar

2.8 Starter culture of milk fermentation

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. LAB are majorly used for fermentation of foods and beverages (Leroy and De Vuyst, 2004). According to FSSAI, India (2006), yoghurt is a symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Erkus, 2007). The symbiotic relationship between this mixed cultures during milk acidification may result in greater bacterial concentration, acid production, flavor development and improved texture, than the use of single culture growth (Pourahmad and Assadi, 2007).

When milk is inoculated with these organisms, they grow in a suspension. Firstly, *Streptococcus thermophilus* grows exponentially (for first 90 to 120 min) while the *Lactobacillus delbrueckii* remains in inoculation stage. After certain time, growth rate of *Streptococcus thermophilus* reduces while *Lactobacillus delbrueckii* starts to grow exponentially. As the milk becomes acidic with a pH of 5.2, *Streptococcus thermophilus* stops growing. On the contrast, *Lactobacillus delbrueckii* continues to grow until the pH drop to 4.4 (Walstra *et al.*, 2006). Moreover, *Lactobacillus delbrueckii* produces small number of peptides and amino acids mainly valine which supports the growth of *Streptococcus thermophilus.* On the other hand, *Streptococcus thermophilus* enhances the growth of the *Lactobacillus delbrueckii* subsp. *bulgaricus* by forming formic acid out of pyruvic acid under anaerobic condition and by rapid production of carbon dioxide (Walstra *et al.*, 2006).

2.8.1 Role of starter culture in *dahi* production

Yoghurt is produced by the symbiotic activity of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* cultures (Aswal *et al.*, 2012). *Streptococcus thermophilus* produces pruvic acid, formic acid, folic acid which reduces the p^H of milk to an optimum level for growth of *Lactobacillus delbrueckii.* At the same time, growth of *Lactobacillus delbrueckii* provides peptides, free amino acids and putrescine that simulate growth of *Streptococcus thermophilus* (Dabija *et al*., 2018).

A variety of volatile organic aroma compounds are produced by *Streptococcus thermophilus* and *lactobacillus delbrueckii* like carbonyl compounds, acetaldehyde, diacetyl and the volatile fatty acids C_3-C_{10}. Lactic acid and volatile fatty acids are the major compounds contributing to the aroma and flavor of yoghurt (Beshkova *et al.*, 1998).

Acid production by LAB results in aggregation of casein molecules due to hydrophobic and electrostatic (+, - charges) interaction as the pH of milk approaches the isoelectric point (pH 4.6). The production of EPS by starter organism is considered to help increase the viscosity of yoghurt and reduce whey separation (Leucy, 2004).

Starter culture also improves nutritional value and digestibility of yoghurt. *Streptococcus thermophilus* enhances lactose digestion of lactose intolerant people. The viable cells of *Streptococcus thermophilus* survive in the stomach and are lysed in the gastrointestinal tract. The intracellular β-galactosidases are released and hydrolyze lactose. Lactose does not reach the large intestine and the symptoms of lactose intolerance does not occur (Erkus, 2007).

Different anti-microbial such as, lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins produced by LAB can inhibit the spoilage and pathogenic microorganisms present in milk thus extending the shelf life and enhancing the food safety of the product. Moreover, the bacteriocins produced by *lactobacillus delbrueckii* ssp. *bulgaricus* had an anti-bacterial potential against some food borne pathogen and spoilage microorganisms like *Vibrio cholera* and *E. Coli*. Thus, they have a preservative effect on the product (Tufail *et al.*, 2011).

2.9 Lactic acid bacteria associated with traditional fermented milk

Lactic acid bacteria that are widely distributed in the nature and occurring naturally as indigenous microflora in raw milk are Gram-positive bacteria that play an important role in many food and feed fermentations (Guessas and Kihal, 2004)**.** The most frequently isolated LAB genera from raw milk and dairy products were *Lactobacillus, Leuconostoc, Streptococcus, Lactococcus* and *Enterococcus* (Karkas-sen and Karkas, 2018). These strains are widely used as starter culture due to their good viability and ability to lower the pH of fermented foods (Akbar *et al.*, 2019). *Dahi* has been reported to contain a mixture of LAB in addition to *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mostly used cultures for yoghurt making (Maqsood *et al.*, 2013). For many centuries, LAB has been an effective form of preservation. In addition, they strongly determine the flavor, texture and also the nutritive value of food and feed products (Azadina *et al.*, 2011).

In the study conducted in indigenous *dahi* from Nepal Himalayas, *Lactobacillus fermentum, Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroids* subsp. *mesenteroides. S. thermophilus* was found as the dominant lactic acid

bacteria (Bhattarai *et al.*, 2016)**.** Additionally, LAB found in *dahi* has been concluded to have the potential to exert probiotic effects in the individual consumer. These microbes also have properties that predicts their ability to survive through upper gut and to modulate gut mucosal innate immune reactions (Balamurugan *et al.*, 2014).

2.10 Calculation of growth parameters by optical density measurement

Microbiology requires a large quantity of data when the growth of one or several strains is studied as a function of different conditions, such as temperature, pH or water activity. Plating methods have been widely used since the early days of microbiology, but they do not give immediate results and are time-consuming, especially when a large quantity of data is required. Turbidimetry is an established method used to study bacterial growth since Optical Density (O.D.) measurements make it possible to follow bacterial population growth in real time. The advent of automated instruments has recently prompted microbiologists to use O.D. measurements. Some authors have attempted to derive growth parameters from O.D. measurements (Begot *et al.*, 1996)**.**

Before turbidimetric measurements can be made, the spectrophotometer must be adjusted to 100% transmittance (0% absorbance). This is done using a sample of uninoculated medium. Percent transmittance of various dilutions of the bacterial culture is then measured and the values converted to optical density. A wavelength of 420 nm is used when the solution is clear, 540 nm when the solution is light yellow, and 600-625 nm is used for yellow to brown solutions (Sutton, 2011).

2.11 Purposive sampling for qualitative data collection

Sampling strategies for quantitative methods used in mixed methods designs in implementation research are generally well-established and based on probability theory. In contrast, sampling strategies for qualitative methods in implementation studies are less explicit and often less evident. Although the samples for qualitative inquiry are generally assumed to be selected purposefully to yield cases that are “information rich” (Patton, 2015).

There are no clear guidelines for conducting purposeful sampling in mixed methods implementation studies, particularly when studies have more than one specific objective. Moreover, it is not entirely clear what forms of purposeful sampling are most appropriate for the challenges of using both quantitative and qualitative methods in the mixed methods designs

used in implementation research. Such a consideration requires a determination of the objectives of each methodology and the potential impact of selecting one strategy to achieve one objective on the selection of other strategies to achieve additional objectives (Palinkas *et al.*, 2013).

2.12 Pool sampling

The tests included in the food businesses' internal control for food safety are done by commercial laboratories. The food legislation allows FBOs to choose their own procedures to fulfil the legal requirements (Katona, 2014). Pooling of samples is an approach that has increasingly been considered for compliance with the microbiological criteria. Pooling reduces both the work load and costs when testing many samples. There are two ways of sample compositing (pooling) described: dry-pooling, which involves the composition of dry sample units, and wet-pooling, which refers to the pooling of pre-enriched samples ((ISO), 2002).

According to the analytical method ISO 6579/2002, pooling of up to ten samples is allowed as long as there is evidence of no negative effect on sensitivity. However, the validity of pooling has not yet been established and that infers uncertainty of whether pooling can be used for compliance with the regulation of microbiological criteria. Since pooling may impair detection of low numbers of microorganisms, the International Commission on microbiological specification of foodstuffs has urged that pooling should be approached with caution and should be validated for each specific combination of food and pathogen (Jarvis, 2007).

Part III

Materials and methods

3.1 Materials

3.1.1 *Theki dahi*

A total of 48 samples of the *theki dahi* were collected from different places around Pokhara valley. The samples were collected during the morning and were brought to refrigeration temperature within 3 h of collection. Sampling was done on the purposive sampling method, the main reason behind this method was to produce a sample that can be logically assumed to be representative sample of the population. The main criterion of sample selection was the people who often or regularly prepare *theki dahi*.

The samples were safely collected in sterile container and safely brought to the laboratory of Pokhara Bigyan tatha Prabidhi Campus, Pokhara. 10 g of each sample was collected and pooled separately to yield 500 g of final sample. Then, they were immediately stored in a sterile container and kept in refrigerator where they were stored at <4 ^{o}C for further use.

3.1.2 Probiotic strain of *Lactobacillus casei* sub sp. Shirota

Probiotic strain of *Lactobacillus casei* was isolated from Yakult manufactured by Yakult Danone India Private Limited which was transported to Nepal by Flipkart through E-Kart logistics. One yakult bottle was of 65 ml containing pure culture of *Lactobacillus casei* sub sp. Shirota with a total shelf life of only 1.5 months.

3.1.3 Laboratory materials

All of the materials required were collected with the help of Pokhara Bigyan Tatha Prabihi Campus (PBPC). The list of chemicals used for the research and the list of instruments is mentioned in appendix A.

3.1.4 Microbiological media

All microbiological media required were used from Pokhara Bigyan Tatha Prabidhi Campus. All the media used in the research is shown in Table 3.1 and the list of ingredients used in formulating SL differential media and Differential media is shown in Table 3.2

Table 3.1 List of microbiological media

Microbiological media	Microbiological media
MRS agar (Himedia Laboratories, Mumbai India)	Mannitol salt agar (Himedia Laboratories, Mumbai India)
M-17 agar (Himedia Laboratories, Mumbai India)	Arginine broth (Himedia Laboratories, Mumbai India)
SL differential agar	Simmon's citrate agar (Himedia Laboratories, Mumbai India)
Differential agar	LcS select medium

Table 3.2 Ingredients for SL differential and differential agar

Media	Ingredients
SL differential agar	1% w/v nonfat milk
	0.25% Milk protein hydrolysate peptone
	0.5% Dextrose
	1.5% Agar
	10% potassium ferricyanide
	Ferric citrate
	Sodium citrate
Differential media for *S. cremoris* and *S. lactics*	0.5% Tryptone
	0.5% Yeast extract
	0.4% L-arginine hydrochloride

	0.1% K_2HPO_4
	0.3% $CaCO_3$
	0.6% Carboxy Methyl Cellulose
	1.5% Agar
	Reconstituted nonfat dry milk
	Bromocresol purple
	Carboxy Methyl cellulose

3.2 Methods

3.2.1 Isolation and characterization of microbes

Ten g of the collected *theki dahi* sample was homogenized with 90 ml of 0.1% (w/v) sterile peptone water to obtain 1:10 (10^{-1}) dilution. Successive decimal dilutions were carried out with 0.1% (w/v) sterile peptone water.

3.2.1.1 Isolation of lactobacilli

One ml of the aliquot from various dilutions was spread plated in duplicate on MRS agar (Badis *et al.*, 2004). The plates were then incubated anaerobically using gas pack system at 37^{o}C for 24-48 h (Goyal *et al.*, 2012).

Based on the morphological property, colonies were selected from the agar plates but if the plate contained less than 4 colonies, all the colonies were isolated. The isolated colonies were streaked and sub-cultured on the fresh agar plates (MRS) followed by microbial examination to obtain pure culture. For short term preservation, the isolated agar plates (MRS) were kept at 4^{o}C. For long term preservation, the isolates were stored in MRS agar supplemented with 20% glycerol (below freezing condition) as cryoprotectant (El Kahlout *et al.*, 2018).

3.2.1.2 Isolation of *Bifidobacterium* sp.

Firstly, 0.1 ml of sample was spread on MRS agar containing 0.25% L-cysteine and incubated at 37°C for 48 h in an anaerobic chamber. The colonies obtained were sub-cultured on other plates to obtain pure cultures (Mishra *et al.*, 2012; Zinedine and Faid, 2007). Further selection, isolation and preservation of isolates were performed as described in sub section 3.2.1.1.

3.2.1.3 Isolation of *Streptococcus thermophilus*

Aliquot of 0.1 ml from various dilutions (10^{-3}, 10^{-4} and 10^{-5}) was spread plated in duplicate on M_{17} agar which was then incubated at 42ºC for 24 h (Giraffa *et al.*, 2001). To prevent yeast contamination, M_{17} agar plates were complemented with 100 mg/L of cycloheximide antibiotic (El Kahlout *et al.*, 2018). Further selection, isolation and preservation of isolates were conducted as described in sub section 3.2.1.1.

3.2.1.4 Isolation of *Leuconostoc*

Aliquot of 0.1 ml from various dilutions (10^{-3}, 10^{-4} and 10^{-5}) was spread plated in duplicate on MRS-vanomycin agar (Vanomycin 20 mg/L) which was then incubated at 30ºC for 24 h (Bhattarai *et al.*, 2016). Further selection, isolation and preservation of isolates were carried out as described in sub section 3.2.1.1.

3.2.1.5 Isolation of lactococci

Aliquot of 0.1 ml from various dilutions (10^{-3}, 10^{-4} and 10^{-5}) was spread plated in duplicate on;

1. *Streptocoocus lactis* differential agar plate (SL) and incubated anaerobically using gas pack system for 48 h to differentiate citrate utilizing and non-utilizing Lactococci (Kempler and McKay, 1980).
2. Differential agar medium (D) plate for differentiating *Streptococcus lactis* and *Streptococcus cremoris* based on their ability to hydrolase arginine, which was incubated at 32ºC for 48 h (Reddy *et al.*, 1969).

Further selection, isolation and preservation of isolates were done as described in sub section 3.2.1.1.

3.2.1.6 Isolation of *Lactobacilli casei* sub sp. Shirota

Firstly, LcS select medium was prepared by dissolving 62 g of MRS agar and 0.5 g L-cysteine hydrochloride (final concentration, 0.05%) in 1 L of distilled water. The pH of this medium was then adjusted to 6.2±0.2 with 1.0 M HCl. Bromophenol blue (0.02 g/L) was then dissolved in 1.0 M NaOH and then added to the medium (Final concentration, 0.002%) and mixed thoroughly. The medium was finally sterilized at 121^{o}C for 15 min. Lastly, sterilized vancomycin (10 µg/L, final concentration 1%) was added to the cooling agar (Sutula *et al.*, 2012).

0.1 ml of aliquot from various dilutions (10^{-3}, 10^{-4} and 10^{-5}) was spread plated in duplicate on LcS select medium which was then incubated anaerobically at 37^{o}C for 72 h (Sutula *et al.*, 2012). Further selection, isolation and preservation of isolates were performed as described in sub section 3.2.1.1.

3.2.2 Identification of the isolated colonies

All the preserved isolated colonies were initially characterized on the basis of their morphology and biochemical tests. All the strains were subjected to gram staining, catalase test and spore formation.

Further identification was carried out by performing following tests;

1. Growth at various temperature (10^{o}C, 37^{o}C and 45^{o}C) for 5 days
2. Growth in the presence of 4% and 6.5% (w/v) NaCl
3. Heat resistance at 60^{o}C for 30 min (Sherman test)
4. Production of CO_2 from glucose
5. Hydrolysis of arginine
6. Citrate utilization
7. Sugar fermentation test

3.2.2.1 Growth at various temperature (10^{o}C, 30^{o}C and 45^{o}C)

The isolated organisms were grown at various temperatures (10, 30 and 45^{o}C) to determine whether the isolated microbe can resist high temperature or not and to detect their optimal growth temperature for best growth (Khanal and Koirala, 2019).

Actively growing isolated bacteria (1.0 ml) in their respective medium broth at their optimum condition were sub cultured to 10 ml of their respective broth which was then incubated at 10, 30 and 45ºC for 5 days. After 5 days, each sample was streaked on their own individual agar plate and incubated at their optimum temperature to determine the presence or absence of growth. Furthermore, OD of the broth was recorded at 620 nm by using a spectrophotometer. The growth index was then determined by comparing the OD of test sample with that of control sample grown at optimum temperature. The experiment was carried out in triplicate and the average OD was used to acquire a bar diagram comparing growth of each organism at different temperatures (Goswami *et al.*, 2017; Menconi *et al.*, 2014; Sharma *et al.*, 2021)

$$\text{Growth index} = \frac{\text{OD at 620 nm of test sample}}{\text{OD at 620 nm of control sample}} \times 100\%$$

3.2.2.2 Growth in the presence of 4.0% and 6.5% (w/v) NaCl

The isolated organisms were grown at various NaCl concentrations (4.0 and 6.5% w/v) to determine whether that microbe can grow in halophilic conditions or not and to detect their optimal growth at different NaCl concentration for best growth (Khanal and Koirala, 2019).

Actively growing isolated bacteria (1.0 ml) in their respective medium broth at their optimum condition were sub cultured to 10 ml of respective broth supplemented with 4.0% (w/v) and 6.5% (w/v) NaCl. These broths were then incubated at their optimum condition. After that, each sample was streaked on their own individual agar plate and incubated at their optimum condition to determine the presence or absence of growth. Furthermore, OD of the broth was recorded at 620 nm by using a spectrophotometer. The growth index was then determined by comparing the OD of test sample with that of control sample. The experiment was carried out in triplicate and the average OD was used to acquire a bar diagram comparing growth of microbes at different NaCl concentration (Goswami *et al.*, 2017; Menconi *et al.*, 2014; Sharma *et al.*, 2021).

$$\text{Growth index} = \frac{\text{OD at 620 nm of test sample (4.0\% and 6.5\% NaCl)}}{\text{OD at 620 nm of control sample (without NaCl)}} \times 100\%$$

3.2.2.3 Hydrolysis of arginine

The isolated bacterial culture was incubated in arginine broth with few drops of Nessler reagent for 24-48 h. A brown coloration of the medium indicated hydrolysis of arginine by the inoculated bacteria (Mehmood *et al.*, 2009).

3.2.2.4 Citrate utilization test

A light inoculum was pricked from the center of a well-isolated bacterial colony which was then streaked back and forth on the slant of Simmon's citrate agar. The test tube was then incubated aerobically at 35 to 37^{o}C for 4 to 7 days. Finally, color change from green to blue along the slant was observed (Aryal, 2019a).

3.2.2.5 Sugar fermentation test

Membrane (0.45 μm) filtered 1.0% (w/v) solutions of different sugars (glucose, fructose, lactose, galactose, sucrose, maltose, and mannitol) were used to study fermentation characteristics of the isolates. Nutrient broth (0.8%) with 1.0 ml phenol red was prepared. 5.0 ml of broth was kept in each test tube with a Durham tube and autoclaved at 121°C for 15 min. Sterilized test tubes with broth were mixed with 100 μL of sugar solutions. The freshly cultured purified colonies were inoculated into test tubes with specific sugar-containing broth and incubated at 37°C for 48 h. The positive test for sugar fermentation was indicated by a color change from red to yellow in the test tubes while gas production was noted in Durham tube (Mahato and Shahani, 2019).

3.2.2.6 Production of CO_2 from glucose

Gas production was noted in the durham tubes used for sugar fermentation test (Mahato and Shahani, 2019).

3.2.3 Test for probiotic potentiality of isolated organisms.

3.2.3.1 Acid resistance test

First, the pH of the broth medium was adjusted to pH of 2.0 and pH of 4.0 with hydrochloric acid (1.0 N). Then, the medium was inoculated with 1.0 ml of actively growing isolated bacteria in their respective medium broth at their optimum condition and was incubated anaerobically for 2 h at 37°C. In the next step, the cells were isolated using centrifugation

at 6000 rpm for 10 min and inoculated in broth cultivation medium (neutral pH). The optical absorption of the samples was accessed at 620 nm using spectrophotometer. Resistance percentage of strains to stomach acid was determined by comparing optical absorption of the samples with the control sample (pH = neutral). This test was repeated three times for each sample (Hoque *et al.*, 2010). A schematic flowchart of acid resistance test is shown in Fig. 3.1.

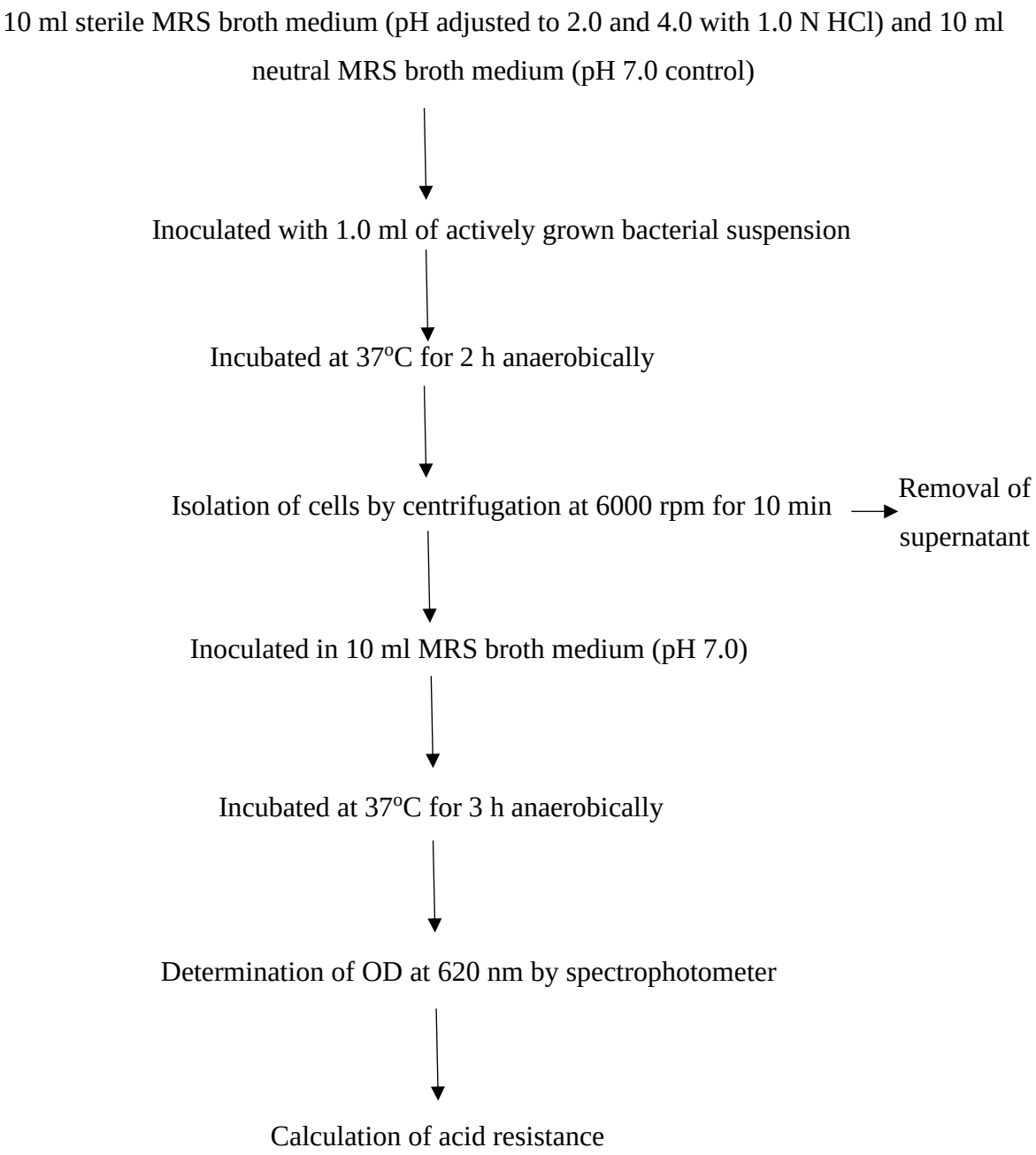

Fig. 3.1 Procedure for acid resistance test

$$\text{Acid Resistance \%} = \frac{\text{OD at 620 nm of sample (pH 2.0)}}{\text{OD at 620 nm of control sample (pH 7.0)}}$$

Source: Hoque *et al.* (2010)

3.2.3.2 Bile salt resistance test

The isolated strains were cultivated in medium containing 0.3% and 0.4% (w/v) bile salt. The level of strains resistance was determined by comparing optical absorption of the sample with the control sample (cultivation medium without bile salts). This test was repeated three times for each sample (Hoque *et al.*, 2010). A schematic flowchart for bile salt resistance test is shown in Fig. 3.2.

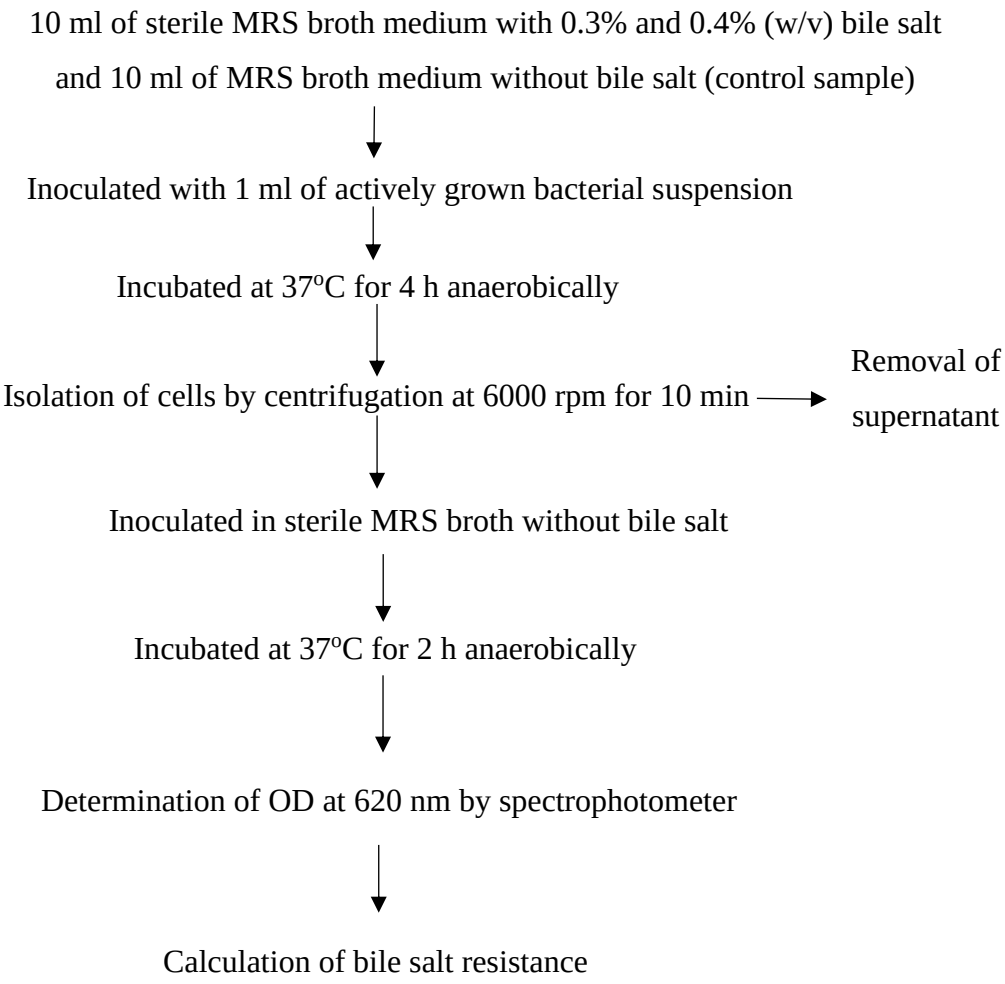

Fig. 3.2 Procedure for bile salt resistance

$$\text{Bile salt resistance \%} = \frac{\text{OD at 620 nm of sample with 0.3\% and 0.4\% bile salt}}{\text{OD at 620 nm of control sample without bile salt}} \times 100\%$$

Source: Hoque *et al.* (2010)

3.2.3.3 Bile salt hydrolysis test

For this test, the isolated strains were cultivated in medium containing 0.5% (w/v) bile salt. The medium was then incubated to allow dissolving. The incubation was performed for 48 to 72 h at 37°C in an incubator containing CO_2. Then, plates were examined for white precipitates. This white precipitate was a sign of bile salt hydrolysis (Hoque *et al.*, 2010). A schematic flowchart for bile salt hydrolysis test is shown in Fig. 3.3.

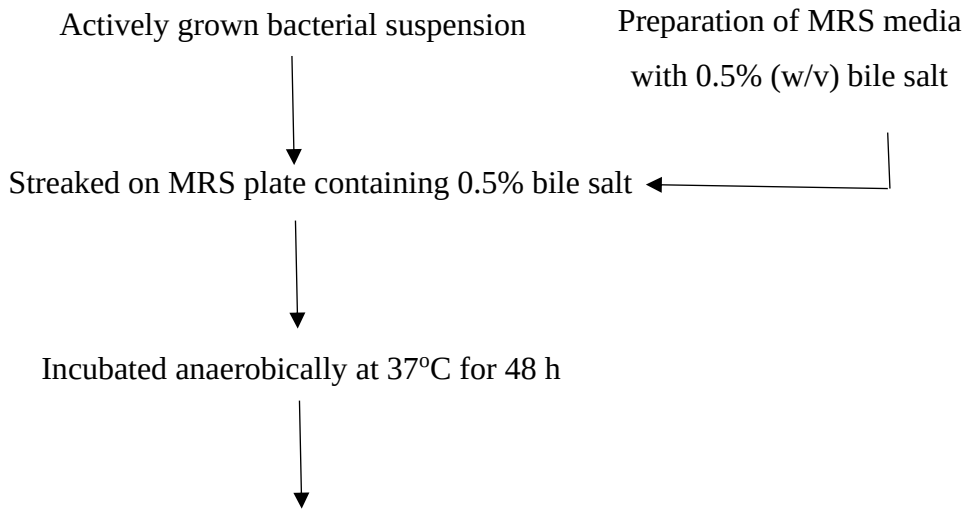

Fig. 3.3 Procedure for bile salt hydrolysis test

Source: Hoque *et al.* (2010)

3.2.3.4 Antibiotic resistance test

In order to determine antibiotic resistance, first 1 ml of actively growing bacteria in their respective medium broth at their optimum condition isolated from newly cultivated samples were added to warm sterile MRS agar. Then, a fixed amount of the medium was divided into plates. After the cultivation medium was made (i.e., became solid), antibiotic disks were placed at regular intervals on plates and incubated in a CO_2 incubator at 37°C. After 24 h, each plate were retrieved and measured using a metric rule for the zone of inhibition

(Liu and Pop, 2009). A schematic flowchart for antibiotic resistance test is shown in Fig. 3.4.

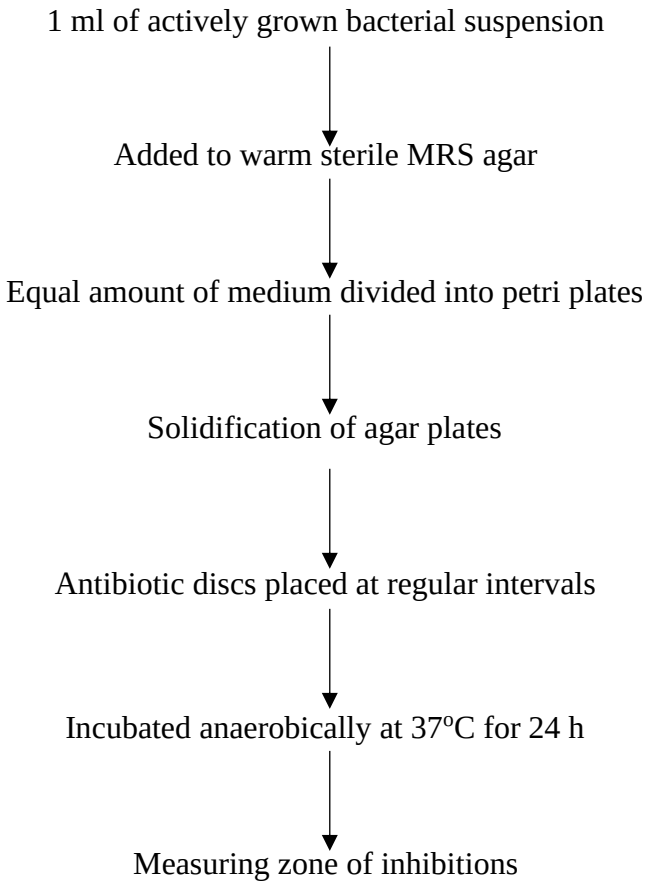

Fig. 3.4 Procedure for antibiotic resistance test

Source: Hoque *et al.* (2010)

3.2.3.5 Cell surface hydrophobicity test

The cell surface hydrophobicity of all the test strains was evaluated by measuring the bacterial cell adhesion to hydrocarbon. Firstly, the overnight grown bacterial cultures in MRS broth were harvested by centrifugation at 8,000 rpm for 10 min, washed twice with PBS and resuspended in the PBS buffer solution followed by absorbance (A_o) measurement at 600 nm. A cell suspension of about 3.0 ml was then mixed with 1.0 ml of hydrocarbon (hexane) and incubated at 37°C for 1 h for aqueous phase and organic phase separation. 1 ml of aqueous phase was removed carefully and the absorbance (A_1) was measured by spectrophotometer at 600 nm. The percentage hydrophobicity was measured by decrease in

the absorbance (Somashekaraiah *et al.*, 2019). A schematic flowchart for cell surface hydrophobicity is shown in Fig. 3.5.

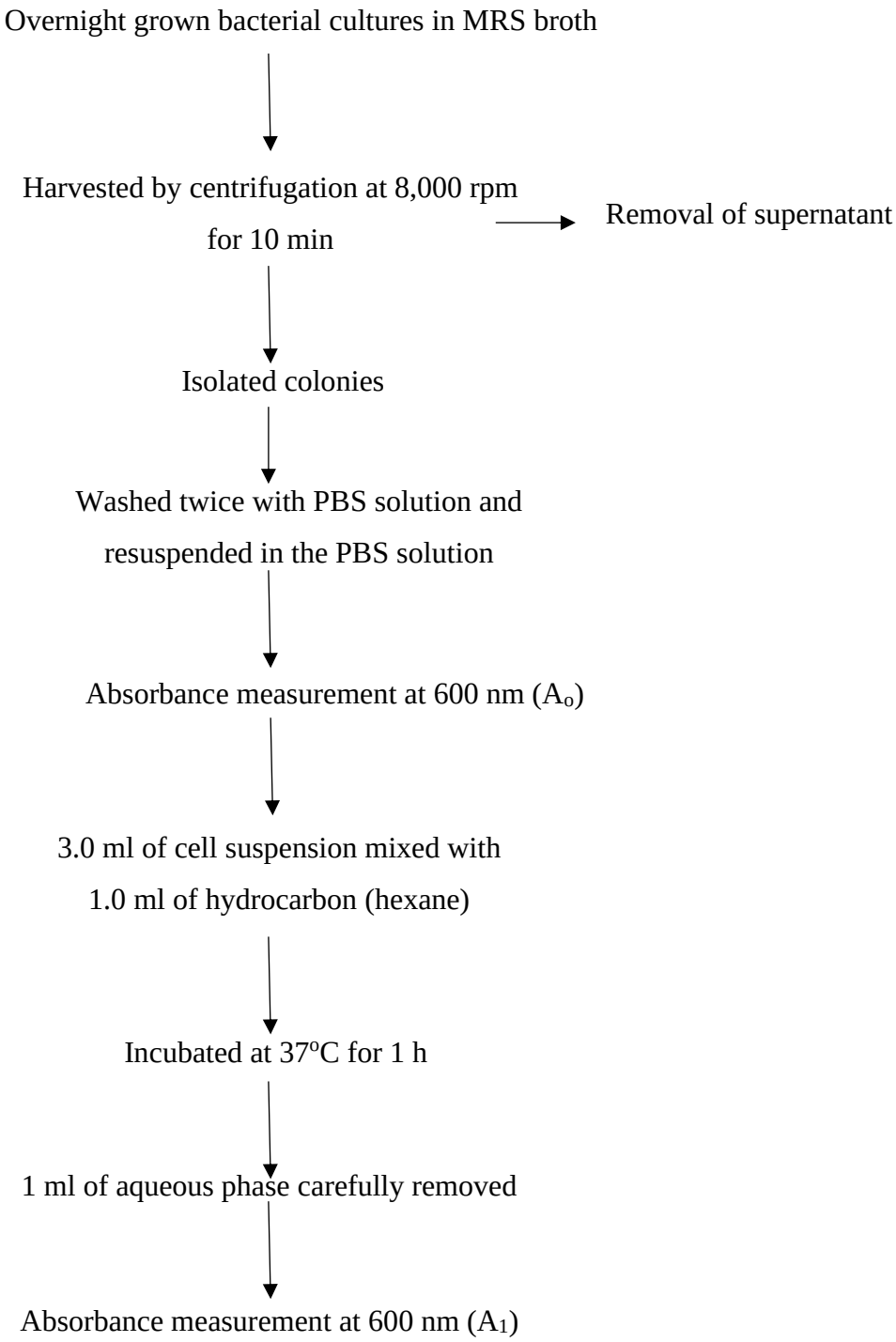

Fig. 3.5 Procedure for cell surface hydrophobicity test

$$\text{Cell surface hydrophobicity \%} = \left(1 - \frac{A_1}{A_0} \times 100\%\right)$$

Source: Somashekaraiah *et al.* (2019)

3.2.4 Data analysis

All the data obtained in this work was analyzed by the statistical program known as GenStat Release 12.1 and IBM-SPSS V-20. From this, mean and Tukey HSD at 5% level of significance was determined by use of ANOVA. Similarly, MS -Excel (2016) was used for data arrangement data management and general graphs and diagram construction.

Part IV

Results and discussion

Purposive sampling was done in order to collect 48 samples of *theki dahi* samples from different places of Pokhara Valley in a sterile container and finally brought safety to laboratory where it was stored under refrigeration. Finally, 10 g of each sample was pooled together to yield 500 g of total sample which was used for further research purpose.

4.1 Isolation and characterization of Lactobacilli

Out of six colonies of lactobacilli isolated from the pooled *theki dahi* sample, only three types were differentiated on the basis of their biochemical and physiological characteristics. They were designated as LAB A, LAB B and LAB C. The isolated lactobacilli were further subjected to various morphological and biochemical tests for identification purpose (Table 4.1). Moreover, the characters of similar isolates are presented at Appendix B.

Morphologically, all the isolated colonies were found to be white colored, round shaped, of creamy texture and small in size with entire margin. From gram staining and microscopic observation, they were found to be gram positive and elongated rod-shaped bacteria. All of them showed negative result for both catalase-oxidase and motility test. In terms of citrate test, only type B showed positive result. All of them did not hydrolyzed arginine but only type C produced CO_2 from glucose. They were found to be resistant to heat at 60^oC for 30 min. In terms of sugar fermentation, only type C failed to ferment mannitol. All of them showed optimum growth at 30^oC and at 0% NaCl. In addition, they were able to grow even at 45^oC but not only type C was able to grow at 10^oC. However, all of them were resistant to NaCl concentration (Table 4.1).

The findings were found to be in agreement with the findings of Bhattarai *et al.* (2016), Dewan and Tamang (2007), Koirala *et al.* (2014) and Mahato and Shahani (2019) which also concluded lactobacilli as dominant lactic acid bacteria in various fermented milk with similar properties. Moreover, Erkus (2007) and Khalil and Anwar (2016) suggested that lactobacilli are resistant of higher temperature but cannot grow at lower temperature (10^oC). Finally, Khanal and Koirala (2019) concluded that lactobacilli were able to survive extreme NaCl conditions.

Table 4.1 Morphological and biochemical characterization of lactobacilli isolated from the pooled *theki dahi* sample

S No.	Test	Lactobacilli isolates		
		LAB A	LAB B	LAB C
1	Colony morphology	White colored, round shaped, of creamy texture and small sized with entire margin	Off-white colored, round shaped, of creamy texture and small sized with entire margin	White colored, round shaped, of creamy texture and small sized with entire margin
2	Gram staining	Gram positive and elongated rod shaped	Gram positive and elongated rod shaped	Gram positive and elongated rod shaped
3	Catalase	-	-	-
4	Oxidase	-	-	-
5	Motility	-	-	-
6	Citrate utilization	-	+	-
7	Heat resistance at $60^{o}C$ for 30 min (Sherman test)	+	+	+
8	Arginine hydrolysis	-	-	-
9	CO_2 production from glucose	-	-	+
10	Sugar fermentation			
	Glucose	+	+	+

	Lactose	+	+	+
	Galactose	+	+	+
	Fructose	+	+	+
	Sucrose	+	+	+
	Maltose	+	+	+
	Mannitol	+	+	-
11	Growth at different temperature			
	10^{o}C	-	-	+
	30^{o}C	+++	+++	+++
	45^{o}C	+	+	++
12	Growth at different NaCl concentration			
	0%	+++	+++	+++
	4.0%	++	++	++
	6.50%	+	+	++

4.2 Isolation and characterization of *Leuconostoc*

Out of four colonies of *Leuconostoc* isolated from the pooled *theki dahi* sample, only two types were differentiated on the basis of their biochemical and physiological characteristics. These were designated as LEU A and LEU B. The isolated *Leuconostoc* were further subjected to various morphological and biochemical tests for identification purpose (Table 4.2). Moreover, the characters of similar isolates are presented at Appendix B.

Morphologically, all the isolated colonies were found to be white colored, round shaped, of creamy texture and slightly bigger in size with entire margin. Based on gram staining and microscopic observation, they were found to be gram positive and cocci shaped bacteria. They showed negative result for both catalase-oxidase and motility test. In terms of citrate test, only

type A showed negative result. Both of them did not hydrolyzed arginine but they produced CO_2 gas from glucose. They were found to be non-resistant to heat at 60^oC for 30 min. Also, they were not able to ferment mannitol. But, only one type failed to ferment sucrose. All of them showed optimum growth at 30^oC, and at 0% NaCl. In addition, they were able to grow even at 10^oC but not at 45^oC. However, all of them were resistant to NaCl concentration (Table 4.2).

Earlier similar observations were made where *Leuconostoc* with similar properties was isolated (Bhattarai *et al.*, 2016; Harun-ur-Rashid *et al.*, 2006; Mathara *et al.*, 2004). They suggested that *Leuconostoc* were able to grow at 10^oC. However, Cardamone *et al.* (2011) reported that some strains of *Leuconostoc* were able to survive even at higher temperature. Moreover, *Leuconostoc* were found to be tolerant to high NaCl concentration (de Paula *et al.*, 2014; Kwon and Son, 2016).

Table 4.2 Morphological and biochemical characterization of *Leuconostoc* isolated from the pooled *theki dahi* sample

S N.	Test	*Leuconostoc isolates*	
		LEU A	LEU B
1	Colony morphology	White colored, round shaped, of creamy texture and slightly bigger in size with entire margin	White colored, round shaped, of creamy texture and slightly bigger in size with entire margin
2	Gram staining	Gram positive and cocci shaped	Gram positive and cocci shaped
3	Catalase	-	-
4	Oxidase	-	-
5	Motility	-	-
6	Citrate utilization	-	+

7	Heat resistance at 60°C for 30 min (Sherman test)	+	+
8	Arginine hydrolysis	-	-
9	CO_2 production from glucose	+	+
10	Sugar fermentation		
	Glucose	+	+
	Lactose	+	+
	Galactose	+	+
	Fructose	+	+
	Sucrose	+	-
	Maltose	+	+
	Mannitol	-	-
11	Growth at different temperature		
	10°C	+	+
	30°C	++	++
	45°C	-	-
12	Growth at different NaCl concentration		
	0%	+++	+++
	4.0%	++	++
	6.5%	++	++

4.3 Isolation and characterization of *Bifidobacterium*

Out of three colonies of *Bifidobacterium* isolated from the pooled *theki dahi* sample, only two types were differentiated on the basis of their biochemical and physiological characteristics. They were designated as BIF A and BIF B. The isolated *Bifidobacterium* were further subjected to various morphological and biochemical tests for identification purpose (Table 4.3). Moreover, the characters of similar isolates are presented at Appendix B.

Morphologically, all the isolated colonies were found to be white colored, round shaped, of creamy texture with entire margin. From gram staining and microscopic observation, all of them were observed to be gram positive, Y and U-shaped bacteria with branches. They both showed negative result for both catalase-oxidase and motility test. Also, they failed to utilize citrate and did not produce ammonia from arginine but they produced CO_2 gas from glucose. Both of them were found to be resistant to heat at 60^{o}C for 30 min. Type A fermented all the sugars while type B was not able to ferment mannitol and sucrose. All of them showed optimum growth at 30^{o}C and at 0% NaCl. However, all of them were resistant to highly resistant to adverse condition of heat and NaCl concentration (Table 4.3).

The obtained results were found to be within agreement with the findings of (Liu *et al.*, 2020; Mishra *et al.*, 2012; Zinedine and Faid, 2007). They also suggested that *Bifidobaterium* were able to grow at 10-42^{o}C. In addition, *Bifidobacterium* have an improved molecular machinery to degrade non-digestible sugars allowing them to survive in adverse stress conditions (Ruiz *et al.*, 2011).

Table 4.3 Morphological and biochemical characterization of *Bifidobacterium* isolated from the pooled *theki dahi* sample

S No.	Tests	*Bifidobacterium* isolates	
		BIF A	BIF B
1	Colony morphology	White colored, round shaped, of creamy texture with entire margin	White colored, round shaped, of creamy texture with entire margin
2	Gram staining	Gram positive, Y and U shaped with branches	Gram positive, Y and U shaped with branches
3	Catalase	-	-
4	Oxidase	-	-
5	Motility	-	-
6	Citrate utilization	-	-
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	+	+
8	Arginine hydrolysis	-	-
9	CO_2 production from glucose	-	-
10	Sugar fermentation		
	Glucose	+	+
	Lactose	+	+

	Galactose	+	+
	Fructose	+	+
	Sucrose	+	-
	Maltose	+	+
	Mannitol	+	-
11	Growth at different temperature		
	10^{o}C	+	-
	30^{o}C	+++	+++
	45^{o}C	++	++
12	Growth at different NaCl concentration		
	0%	+++	+++
	4.0%	++	++
	6.5%	++	++

4.4 Isolation and characterization of *Streptococcus thermophilus*

Only one type of *Streptococcus thermophilus* was isolated from the pooled *theki dahi* sample. The isolate was further subjected to various morphological and biochemical tests for identification purpose.

Morphologically, the isolated colonies were found to be creamy white colored, round shaped with entire margin. From gram staining and microscopic observation, they were gram positive, cocci in pairs or forming chains. It showed negative result for catalase-oxidase, motility and citrate utilization test. It neither hydrolyzed arginine nor produce CO_2 gas from glucose. In terms of sugar fermentation, it showed positive result for glucose, lactose, fructose and sucrose but negative result for the rest. Furthermore, the isolated *Streptococcus thermophilus* was not able to grow at 10^{o}C, but it grew well at 30^{o}C and demonstrated maximum growth at 45^{o}C. Also, it was found to be resistant to heat at 60^{o}C for 30 min

(Sherman test). It was not able to grow at 4.0% and 6.5% NaCl. Finally, the isolated strain demonstrated maximum growth at pH 7 followed by at pH 4 and at pH 2.

These findings were found to be concurrent with the findings reported on Bhattarai *et al.* (2016), Dan *et al.* (2018)and Erkus (2007) which were conducted on various fermented milk products.

4.5 Isolation and characterization of Lactococci

Out of six colonies of lactococci isolated from the pooled *theki dahi* sample, only three types were differentiated on the basis of their biochemical and physiological characteristics. These types were designated as LCC A, LCC B and LCC C. The isolated lactococci were further subjected to various morphological and biochemical tests for identification purpose (Table 4.4). Moreover, the characters of similar isolates are presented at Appendix B.

Morphologically, type A was observed as Prussian blue colored colonies on SL differentiating agar plate, type B as yellow-colored colonies surrounded by yellow zones and type C as white colored colonies with no surrounding zones on purple media. From gram staining and microscopic observation, they were gram positive and cocci forming short chains. All of them showed negative result for both catalase-oxidase and motility test. In terms of citrate test, only type A showed positive result. Among the three, type B did not hydrolyzed arginine. While, all of them did not produce CO_2 from glucose. They were found to be non-resistant to heat at 60^{o}C for 30 min. For sugar fermentation, they showed variety of result for different sugars. All of them showed optimum growth at 30^{o}C and at 0% NaCl. In addition, they were able to grow even at 10^{o}C but not at 45^{o}C. However, only type A and B were resistant to NaCl concentration (Table 4.4).

Similar results were reported by Bhattarai *et al.* (2016), Dewan and Tamang (2007), Guessas and Kihal (2004) and Maqsood *et al.* (2013) which isolated lactococci from various milk samples with similar properties. They reported that lactococci are mesophile growing well at 10^{o}C but not at 45^{o}C.

Table 4.4 Morphological and biochemical characterization of lactococci isolated from the pooled *theki dahi* sample

S N.	Test	Lactococci isolates		
		LCC A	LCC B	LCC C
1	Colony morphology	Prussian blue colored colonies in SL differentiating agar plates	Yellow colonies surrounded by yellow zones on purple media	White colonies with no surrounding zones
2	Gram staining	Gram Positive and cocci in small chains	Gram positive, cocci in short chains	Gram positive, cocci in short chains
3	Catalase	-	-	-
4	Oxidase	-	-	-
5	Motility	-	-	-
6	Citrate utilization	+	-	-
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	-	-	-
8	Arginine hydrolysis	+	-	+
9	CO_2 production from glucose	-	-	+
10	Sugar fermentation			
	Glucose	+	+	+
	Lactose	+	+	+

	Galactose	+	-	+
	Fructose	+	-	+
	Sucrose	+	-	+
	Maltose	-	+	+
	Mannitol	+	-	-
11	Growth at different temperatures			
	10^{o}C	++	++	++
	30^{o}C	+++	+++	+++
	45^{o}C	-	-	-
12	Growth at different NaCl concentration			
	0%	+++	+++	+++
	4.0%	++	++	++
	6.5%	-	-	-

4.6 Isolation and characterization of *Lactobacillus casei* sub sp. Shirota

Lactobacillus casei sub sp. Shirota was isolated from the yakult sample. The isolated strain was further subjected to various morphological and biochemical tests for identification purpose (Table 4.5).

Similar results were recorded in previous reports (Das *et al.,* 2019). Its general characterization and sugar fermentation tests were found to collide with our findings. According to Haddaji *et al.* (2015a)**,** *Lactobacillus casei* are able to survive heat shock up to 75^{o}C. In addition, it concluded that, they remained cultivable at 65^{o}C, which demonstrates that the bacteria are able to withstand adverse environment. Furthermore, Das *et al.* (2019) also suggested that *Lactobacillus casei* group were able to survive in adverse stress conditions of pH, temperature and osmotic stress.

Table 4.5 Morphological and biochemical characterization of isolated *Lactobacillus casei* sub sp. Shirota from the yakult sample

S No.	Tests	Isolated strain of *Lactobacillus casei* sub sp. Shirota
1	Colony morphology	Creamy blue colored colonies, round shaped with entire margin
2	Gram staining	Gram positive, small rod shaped
3	Catalase	-
4	Oxidase	-
5	Motility	-
6	Citrate utilization	-
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	+
8	Arginine hydrolysis	-
9	CO_2 production from glucose	-
10	Sugar fermentation	
	Glucose	+
	Lactose	-
	Galactose	+
	Fructose	+
	Sucrose	-
	Maltose	+
	Mannitol	-

11	Growth at different temperatures	
	10ºC	+
	30ºC	+++
	45ºC	++
12	Growth at different NaCl concentration	
	0%	+++
	4.0%	+++
	6.5%	++

4.7 Probiotic potentiality tests of isolated organism

4.7.1 Acid resistance test

Acid resistance at pH 2.0 and 4.0 of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. All the tested isolates were able to survive well at both pH 2.0 and 4.0 for 2 h. Moreover, a bar diagram comparing acid resistance of all the isolates at both pH is shown in Fig. 4.1. From the figure, it is clear that LB casei showed highest resistance to acid while *Leuconostoc* showed lowest degree of resistance. In addition, from statistical analysis (Appendix C), it is noted that there is no significant difference ($p<0.5$) among LB *casei*, LAB A and BIF B at both pH 2.0 and 4.0.

According to Khanal and Koirala (2019), Lactobacilli were able to grow at various pH ranges 2.5 to 8.5 indicating that they were resistant to acidic condition. In case of *Leuconostoc*, Bhattarai *et al.* (2016) also suggested that they were very sensitive to low pH which may be attributed to their low acidification ability. Also, Awasti *et al.* (2016) has reported that *Bifidobacterium* showed appreciable acid tolerance. As per Ruiz *et al.* (2011), they have been shown to possess a wide molecular machinery allowing degradation of many non-digestible sugars which in turn supports their survival in different stress conditions. Moreover, our result

of low acid tolerance of Lactococci collides with the findings of (Akbar *et al.*, 2019; Bhattarai *et al.*, 2016).

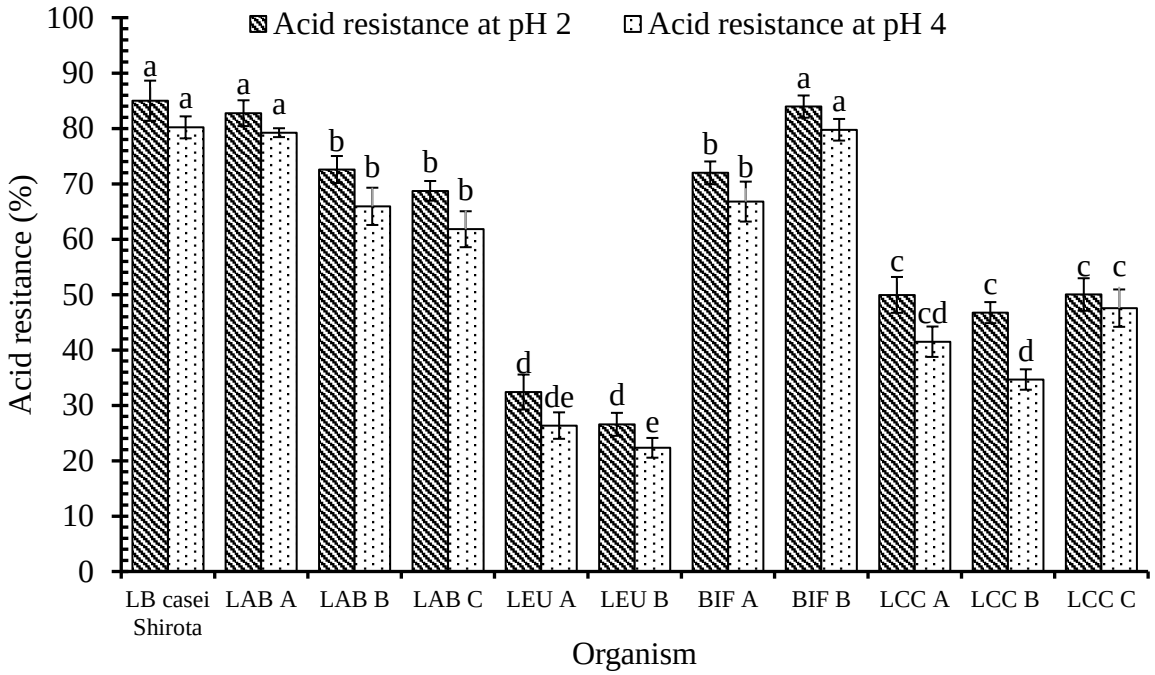

Fig. 4.1 Acid resistance of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation of the triplicate value. Also, the similar subscript denotes that they are significantly similar ($p<0.5$) while the different subscript represents significant different ($p<0.5$) between them.

4.7.2 Bile salt resistance test

Bile salt resistance at 0.3% and 0.4% (w/v) bile salt of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. All the tested isolates were able to survive well at bile salt 0.3% and 0.4% for 4 h. Moreover, a bar diagram comparing bile salt resistance of all the isolates at both conditions is shown in Fig. 4.2. From the figure, it is clear that LB *casei* showed highest resistance to bile salt while *Leuconostoc* showed lowest degree of resistance. In addition, from statistical analysis (Appendix D), it is noted that there is no significant difference ($p<0.5$) among LB *casei*, LAB A and BIF B at both conditions.

The reports were found to be in agreement with the findings reported in Balamurugan *et al.* (2014) carried on homemade curd in Southern India. Moreover, it was found that LAB were able to survive well even at 0.4%, 0.5% and 0.6% bile salt concentration for 2, 4 and 24 h

(Menconi *et al.*, 2014). Furthermore, as being popularly known a probiotic organism, *Bifidobacterium* showed appreciable bile salt tolerance even at 1.0% and 2.0% concentration (Awasti *et al.*, 2016). On the other hand, de Paula *et al.* (2014), reported decreased growth of *Leuconostoc* on higher bile salt concentration. It suggested that bile salt affected their growth and limited its viability.

Similarly, our results were found to be concurrent with the findings of Ozdogan *et al.* (2014), which suggested their similar growth rate at 0.3% bile salt. However, in contrast to our findings Yerlikaya (2019) suggested that Lactococci were not able to survive at 0.3% and higher bile salt concentration supporting our finding of lower viability of Lactococci at higher bile salt.

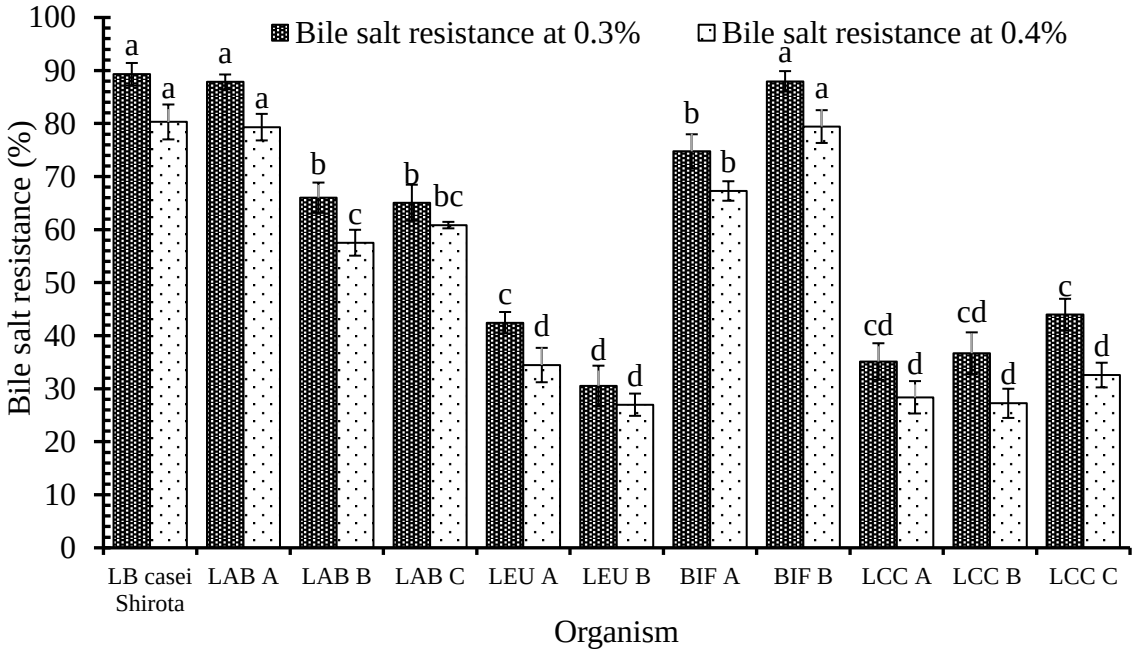

Fig. 4.2 Bile salt resistance of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation of the triplicate value. Also, the similar subscript denotes that they are significantly similar ($p<0.5$) while the different subscript represents significant different ($p<0.5$) between them.

4.7.3 Bile salt hydrolysis test

Bile salt hydrolysis of all the isolates were determined. It was observed that all the isolates including *Lactobacillus casei* sub sp. Shirota was able to hydrolyze bile salt at the concentration of 0.5% (w/v).

Similar reports were concluded for LAB from camel milk (Sharma *et al.*, 2021), for *Bifidobacterium* of Indian human origin, for *Leuconostoc* from Brazilian mozzarella cheese de Paula *et al.* (2014), and also for Lactococci isolated from raw milk and kefir grains (Yerlikaya, 2019). This signifies as a good indication for probiotics as it can reduce the serum cholesterol accumulation in humans (Sharma *et al.*, 2021).

4.7.4 Antibiotic resistance test

Antibiotic resistance of all the 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. The various result obtained from various isolates in shown in the Table 4.6. It is observed that all the isolates were found to be sensitive to amoxicillin, ampicillin and penicillin-G. Lactobacilli and *Bifidobacterium* were found to be resistant to ciproflaxacin whereas, *Leuconostoc* were found to be sensitive to it and Lactococci demonstrated intermediary sensitivity.

Table 4.6 Antibiotic resistance for all the isolates

Organisms	Antibiotics			
	AMX	AMP	CIP	PEN- G
LB *casei*	--	--	+	--
LAB A	--	--	+	--
LAB B	--	--	+	--
LAB C	--	--	+	--
LEU A	--	--	--	--
LEU B	--	--	--	--
BIF A	--	--	+	--
BIF B	--	--	+	--
LCC A	--	--	-	--
LCC B	--	--	-	--
LCC C	--	--	-	--

(+) =Resistant, (-) =Intermediate and (--) =Sensitive

Similar results were recorded in the previous findings also. LcS was found to be sensitive to penicillins but quite resistant to ciprofloxacin (Danielsen and Wind, 2003; Shao *et al.*, 2015; Shokryazdan *et al.*, 2014). Lactobacilli were found to be inhibited by penicillin, ampicillin, amoxicillin, tetracycline, erythromycin nalidixic acid and chloramphenicol, but only resistance to ciprofloxacin (Khanal and Koirala, 2019). It also suggested that resistance of lactobacilli to ciprofloxacin might be attributed to their natural and intrinsic resistance, probably due to the cell wall structure and membrane impermeability, complemented in some cases by potential efflux mechanisms.

According to de Paula *et al.* (2014), among 35 antibiotic tested, *Leuconostoc* were found to be resistant to only five different types of antibiotic namely; nalidixic acid, sulfamethoxazole/trimethoprim, sulfonamide, teicoplanin, and vancomycin. Similar to this

study, they were found to be sensitive to all tested antibiotics. Moreover, it suggested that antibiotic resistance could be strain-dependent and related to the environment in which the strain was isolated.

In a previous study, none of the isolated *Bifidobacterium* strains were found to be resistant to penicillin-G, amoxicillin and ampicillin (Moubareck *et al.*, 2005). However, *Bifidobacterium* strains displayed smaller inhibition zone for ciprofloxacin and thus they were concluded as resistant to this agent (Masco *et al.*, 2006). However, Moubareck *et al.* (2005) suggested that, since antibiotics are mainly absorbed in the ileum, the therapeutic dosage that reaches the colon might therefore be low compared with the initial dose. Hence, bifidobacteria and lactobacilli might survive better in vivo than in vitro.

Similar results were recorded in previous report while studying the probiotic potentiality of *Lactococcus lactis* which also suggested that Lactococci were susceptible to most of the tested antibiotic including penicillin-G, ampicillin amoxicillin and ciprofloxacin (Yerlikaya, 2019).

4.7.5 Cell surface hydrophobicity test

Cell surface hydrophobicity of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. All the tested isolates were able to adhere to the tested hydrocarbon (hexane). Moreover, a bar diagram comparing cell surface hydrophobicity of all the isolates is shown in Fig. 4.3. From the figure, it is clear that the isolate demonstrated varying hydrophobic spectrum ranging from 82.887% (LB *casei*) to 43.755% (LCC B). In addition, from statistical analysis (Appendix E), it is noted that there is no significant difference ($p<0.5$) among LB *casei*, LAB A, BIF A and BIF B.

The result was found to be in agreement with the findings of Sharma *et al.* (2021), which also reported that lactobacilli showed high surface hydrophobicity while Lactococci showed low hydrophobicity. Furthermore, Awasti *et al.* (2016) also suggested that *Bifidobacterium* showed high hydrophobicity to different hydrocarbons. However, our results were higher than that reported in the literature. As, cell surface hydrophobicity is strain-specific and the presence of different nutrients or carrier food matrices may influence the expression of adhesion genes in the microorganisms. Moreover, different compounds used to evaluate the hydrophobicity can also lead to difference in results (de Paula *et al.*, 2014).

In case of *Leuconostoc*, similar results were reported by de Paula *et al.* (2014), studying probiotic potentiality of *Leuconostoc* on mozzarella cheese. Lastly, Guan *et al.* (2020)

suggested that the different hydrophobicity might be attributed to the structurally and chemically heterogeneous bacterial surface, such as the unique hydrophobic amino acids, polysaccharides and other constitutions on the cell surface.

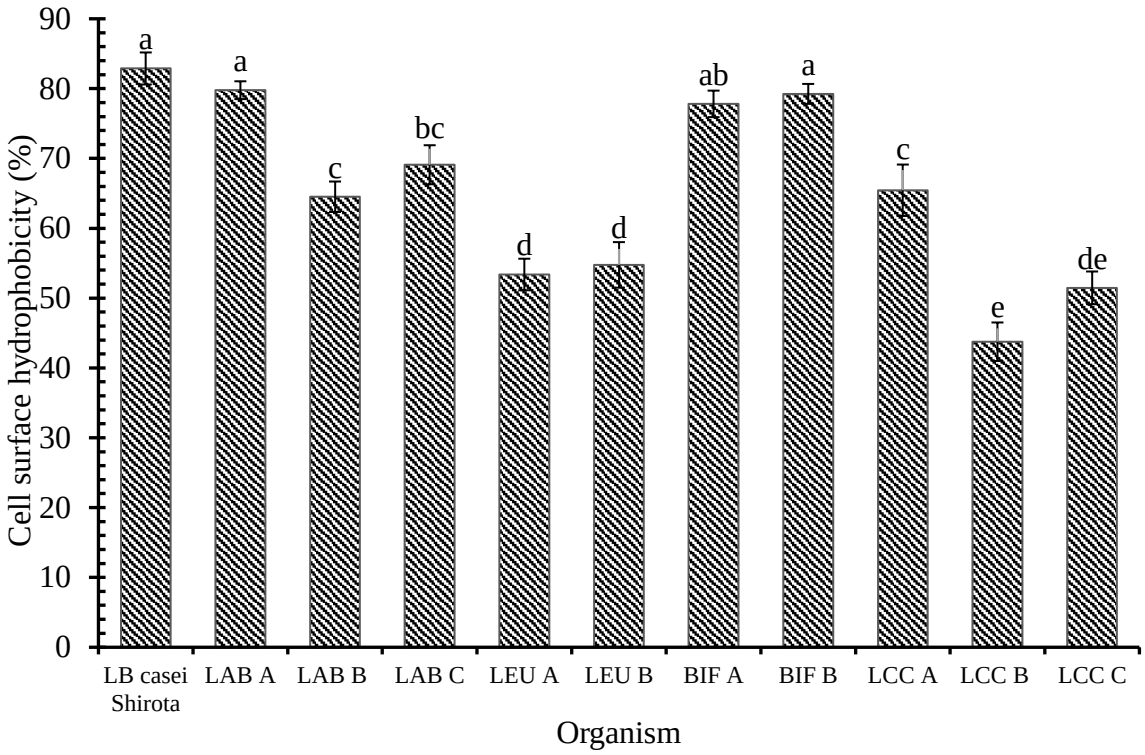

Fig. 4.3 Cell surface hydrophobicity of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation of the triplicate value. Also, the similar subscript denotes that they are significantly similar ($p<0.5$) while the different subscript represents significant different ($p<0.5$) between them.

Part V

Conclusions and recommendations

5.1 Conclusions

- *Theki dahi* of Pokhara Valley comprises of varieties of LAB viz; Lactobacilli, *Leuconostoc*, *Bifidobacterium*, Streptococci and Lactococci.
- These organisms associated with *theki dahi* demonstrated potential probiotic properties.
- Almost all the bacteria except *Leuconostocs* were able to resist high acidic condition of pH 2 and pH 4.
- The microbes were able to resist bile salt of 0.4 % (w/v) as well as hydrolyze it (0.5 % w/v). Therefore, they can pass through gastric transit; high acid and bile condition.
- Out of all the isolated organisms, Lactobacilli and *Bifidobacterium* demonstrated resistivity against ciprofloxacin and hence effective against it.
- All the tested isolates were able to adhere to the tested hydrocarbon (hexane). This implies that they can adhere to mucosal membrane and colonize in intestine.

5.2 Recommendations

Based on the present study, following recommendations are made for further research work

- The two most effective probiotic potential isolates of *theki dahi*; LAB A and BIF B from Pokhara Valley can be recommended as its potential use for probiotic drinks.
- Genomic characterization of isolated strains can be done for further documentation.
- In vivo study for probiotic potentiality of most potent isolated strain; LAB A and BIF B can be done.
- Probiotic potentiality of other indigenous drinks like *jaad, nigar, tongba* etc. can be compared with organisms isolated from *theki dahi*.
- Impact of using variety of wood to make *theki* on their microflora can be studied.

Part VI

Summary

Probiotics refers to viable, nonpathogenic microorganisms which when ingested confer health benefits to the host. Most probiotic microorganisms belong LAB such as, *Lactobacillus, Leuconostoc, Lactococcus* and also *Bifidobacteirum.* These organisms must survive adverse conditions during its transit through gastro intestinal tract and must adhere to mucosal surface and colonize the intestine in order to exert beneficial effects to the consumer. *Dahi* is one of the most popular fermented dairy products of South Asia. *Theki* refers to a close-necked wooden vessel. The main purpose of using *theki* is to give flavorful *dahi* and to serve as natural microflora reservoir.

For this study, 48 samples of *theki dahi* were collected from various places of Pokhara valley. 10g of all the samples were pooled together to yield 500g of final sample. And finally stored in refrigerated condition. Similarly, yakult sample was imported from India for isolation of LcS. Firstly, isolation of microbes was conducted on their respective agar plates. The isolated colonies were streaked on another agar plate for purification.

Based on the morphological, biochemical and physiological characteristics, three types of lactobacilli, two types of Leuconostoc, two types of *Bifidobacterium*, one type of streptococci and three types of Lactococci were identified. Also, *Lactobacillus casei* strain Shirota was isolated from yakult sample. Finally, probiotic potentiality of these organisms was examined and compared to that of LcS.

All the isolated LAB were found to be gram positive, catalase-oxidase negative, non-motile bacteria, growing optimally at 30^{o}C (expect *Streptococcus thermophilus*) and at 0% NaCl. Moreover, Lactobacilli grew well at 45^{o}C but only LAB C was able to grow at 10^{o}C. All the isolates of Leuconostoc and Lactococci were found to be mesophilic. Bifidobacterium grew well at 45^{o}C but only type A was able to grow at 10^{o}C. Among 11 organisms, only LCC C of Lactococci did not grow at 6.5% NaCl.

In terms of acid resistance test, all the isolated organisms were found to be acid resistant (pH 2 and 4) thus able to survive adverse condition of low pH during GIT. Among them, LAB A and BIF B showed high acid resistance. Similarly, all the isolates were able to resist bile salt of 0.3% and 0.4% (w/v) and LAB A and BIF B showed high bile salt resistance. Moreover, all

the isolates were able to hydrolyze bile salt and hence may reduce serum cholesterol accumulation in humans. In regards of antibiotic resistance, they were found to be sensitive to penicillin G, ampicillin and amoxicillin while lactobacilli and *Bifidobacterium* showed resistance to ciprofloxacin. Finally, all the isolates were able to adhere to tested hydrocarbon (n-hexane) while LAB A, BIF A and BIF B showed high cell surface hydrophobicity.

This study implies that *theki dahi* comprises large variety of lactic acid bacteria. These organisms exhibit probiotic properties as well. This study helps in the proper documentation of traditional fermented food of Nepal thereby preserving it. Consequently, this will help to commercialize *theki dahi* as a probiotic food which is easily accessible to many Nepalese society.

References

(ISO), I. O. f. S. (2002). Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella spp.* In I. O. f. S. (ISO) (Ed.). Geneva, Switzerland.

Akbar, A., Sadiq, M. B., Ali, I., Anwar, M., Muhammad, N., Muhammad, J., Shafee, M., Ullah, S., Gul, Z., Qasim, S., Ahmad, S. and Anal, A. K. (2019). *Lactococcus lactis* subsp. *lactis* isolated from fermented milk products and its antimicrobial potential. *CyTA- J. Food.* **14** (214-220).

Anagnostopoulos, D. A., Goulas, V., Xenofontos, E., Vouras, C., Nikoloudakis, N. and Tsaltas, D. (2019). Benefits of the use of lactic acid bacteria starter in green cracked cypriot table olives fermentation. *Foods.* **9** (1). [doi:10.3390/foods9010017].

Aryal, S. (2019a). Citrate Utilization Test- Principle, Media, Procedure and Result. Retrieved from https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result/. (Last update 14 June, 2019). [Accessed 2021].

Aryal, S. (2019b). Fermentation Test – Principle, Procedure, Uses and Interpretation. Retrieved from https://microbiologyinfo.com/fermentation-test/. (Last update 14 June, 2019). [Accessed 2021].

Aswal, P., Shukla, A. and Priyadarshi, S. (2012). Yoghurt: preparation, characteristics and recent advancements. *Cibtech J. Bio-Protocols.* **1** (2), 32-44.

Awasti, N., Tomar, S. K., Pophaly, S. D., Poonam, Lule, V. K., Singh, T. P. and Anand, S. (2016). Probiotic and functional characterization of bifidobacteria of Indian human origin. *J. Appl. Microbiol.* **120** (4), 1021-1032. [doi:10.1111/jam.13086].

Azadina, P., Gashemi, M. A. S., Mohaghegh, M. D., Jashni, M. K., Zamani, M. H., Babaki, A. K. a. and Taarof, N. (2011). Isolation and identification of lactococci from traditional yoghurts in tribe of Kazerun *J. Animal Vet. Adv.* **10** (6), 698-700.

Badis, A., Guetarni, D., Moussa-Boudjemâa, B., Henni, D. E., Tornadijo, M. E. and Kihal, M. (2004). Identification of cultivable lactic acid bacteria isolated from Algerian raw goat's milk and evaluation of their technological properties. *Food Microbiol.* **21** (3), 343-349. [doi:10.1016/s0740-0020(03)00072-8].

Balamurugan, R., Chandragunasekaran, A. S., Chellappan, G., Rajaram, K., Ramamoorthi, G. and Ramakrishna, B. S. (2014). Probiotic potential of lactic acid bacteria present in home made curd in southern india. *Indian J. Med. Res.* **140**, 345-355.

Begot, C., Desnier, I., Duadin, J. D., Labadie, J. C. and Lebert, A. (1996). Recommendations for calculating growth parameters by optical density measurements. *J. Microbiol. Methods.* **25**, 225-232.

Beshkova, D., Simova, E., Frengova, G. a. and Simov, Z. (1998). Production of flavour compounds by yogurt starter cultures. *J. Indust. Microbiol. Biotechnol.* **20**, 7.

Bhattarai, R. R. and Das, S. K. L. (2013). Scientific study on indigenous technology of dahi making of eastern Nepal. *J. Food Process. Tech.* . **04** (08), 253-300. [doi:10.4172/2157-7110.1000253].

Bhattarai, R. R., Gautam, N., Nawaz, M. A. and Lal Das, S. K. (2016). Isolation and identification of dominant lactic acid bacteria from dahi: an indigenous dairy product of Nepal Himalayas. *J. Microbiol. Biotech. Food Sci.* **05** (04), 358-363. [doi:10.15414/jmbfs.2016.5.4.358-363].

Brook, I. and Frazier, E. H. (1993). Significant recovery of nonsporulating anaerobic rods from clinical specimens. *Clin. Infect. Dis.* **16**, 476-481.

Burns, P., Sanchez, B., Vinderola, G., Ruas-Madiedo, P., Ruiz, L., Margolles, A., Reinheimer, J. and de los Reyes-Gavilan, C. G. (2010). Inside the adaptation process of *Lactobacillus delbrueckii* subsp. *lactis* to bile. *Int. J. Food Microbiol.* **142** (1-2), 132-141. [doi:10.1016/j.ijfoodmicro.2010.06.013].

Buyukyoruk, S., Cibik, R., Cetinkaya, F., Soyutemiz, G. E., Goksoy, E. O. and Kirkan, S. (2010). Isolation, phenotypic and molecular identification of *Lactococcus lactis* isolates from traditionally produced village cheeses. *J. Anim. Vet. Adv.* **9** (16), 2154-2158. [doi:10.3923/javaa.2010.2154.2158].

Cardamone, Quiberoni, D., M., Fornasari, M., Reinherimer, J. and Guglielmotti, D. (2011). Adventitious dairy *Leuconostoc* strains with interesting technological and biological properties useful for adjunct starters. *Dairy Sci. Technol.* **91** (4), 457-470.

Cheng, H. (2010). Volatile flavor compounds in yogurt: a review. *Crit. Rev. Food Sci. Nutr.* **50** (10), 938-950. [doi:10.1080/10408390903044081].

Cronin, M., Ventura, M., Fitzgerald, G. F. and van Sinderen, D. (2011). Progress in genomics, metabolism and biotechnology of bifidobacteria. *Int. J. Food Microbiol.* **149** (1), 4-18. [doi:10.1016/j.ijfoodmicro.2011.01.019].

Dabija, A., Codina, G. G. and Gatlan, A. M. (2018). Influence of different commercial starter cultures on quality of yogurt. *J. Environ. Sci. Toxicol. Food Technol.* . **12** (2), 17-24. [doi:10.9790/2402-1202021724].

Dahal, N. R., Karki, T. B., Swamylingappa, B., Li, Q. and Gu, G. (2005). Traditional foods and beverages of Nepal—A review. *Food Rev. Int.* **21** (1), 1-25. [doi:10.1081/fri-200040579].

Daly, C. and Davis, R. (1998). The biotechnology of lactic add bacteria with emphasis on applications in food safety and human health. *Agri. Food Sci. Finland.* **7**, 251-265.

Dan, T., Jin, R., Ren, W., Li, T., Chen, H. and Sun, T. (2018). Characteristics of milk fermented by *Streptococcus thermophilus* MGA45-4 and the profiles of associated volatile compounds during fermentation and storage. *Molecules.* **23** (4), 878-892. [doi:10.3390/molecules23040878].

Danielsen, M. and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. . *Int. J. Food Microbiol.* **82**, 1-11.

Das, Das, M. J., Miyaji, T. a. and Deka, S. C. (2019). Growth and metabolic characterization of four lactic acid bacteria species isolated from rice beer prepared in Assam, India. *Access Microbiol.* **1** (4), e000028. [doi:10.1099/acmi.0.000028].

Das, khowala, a. and Biswas. (2016). In vitro probiotic characterization of *Lactobacillus casei* isolated from marine samples. *LWT- Food Sci.Technol.* 73 383-390.

de Paula, Jeronymo-Ceneviva, A. B., Silva, L. F., Todorov, S. D., Franco, B. D. G. M. a. and Penna, A. L. B. (2014). *Leuconostoc mesenteroides* SJRP55: a potential probiotic strain isolated from Brazilian water buffalo mozzarella cheese. *Ann. Microbiol.* **65** (2), 899-910. [doi:10.1007/s13213-014-0933-9].

Dewan, S. and Tamang, J. P. (2007). Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. *Antonie Van Leeuwenhoek.* **92** (3), 343-352. [doi:10.1007/s10482-007-9163-5].

Durust, P. and Bayasgalanbat, N. (2014). "Promotion of Underutilized Indigenous Food Resources for Food Security and Nutrition in Asia and the Pacific". FAO. Thailand.

El Kahlout, K. E. M., El Quqa, I. M., El Hindi, M. W. and El Bashiti, T. A. (2018). Isolation, biochemical characterization and DNA identification of yogurt starters *Streptococcus thermophilus* & *Lactobacillus delbrueckii* ssp. *bulgaricus* in Gaza Strip. *Adv. Microbiol.* **08** (12), 1005-1020. [doi:10.4236/aim.2018.812068].

Erkmen, O. and Bozoglu, T. F. (2016). Factors affecting microbial growth in foods. *In:* "Food Microbiology: Principles into Practice" (Vol. 1). (O. Erkmen and T. F. Bozoglu, Eds.). The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK. John Wiley & Sons, Ltd.

Erkus, O. (2007). Isolation, phenotypic and genotypic characterization of yoghurt starter culture bacteria *Izmir Instituition of Technology* (Vol. Master of Science, pp. 117). Izmir: Graduate School of Engineering and Sciences.

FAO. (1990). The technology of traditional milk products in developing countries. FAO Animal production and health paper *Rome: Food Agri. Org. Uni. Nations.*

Faudzi, H., Faroque, H., Chia, S.-L., Abdul Rahim, R. and Othman, S. (2018). *Lactococcus lactis*: LAB model organism for bacteria-mediated therapeutic strategies. *Asia Pacific J. Mol. Biol. Biotechol.*, 1-10. [doi:10.35118/apjmbb.2018.026.1.01].

Giraffa, G., Paris, A., Valcavi, L., Gatti, M. and Neviani, E. (2001). Genotypic and phenotypic heterogeneity of *Streptococcus thermophilus* strains isolated from dairy products. *J. App. Microbiol.* **91**, 937-943.

Goswami, G., Bora, S. S., Parveen, A., Chandra Boro, R. and Barooah, M. (2017). Identification and functional properties of dominant lactic acid bacteria isolated from Kahudi , a traditional rapeseed fermented food product of Assam, India. *J. Ethnic Foods.* **4** (3), 187-197. [doi:10.1016/j.jef.2017.08.008].

Goyal, R., Dhingra, H., Bajpai, P. and Joshi, N. (2012). Characterization of the *Lactobacillus* isolated from different curd samples. *Afr. J. Biotech.* . **11** (79), 14448-14452. [doi:10.5897/ajb11.310].

Guan, C., Chen, X., Jiang, X., Zhao, R., Yuan, Y., Chen, D., Zhang, C., Lu, M., Lu, Z. and Gu, R. (2020). In vitro studies of adhesion properties of six lactic acid bacteria isolated from the longevous population of China. *RSC Adv.* **10** (41), 24234-24240. [doi:10.1039/d0ra03517c].

Guessas, B. and Kihal, M. (2004). Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats' milk. *Afri. J. Biotechnol.* **3** (6), 339-342.

Guo, Z., Wang, J., Yan, L., Chen, W., Liu, X.-m. and Zhang, H.-p. (2009). In vitro comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. *LWT - Food Sci. Technol.* **42** (10), 1640-1646. [doi:10.1016/j.lwt.2009.05.025].

Gutierrez-Mendez, N., Vallejo-Cordoba, B., Gonzalez-Cordova, A. F., Nevarez-Moorillon, G. V. and Rivera-Chavira, B. (2008). Evaluation of aroma generation of *Lactococcus lactis* with an electronic nose and sensory analysis. *J. Dairy Sci.* . **91** (1), 49-57. [doi:10.3168/jds.2007-0193].

Haddaji, N., Krifi, B., Lagha, R., Khouadja, S. and Bakhrouf, A. (2015a). Effect of high temperature on viability of *Lactobacillus casei* and analysis of secreted and GroEL proteins profiles. *Afri. J. Biotechnol. Res.* **7** (3), 29-34. [doi:10.5897/JBR2015.0155].

Haddaji, N., Mahdhi, A. K., Krifi, B., Ismail, M. B. and Bakhrouf, A. (2015b). Change in cell surface properties of *Lactobacillus casei* under heat shock treatment. *FEMS Microbiol. Lett.* . **362** (9). [doi:10.1093/femsle/fnv047].

Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D. and Ennahar, S. (2011). Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiol.* **11** (63), 63. [doi:10.1186/1471-2180-11-63].

Harding, C. D. and Shaw, B. G. (1990). Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. *J. Appl. Bacteriol.* **69** (5), 648-654. [doi:10.1111/j.1365-2672.1990.tb01558.x].

Harun-ur-Rashid, M., Togo, K., Ueda, M. and Miyamoto, T. (2006). Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk *Dahi* in Bangladesh. *World J. Microbiol. Biotechnol.* **23** (1), 125-133. [doi:10.1007/s11274-006-9201-x].

Hawaz, E. (2014). Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. *Afr. J. Microbiol. Res.* . **8** (13), 1419-1425. [doi:10.5897/ajmr2014.6639].

Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *Am. J. Clin. Nutr.* . **73**, 374-379.

Hemme, D. and Foucaud-Scheunemann, C. (2004). *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. *Int. Dairy J.* . **14** (6), 467-494. [doi:10.1016/j.idairyj.2003.10.005].

Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S. M., Billlah, M. M. and Islam, K. M. D. (2010). Isolation, identification and analysis of probiotic properties of *Lactobacillus* spp. from selective regional yoghurts. *World J. Dairy Food Sci.* **5** (1), 39-46.

Huang, C. H., Li, S. W., Huang, L. and Watanabe, K. (2018). Identification and classification for the *Lactobacillus casei* group. *Front Microbiol.* **9**, 1-13. [doi:10.3389/fmicb.2018.01974].

Hussain, M., Khan, M. T., Wajid, A. and Sheikk, A. R. (2008). Technological characterization of indigenous enterococcal population for probiotic potential. *Pak. J. Bot.* **40** (2), 867-875.

Jarvis, B. (2007). On the compositing of samples for qualitative microbiological testing. *Lett. in Appl. Microbiol.* **45** (6), 592-598. [doi:10.1111/j.1472-765X.2007.02237.x].

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B. and Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharm. Res.* **23** (1), 165-176. [doi:10.1007/s11095-005-8476-1].

Karkas-sen, A. a. and Karkas, E. (2018). Isolation, identification, technological properties of lactic acid batceria from raw cow milk. *Biosci. J.* . **34** (2), 385-399.

Kato-Kataoka, A., Nishida, K., Takada, M., Kawai, M., Kikuchi-Hayakawa, H., Suda, K., Ishikawa, H., Gondo, Y., Shimizu, K., Matsuki, T., Kushiro, A., Hoshi, R., Watanabe, O., Igarashi, T., Miyazaki, K., Kuwano, Y., Rokutan, K. and Björkroth, J. (2016). Fermented milk containing *Lactobacillus casei* strain Shirota preserves the diversity of the gut microbiota and relieves abdominal dysfunction in healthy medical students exposed to academic stress. *Appl. Environ. Microbiol.* **82** (12), 3649-3658. [doi:10.1128/aem.04134-15].

Katona, B. (2014). Pooling of ground meat samples during analysis of Salmonella for compliance with regulations on microbiological criteria for foodstuffs. Master Degree Project in Infection Biology Uppsala Universitet,

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N. and Fakiri, E. M. (2013). Health benefits of probiotics: a review. *ISRN Nutr.* **68** (3), 1-7. [doi:10.5402/2013/481651].

Kempler, G. M. and McKay, L. L. (1980). Improved medium for detection of citrate-fermenting *Streptococcus lactic* subsp. *diacetylactics*. *App. Environ. Microbiol.* . **39** (4), 926-927.

Khalil, I. and Anwar, N. (2016). Isolation, identification and characterization of lactic acid bacteria from milk and yoghurts. *J. Food Dairy Technol.* **4** (3), 17-26.

Khanal, A. and Koirala, N. (2019). Screening and analysis of probiotic properties of *Lactobacillus* Spp. from famous commercial brand of yogurt found in Kathmandu valley. *Curr. Trends Biomedical Eng. Biosci.* **18** (4), 75-82. [doi:10.19080/CTBEB.2019.18.555992].

Koirala, R., Ricci, G., Taverniti, V., Ferrario, C., Malla, R., Shrestha, S., Fortina, M. G. and Guglielmetti, S. (2014). Isolation and molecular characterization of lactobacilli from traditional fermented dahi produced at different altitudes in Nepal. *Dairy Sci. Technol.* . **94** (4), 397-408. [doi:10.1007/s13594-014-0167-4].

Kosin, B. and Rakshit, S. K. (2006). Microbial and processing criteria for production of probiotic: A review. *J. Food Technol. Biotechnol.* . **44**, 371-379.

Kumar, R. S., Brannigan, J. A., Prabhune, A. A., Pundle, A. V., Dodson, G. G., Dodson, E. J. and Suresh, C. G. (2006). Structural and functional analysis of a conjugated bile salt hydrolase from *Bifidobacterium longum* reveals an evolutionary relationship with penicillin V acylase. *J. Biol. Chem.* **281** (43), 32516-32525. [doi:10.1074/jbc.M604172200].

Kwon, O. a. and Son, M. (2016). NaCl effect on dextran producing *Leuconostoc mesenteroides* CS-5 that isolated from Chonggak Kimchi. *Quant. Biosci.* **35** (1), 11-17.

Law, S. V., Bakar, F. A., Hashim, D. M. and Hamid, A. A. (2011). Popular fermented foods and beverages in Southeast Asia. *Int. Food Res. J.* . **18**, 475-485.

Lee, J. H. and O'Sullivan, D. J. (2010). Genomic insights into bifidobacteria. *Microbiol Mol Biol Rev.* **74** (3), 378-416. [doi:10.1128/MMBR.00004-10].

Leroy, F. and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* **15** (2), 67-78. [doi:10.1016/j.tifs.2003.09.004].

Leucy, J. A. (2004). Cultured dairy products: an overview of their gelation and texture properties. *Int. J. Dairy Technol.* **57** (2-3), 77-84.

Liu, Chen, M., Duo, L., Wang, J., Guo, S., Sun, H., Menghe, B. a. and Zhang, H. (2020). Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. *J. Dairy Sci.* **103** (5), 4013-4025. [doi:10.3168/jds.2019-17602].

Liu and Pop, M. (2009). ARDB--Antibiotic Resistance Genes Database. *Nucleic Acids Res.* **37** (Database issue), D443-447. [doi:10.1093/nar/gkn656].

Ljungh, A. and Wadström, T. (2006). Lactic acid bacteria as probiotics. *Curr. Issues Intestinal Microbiol.* **7**, 73-90.

Mahato, S. and Shahani, A. K. (2019). Identifying the diversity of dominant LABs from fermented dairy products dahi and yoghurt in eastern region of Nepal. *J. Food Sci. Technol. Nepal.* **11**, 60-64.

Maqsood, S., Hasan, F. and Masud, T. (2013). Characterization of lactic acid bacteria isolated from indigenous dahi samples from potential source of starter culture. *Afr. J. Biotechnol.* **12** (33), 5226-5231. [doi:10.5897/AJB09.1172].

Marteau, P., Minekus, M., Havenaar, R. and Huis, J. H. J. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J. Dairy Sci.* . **80**, 1031–1037.

Masco, L., Van Hoorde, K., De Brandt, E., Swings, J. and Huys, G. (2006). Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. *J. Antimicrob. Chemother.* **58** (1), 85-94. [doi:10.1093/jac/dkl197].

Masdea, L., Kulik, E. M., Hauser-Gerspach, I., Ramseier, A. M., Filippi, A. and Waltimo, T. (2012). Antimicrobial activity of *Streptococcus salivarius* K12 on bacteria involved in oral malodour. *Arch. Oral Biol.* **57** (8), 1041-1047. [doi:10.1016/j.archoralbio.2012.02.011].

Mater, D. D., Bretigny, L., Firmesse, O., Flores, M. J., Mogenet, A., Bresson, J. L. and Corthier, G. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiol. Lett.* **250** (2), 185-187. [doi:10.1016/j.femsle.2005.07.006].

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K. and Holzapfel, W. H. (2004). Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *Int. J. Food Microbiol.* **94** (3), 269-278. [doi:10.1016/j.ijfoodmicro.2004.01.008].

Mathur, S. and Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria--a review. *Int. J. Food Microbiol.* **105** (3), 281-295. [doi:10.1016/j.ijfoodmicro.2005.03.008].

Mayer, M. (2018). The effects of salt concentration on bacterial growth. Retrieved from https://sciencing.com/effects-salt-concentration-bacterial-growth-5924409.html. (Last update 7 May, 2018). [Accessed 2021].

Mehmood, T., Masud, T., Abbass, S. A. and Maqsud, S. (2009). Isolation and identification of wild strains of lactic acid bacteria for yoghurt preparation from indigenous dahi. *Pak. J. Nutri*. **8**, 866-871.

Menconi, A., Kallapura, G., Latorre, J. D., Morgan, M. J., Pumford, N. R., Hargis, B. M. and Tellez, G. (2014). Identification and characterization of lactic acid bacteria in a commercial probiotic culture. *Biosci. Microbiota Food Health.* **33** (1), 25-30.

Mishra, M., Paliwal, J. S., Singh, S. K. and Abharam, J. (2012). Isolation and characterization of *Bifidobacterium* from fermented milk products and their antimicrobial studies. [doi:10.13140/RG.2.2.20812.67208].

Moubareck, C., Gavini, F., Vaugien, L., Butel, M. J. and Doucet-Populaire, F. (2005). Antimicrobial susceptibility of bifidobacteria. *J Antimicro Chemotherapy.* **55** (1), 38-44. [doi:10.1093/jac/dkh495].

Moyes, R. B., Reynolds, J. and Breakwell, D. P. (2009). Differential staining of bacteria: gram stain. *Curr. Protoc. Microbiol.* **Appendix 3**. [doi:10.1002/9780471729259.mca03cs15].

Mudie, D. M., Amidon, G. L. and Amidon, G. E. (2010). Physiological parameters for oral delivery and in vitro testing. *Mol. Pharm.* **7** (5), 1388-1405.

Nehal, F., Sahnoun, M., Smaoui, S., Jaouadi, B., Bejar, S. and Mohammed, S. (2019). Characterization, high production and antimicrobial activity of exopolysaccharides from *Lactococcus lactis* *Micro. Patho.* **132**, 10-19. [doi:10.1016/j.micpath.2019.04.018].

Niazi Amraii, H., Abtahi, H., Jafari, P., Mohajerani, H. R., Fakhroleslam, M. R. and Akbari, N. (2014). In vitro study of potentially probiotic lactic acid bacteria strains isolated from traditional dairy products. *Jundishapur J. Microbiol.* **7** (6), e10168. [doi:10.5812/jjm.10168].

Orel, R. and Kamhi Trop, T. (2014). Intestinal microbiota, probiotics and prebiotics in inflammatory bowel disease. *World J. Gastroenterol.* **20** (33), 11505-11524. [doi:10.3748/wjg.v20.i33.11505].

Ozdogan, D. K., Akcelik, N., Aslim, B., Suludere, Z. and Akcelik, M. (2014). Probiotic and antioxidative properties of *L. Lactis* LL27 isolated from milk. *Biotechnol. Biotechnol. Equip.* **26** (1), 2750-2758. [doi:10.5504/bbeq.2011.0091].

Palinkas, L. A., Horwitz, S. M., Green, C. A., Wisdom, J. P., Duan, N. and Hoagwood, K. (2013). Purposeful sampling for qualitative data collection and analysis in mixed method implementation research. *Administration and Policy in Mental Health and Mental Health Services Res.* **42** (5), 533-544. [doi:10.1007/s10488-013-0528-y].

Pan, W. H., Li, P. L. and Liu, Z. (2006). The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. *Anaerobe.* **12** (3), 148-152. [doi:10.1016/j.anaerobe.2006.03.001].

Parvez, S., Malik, K. A., Ah Kang, S. and Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* **100** (6), 1171-1185. [doi:10.1111/j.1365-2672.2006.02963.x].

Patton, M. Q. (2015). "Qualitative Research Evaluation Methods Integrating Theory and Practice" (4th ed.). SAGE Publications, Inc. United States of America.

Pfeiler, E. A. and Klaenhammer, T. R. (2009). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **75** (18), 6013-6016. [doi:10.1128/AEM.00495-09].

Pisano, M. B., Fadda, M. E., Melis, R., Ciusa, M. L., Viale, S., Deplano, M. and Cosentino, S. (2015). Molecular identification of bacteriocins produced by *Lactococcus lactis* dairy strains and their technological and genotypic characterization. *Food Control.* **51**, 1-8. [doi:10.1016/j.foodcont.2014.11.005].

Pourahmad, R. and Assadi, M. M. (2007). Use of isolated autochthonous starter cultures in yogurt production. *Int. J. Dairy Technol.* **60** (4), 259-262.

Pradhan, J. B. (2017). Mathematical ideas in chundara culture: unfolding a Nepalese teaching and learning system. *In:* "Ethnomathematics and its Diverse Approaches for Mathematics Education". (M. R. *e. al.*, Ed.). pp. 125-152. Springer International Publishing. [978-3-319-59219-0 and 978-3-319-59220-6].

Rai, R., Shangpliang, H. N. J. and Tamang, J. P. (2016). Naturally fermented milk products of the Eastern Himalayas. *J. Ethnic Foods.* **3** (4), 270-275. [doi:10.1016/j.jef.2016.11.006].

Rakhmanova, A., Khan, Z. A. and Shah, K. (2018). A mini review fermentation and preservation: role of lactic acid bacteria. *MOJ Food Process. Technol.* . **6** (5), 414-417. [doi:10.15406/mojfpt.2018.06.00197].

Reddy, M. S., Vedamuthu, E. R., Washam, C. J. and Reinbold, G. W. (1969). Differential agar medium for separating *Streptococcus lactis* and *Streptococcus cremoris*. *Appl. Microbiol.* **18** (5), 755-759. [doi:10.1128/am.18.5.755-759.1969].

Reiner, K. (2010). Catalase Test Protocol. *Am. Society Microbiol.*1-7.

Rodriguez, I. C. O. (2017). Heat, acid and osmotic stress tolerance of *Leuconostoc mesenteroides* as influenced by prior exposure to various mild stress conditions. Master of Science in The School of Animal Science Louisiana State University

Ruiz, L., Ruas-Madiedo, P., Gueimonde, M., de Los Reyes-Gavilan, C. G., Margolles, A. and Sanchez, B. (2011). How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. *Genes Nutr.* **6** (3), 307-318. [doi:10.1007/s12263-010-0207-5].

Saarela, M., Mogensen, G., Fonden, R., Matto, J. and Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* **84** (3), 197-215. [doi:10.1016/s0168-1656(00)00375-8].

Sahadeva, R. P. K., Leong, S. F., Chua, K. H., Tan, C. H., H. Y, C., Tong, E. V., Wong, S. Y. W. and Chan, H. K. (2011). Survival of commercial probiotic strains to pH and bile. *Int. Food Res. J.* **18** (4), 1515-1522.

Salminen, S. and Wright, A. V. (1998). Current probiotics - safety assured? *Microb. Eco. Health Disease.* **10** (2), 68-77.

Samaržija, D., Havranek, J. L., Antunac, N. and Sikora, S. (2001). Characteristics and role of mesophilic lactic cultures. *Agriculturae Conspectus Scientificus.* **66** (2), 113-120.

Schmid, K., Schlothauer, R., Friedrich, U., Staudt, C., Apajalahti, J. and Hansen, B. E. (2006). Development of probiotic food ingridients. . *In:* "Probiotics In Food Safety and Human Health". (I. Goktepe, V. K. Juneja and M. Ahmedna, Eds.). pp. 65-72 6000 Broken Sound Parkway NW, Suite 300 Boca Raton. Taylor & Francis Group,.

Semwal, D. K., Rawat, U., Semwal, R., Singh, R., Krishan, P., Singh, M. and Singh, G. J. (2009). Chemical constituents from the leaves of *Boehmeria rugulosa* with antidiabetic and antimicrobial activities. *J. Asian Nat. Prod. Res.* **11** (12), 1045-1055. [doi:10.1080/10286020903352526].

Shao, Y., Zhang, W., Guo, H., Pan, L., Zhang, H. and Sun, T. (2015). Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*. *Food Control.* **50**, 250-258. [doi:10.1016/j.foodcont.2014.09.003].

Sharma, A., Lavania, M., Singh, R. a. and Lal, B. (2021). Identification and probiotic potential of lactic acid bacteria from camel milk. *Saudi J. Biol. Sci.* **28** (3), 1622-1632. [doi:10.1016/j.sjbs.2020.11.062].

Shewale, R. N., Sawale, P. D., Khedkar, C. D. and Singh, A. (2014). Selectrion criteria for probiotics: A review. *Int. J. Probiotics Prebiotics.* **9** (1).

Shokryazdan, P., Sieo, C. C., Kalavathy, R., Liang, J. B., Alitheen, N. B., Faseleh Jahromi, M. and Ho, Y. W. (2014). Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. *Biomed. Res. Int.* **2014**, 927268. [doi:10.1155/2014/927268].

Silva, M. S., Ramos, C. L., González-Avila, M., Gschaedler, A., Arrizon, J., Schwan, R. F. and Dias, D. R. (2017). Probiotic properties of *Weissella cibaria* and *Leuconostoc citreum* isolated from tejuino – A typical Mexican beverage. *LWT- Food Sci. Technol.* **86**, 227-232. [doi:10.1016/j.lwt.2017.08.009].

Somashekaraiah, R., Shruthi, B., Deepthi, B. V. and Sreenivasa, M. Y. (2019). Probiotic properties of lactic acid bacteria isolated from neera: A naturally fermenting coconut palm nectar. *Front. Microbiol.* **10**. [doi:10.3389/fmicb.2019.01382].

Sthapit, B. R., Upadhyay, M. P., Baniya, B. K., Subedi, A. and Joshi, B. K. (2001). "On-farm Management of Agricultural Biodiveristy in Nepal". Proceedings of a National Workshop. Lumle, Nepal.

Sutton, S. (2011). Measurement of microbial cells by optical density. *J. Validation Technol.* **Winter**, 46-50.

Sutula, J., Ann Coulthwaite, L., Thomas, L. V. and Verran, J. (2013). The effect of a commercial probiotic drink containing *Lactobacillus casei* strain Shirota on oral health in healthy dentate people. *Microbial Ecol. Health Disease.* **24** (0). [doi:10.3402/mehd.v24i0.21003].

Sutula, J., Coulthwaite, L. and Verran, J. (2012). Culture media for differential isolation of *Lactobacillus casei* Shirota from oral samples. *J. Microbiol. Methods.* **90** (1), 65-71. [doi:10.1016/j.mimet.2012.03.015].

Taimme, A. Y. and Robinson, R. K. (2002). Microbiology of Starter Culture. *In:* "Dairy Microbiology Handbook" (3rd ed.). (R. K. Robinson, Ed.). pp. 261-366. New York. . John Wiley and Sons. [9780471385967].

Tamang, J. P., Thapa, N., Tamang, B., Rai, A. and Chettri, R. (2015). Microorganisms in fermented foods and beverages. *In:* "Health Benefits of Fermented Foods and Beverages" (1st ed.). (J. P. Tamang, Ed.). pp. 1-110. CRC Press, Taylor and Francis Group. . [9780367268213].

Tufail, M., Hussain, S., Malik, F., Mirza, T., Parveen, G., Shafaat, S., Wajid, A., Mahmood, R., Channa, R. A. and A, S. (2011). Isolation and evaluation of antibacterial activity of bacteriocin produced by *Lactobacillus bulgaricus* from yogurt. *Afr. J. Microbiol. Res.* . **5** (22), 3842-3847. [doi:10.5897/ajmr11.846].

Vedamuthu, E. R. (2013). Other fermented and culture-containing milks. *In:* "Manufacturing Yoghurt and Fermented Milks". (R. C. Chandan and A. Kilara, Eds.). pp. 393-410. The

Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK. Johns Wiley and Sons, Ltd.

Waddington, L., Cyr, T., Hefford, M., Hansen, L. T. and Kalmokoff, M. (2010). Understanding the acid tolerance response of bifidobacteria. *J. Appl. Microbiol.* **108** (4), 1408-1420. [doi:10.1111/j.1365-2672.2009.04540.x].

Walstra, P., Wouters, J. T. M. and Geurts, T. J. (2006). "Dairy Science and Technology" (2nd ed.). CRC Press, Taylor and Francis Group. . Boca Raton, USA.

Wang, R., Chen, S., Jin, J., Ren, F., Li, Y., Qiao, Z., Wang, Y. and Zhao, L. (2015). Survival of *Lactobacillus casei* strain Shirota in the intestines of healthy Chinese adults. *Microbiol. Immunol.* **59** (5), 268-276. [doi:10.1111/1348-0421.12249].

Watson, D., Sleator, R. D., Hill, C. and Gahan, C. G. (2008). Enhancing bile tolerance improves survival and persistence of *Bifidobacterium* and *Lactococcus* in the murine gastrointestinal tract. *BMC Microbiol.* **8**, 176-186. [doi:10.1186/1471-2180-8-176].

Yadav, H., Jain, S. and Sinha, P. R. (2006). Effect of dahi containing *Lactococcus lactis* on the progression of diabetes induced by a high-fructose diet in rats. *Biosci. Biotechnol. Biochem.* **70** (5), 1255-1258. [doi:10.1271/bbb.70.1255].

Yerlikaya, O. (2019). Probiotic potential and biochemical and technological properties of *Lactococcus lactis* ssp. lactis strains isolated from raw milk and kefir grains. *J. Dairy Sci.* **102** (1), 124-134. [doi:10.3168/jds.2018-14983].

Zhang, W., Wang, H., Liu, J., Zhao, Y., Gao, K. and Zhang, J. (2013). Adhesive ability means inhibition activities for *Lactobacillus* against pathogens and S-layer protein plays an important role in adhesion. *Anaerobe.* **22**, 97-103. [doi:10.1016/j.anaerobe.2013.06.005].

Zinedine, A. and Faid, M. (2007). Isolation and characterization of strains of bifidobacteria with probiotic proprieties. *World J. Dairy Food Sci.* **2** (1), 28-34.

Appendices

Appendix A

Table A.1 List of chemicals

Chemicals	Chemicals
Sodium chloride (NaCl) (Merck Specialities Pvt. Ltd. Mumbai, India)	Buffer solutionsx (4.0 and 7.0) (Fisher scientist India Pvt. Ltd. Dehli India)
Hydrochloric acid (HClx) (Qualigens Fine Chemicals, Mumbai, India)	Glucose (Qualigens Fine Chemicals, Mumbai, India)
Distilled water	Sucrose (Qualigens Fine Chemicals, Mumbai, India)
Glycerol (Qualigens Fine Chemicals, Mumbai, India)	Lactose (Qualigens Fine Chemicals, Mumbai, India)
L-cysteine (Leo Chemical Pvt. Ltd. Mumbai, India)	Galactose (Qualigens Fine Chemicals, Mumbai, India)
Vancomycin (Lxir Medilabs Pvt. Ltd. Haryana, India)	Fructose (Qualigens Fine Chemicals, Mumbai, India)
Sodium hydroxide (NaOH) (Merck Specialities Pvt. Ltd. Mumbai, India)	Maltose (Qualigens Fine Chemicals, Mumbai, India)
Mannitol (R. P Chemicals Pvt. Ltd, Titwala East, India)	Nessler reagent (Fisher scientist India Pvt. Ltd. Dehli India)
Phenol red (Hi Media Laboratories Pvt. Ltd. Mumbai, India)	Hydrogen peroxide (H_2O_2) (Qualigens Fine Chemicals, Mumbai, India)
Safranin (Alpha Chemica Pvt. Ltd. Mumbai India)	Crystal violet (Alpha Chemica Pvt. Ltd. Mumbai India)
Gram’s iodine (Alpha Chemica Pvt. Ltd. Mumbai India)	Ethanol (Mount Everest Industrial Works, Lucknow, India)

Bile salt (Sodium Tauroglycocholate) (Loba Chemie Pvt. Ltd. Mumbai, India)	PBS solution (Mount Everest Industrial Works, Lucknow, India)
Hexane (Alpha Chemica Pvt. Ltd. Mumbai India)	

Table A.2 List of instruments

Instruments	Instruments
Incubator (Bio-Techno Lab Pvt. Ltd, Mumbai, India)	Heating arrangement (Aaviskar India Pvt. Ltd. Mumbai, India)
Hot air Oven (Samarth Electrotonic, Mumbai India)	pH meter (Labtroni Pvt. Ltd, Harayana, Inida)
Autoclave (HMG India Pvt. Ltd, Mumbai India)	Anaerobic chamber
Microscope and slides (Lablink Instrument, Hyderabad, India	Centrifuge (Jin scientific glassworks, India)
Spectrophotometer (Aaviskar India Pvt. Ltd. Mumbai, India)	Micropipette (Jain scientific glassworks, India)
Durham tube (Jain scientific glassworks, India)	Refrigerator
Glass wares (Jain scientific glassworks, India)	Water bath (Samarth Electrotonic, Mumbai India)
Antibiotic disc (AMX, AMP, CIP, PEN G) (EOS laboratories Pvt. Ltd, Maharashtra, India)	

Appendix B

Table B.1 Morphological and biochemical characterization of similar isolates of lactobacilli isolated from the pooled *theki dahi* sample

S No.	Test	Lactobacilli isolates		
		Isolate 4	Isolate 5	Isolate 6
1	Colony morphology	White colored, round shaped, of creamy texture and small sized with entire margin		
2	Gram staining	Gram positive and elongated rod shaped		
3	Catalase	-	-	-
4	Oxidase	-	-	-
5	Motility	-	-	-
6	Citrate utilization	-	-	-
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	+	+	+
8	Arginine hydrolysis	-	-	-
9	CO_2 production from glucose	-	-	+
10	Sugar fermentation			
	Glucose	+	+	+
	Lactose	+	+	+
	Galactose	+	+	+

	Fructose	+	+	+
	Sucrose	+	+	+
	Maltose	+	+	+
	Mannitol	+	+	-
11	Growth at different temperature			
	10^{o}C	-	-	+
	30^{o}C	+++	+++	+++
	45^{o}C	+	+	++
12	Growth at different NaCl concentration			
	0%	+++	+++	+++
	4.0%	++	++	++
	6.50%	+	+	++

Table B.2 Morphological and biochemical characterization of similar isolates of *Leuconostoc* isolated from the pooled *theki dahi* sample

S N.	Test	*Leuconostoc* isolates	
		Isolate 3	Isolate 4
1	Colony morphology	White colored, round shaped, of creamy texture and slightly bigger in size with entire margin	
2	Gram staining	Gram positive and cocci shaped	
3	Catalase	-	-
4	Oxidase	-	-
5	Motility	-	-

6	Citrate utilization	-	+
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	+	+
8	Arginine hydrolysis	-	-
9	CO_2 production from glucose	+	+
10	Sugar fermentation		
	Glucose	+	+
	Lactose	+	+
	Galactose	+	+
	Fructose	+	+
	Sucrose	+	-
	Maltose	+	+
	Mannitol	-	-
11	Growth at different temperature		
	10^{o}C	+	+
	30^{o}C	++	++
	45^{o}C	-	-
12	Growth at different NaCl concentration		
	0%	+++	+++
	4.0%	++	++
	6.50%	++	++

Table B.3 Morphological and biochemical characterization of similar isolates of *Bifidobacteria* isolated from the pooled *theki dahi* sample

S No.	Tests	Isolate 3 of *Bifidobacterium*
1	Colony morphology	White colored, round shaped, of creamy texture with entire margin
2	Gram staining	Gram positive, Y and U shaped with branches
3	Catalase	-
4	Oxidase	-
5	Motility	-
6	Citrate utilization	-
7	Heat resistance at 60^{o}C for 30 min (Sherman test)	+
8	Arginine hydrolysis	-
9	CO_2 production from glucose	-
10	Sugar Fermentation	
	Glucose	+
	Lactose	+
	Galactose	+
	Fructose	+
	Sucrose	+
	Maltose	+
	Mannitol	+

11	Growth at different temperatures	
	10°C	+
	30°C	+++
	45°C	++
12	Growth at different NaCl concentration	
	0%	+++
	4.0%	++
	6.5%	++

Table B.4 Morphological and biochemical characterization of similar isolates of *Lactococci* isolated from the pooled *theki dahi* sample

S N.	Test	Lactococci isolates		
		Isolate 4	Isolate 5	Isolate 6
1	Colony morphology	Prussian blue colored colonies in SL differentiating agar plates	Yellow colonies surrounded by yellow zones on purple media	White colonies with no surrounding zones
2	Gram staining	Gram Positive and cocci in small chains		
3	Catalase	-	-	-
4	Oxidase	-	-	-
5	Motility	-	-	-
6	Citrate utilization	+	-	-

7	Heat resistance at 60°C for 30 min (Sherman test)	-	-	-
8	Arginine hydrolysis	+	-	+
9	CO_2 production from glucose	-	-	+
10	Sugar fermentation			
	Glucose	+	+	+
	Lactose	+	+	+
	Galactose	+	-	+
	Fructose	+	-	+
	Sucrose	+	-	+
	Maltose	-	+	+
	Mannitol	+	-	-
11	Growth at different temperatures			
	10°C	++	++	++
	30°C	+++	+++	+++
	45°C	-	-	-
12	Growth at different NaCl concentration			
	0%	+++	+++	+++
	4%	++	++	++
	6.50%	-	-	-

Appendix C

ANOVA for acid resistance test

Table C.1 One way ANOVA for acid resistance test of all the isolated organisms

		Sum of Squares	df	Mean Square	F
Acid resistance 2.0	Between Groups	13018.682	10	1301.868	130.898
	Within Groups	218.805	22	9.946	
	Total	13237.486	32		
Acid resistance 4.0	Between Groups	14037.083	10	1403.708	138.351
	Within Groups	223.212	22	10.146	
	Total	14260.295	32		

Table C.2 Post hoc test for acid resistance at 2.0

Name of isolates	N	Subset for alpha = 0.05			
		1	2	3	4
LEU B	3	26.57200			
LEU A	3	32.40267			
LCC B	3		46.74367		
LCC A	3		49.94433		
LCC C	3		50.01367		
LAB C	3			68.72933	
BIF A	3			71.98667	

LAB B	3			72.58167		
LAB A	3					82.75567
BIF B	3					83.94367
LB *Casei Sirota*	3					85.00000

Table C.3 Post hoc test for acid resistance at 4.0

Name of isolates	N	Subset for alpha = 0.05				
		1	2	3	4	5
LEU B	3	22.33500				
LEU A	3	26.34833				
LCC B	3		34.66467			
LCC A	3		41.48933	41.48933		
LCC C	3			47.55300		
LAB C	3				61.80133	
LAB B	3				65.93300	
BIF A	3				66.78800	
LAB A	3					79.23367
BIF B	3					79.75833
LB *Casei Sirota*	3					80.19333

Appendix D

ANOVA for bile salt resistance test

Table D.1 One way ANOVA for bile salt resistance test of all the isolated organisms

		Sum of Squares	df	Mean Square	F
Bile salt resistance 0.3%	Between Groups	15872.400	10	1587.240	122.592
	Within Groups	284.842	22	12.947	
	Total	16157.242	32		
Bile salt resistance 0.4%	Between Groups	15370.996	10	1537.100	153.144
	Within Groups	220.814	22	10.037	
	Total	15591.809	32		

Table D.2 Post hoc test for bile salt resistance at 0.3%

Name of isolates	N	Subset for alpha = 0.05				
		1	2	3	4	5
LEU B	3	30.53833				
LCC A	3	35.12400	35.12400			
LCC B	3	36.69400	36.69400			
LEU A	3		42.44667			
LCC C	3		43.97400			
LAB C	3			65.07500		
LAB B	3			66.01667		
BIF A	3				74.76067	

LAB A	3				87.85667
BIF B	3				87.96167
LB *Casei Sirota*	3				89.29500

Table D.3 Post hoc test for bile salt resistance at 0.4%

Name of isolates	N	Subset for alpha = 0.05			
		1	2	3	4
LEU B	3	26.98233			
LCC B	3	27.24533			
LCC A	3	28.38000			
LCC C	3	32.57333			
LEU A	3	34.46000			
LAB B	3		57.52667		
LAB C	3		60.85000	60.85000	
BIF A	3			67.28500	
LAB A	3				79.32200
BIF B	3				79.43333
LB *Casei Sirota*	3				80.30133

Appendix E

ANOVA for cell surface hydrophobicity test

Table E.1 One way ANOVA for cell surface hydrophobicity test of all the isolated organisms

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	5377.085	10	537.708	58.436	.000
Within Groups	202.437	22	9.202		
Total	5579.522	32			

Table E.2 Post hoc test for cell surface hydrophobicity

Name of isolates	N	Subset for alpha = 0.05			
		1	2	3	4
LCC B	3	43.7547			
LCC C	3		51.4650		
LEU A	3		53.3847		
LEU B	3		54.7540		
LAB B	3			64.5150	
LCC A	3			65.4440	
LAB C	3			69.0940	
BIF A	3				77.8140
BIF B	3				79.2467
LAB A	3				79.7720
LB *Casei Sirota*	3				82.8873

List of Plates

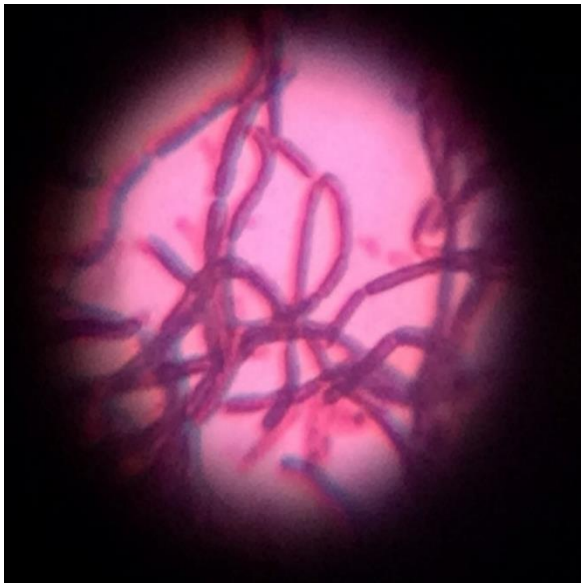

Plate 1 Microscopic observation of Lactobacilli

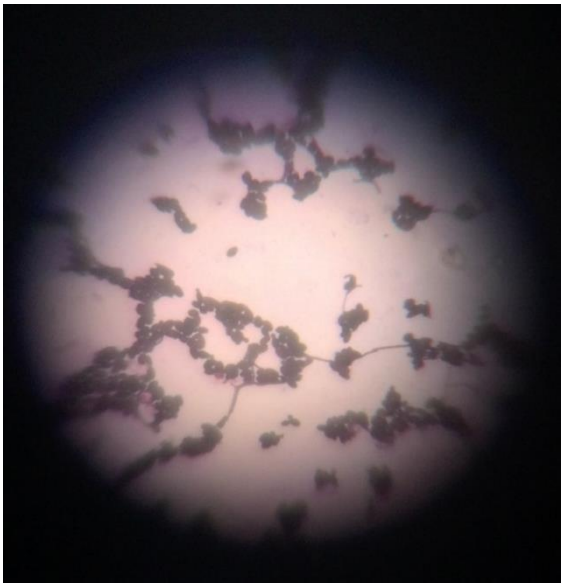

Plate 2 Microscopic observation of *Bifidobacterium*

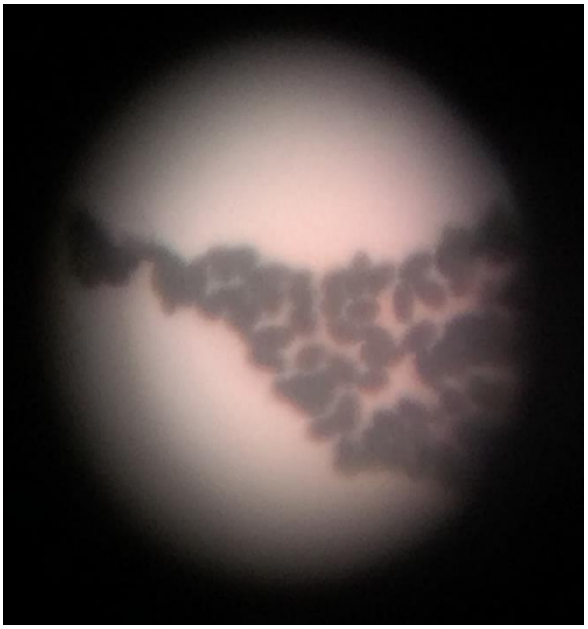

Plate 3 Microscopic observation of Leuconostocs

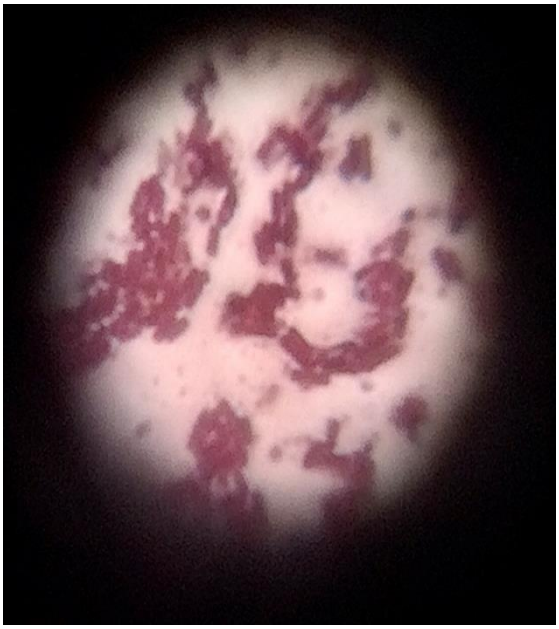

Plate 4 Microscopic observation of Streptococci

Plate 7 Performing laboratory work

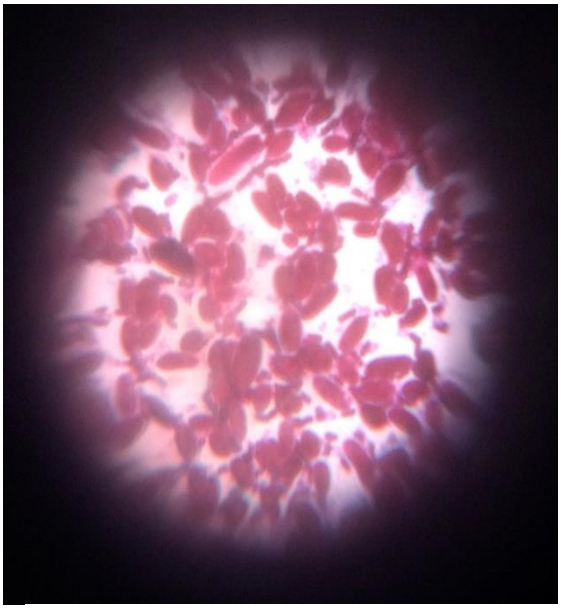

Plate 6 Microscopic observation of Lactococci

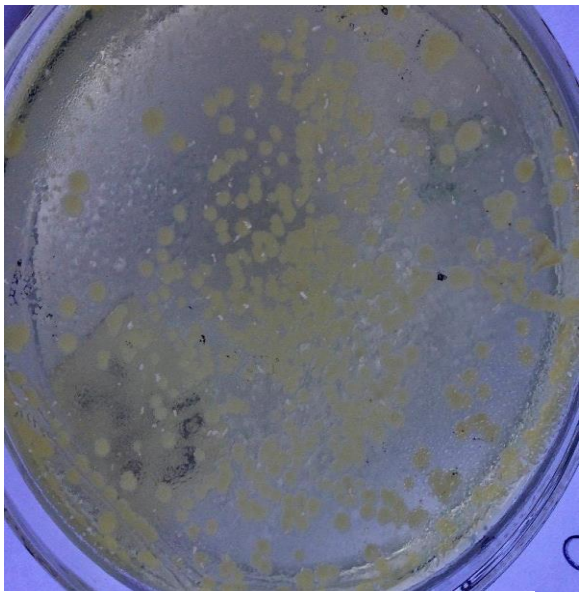

Plate 7 Lactobacilli on MRS plate

Plate 8 Lactobacilli streaked plated on MRS agar

Name of the Student: Pawan Khanal

TU Reregistration No: 5-2-768-14-2009

Roll No: FT59/073

Contact No: 00977-9849608098

Email address: khpawan51@gmail.com

Photo: