MOLECULAR AND PROBIOTIC CHARACTERIZATION *OF LACTOBACILLUS* SPP. ISOLATED FROM TRADITIONALLY PREPARED CURD (*DAHI*) AT DIFFERENT GEO-CLIMATIC CONDITIONS OF NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY
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NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

RANJAN KOIRALA

MARCH, 2016

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DECLARATION

"**Molecular and Probiotic Characterization of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) at different geo-climatic conditions of Nepal**" is being submitted to the Central Department of Biotechnology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Rajani Malla, Central Department of Biotechnology, Tribhuvan University and co-supervised by Dr. Sangita Shrestha, Nepal Academy of Science and Technology (NAST), Nepal.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

…….

Ranjan Koirala

RECOMMENDATION

This is to recommend that **Ranjan Koirala** has carried out research entitled "**Molecular and Probiotic Characterization of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) at different geo-climatic conditions of Nepal**" for the award of Doctor of Philosophy (Ph.D.) in Biotechnology under our supervision. To our knowledge, this work has not been submitted for any other degree. He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

……
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MARCH, 2016

LETTER OF APPROAVAL

Date: DD/MM/YY

On the recommendation of Dr. Rajani Malla and Dr. Sangita Shrestha, this Ph.D. thesis submitted by Ranjan Koirala entitled “Molecular and Probiotic Characterization of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) at different geo-climatic conditions of Nepal” is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U.

……

Prof. Dr. Rajani Malla
Head
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

DEDICATION

Dedicated to my beloved mother

Late Nirmala Koirala

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Ranjan Koirala

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ABSTRACT

Dairy products are an important part of daily food in the Himalayan country Nepal. A wide variety of traditional fermented milk products are prepared from the milk of different animals in different geo-climatic conditions of the country by different ethnic groups. Locally called, *Dahi* (curd) is the yoghurt like most popular product, but little data are available on the autochthonous Lactic Acid Bacteria (LAB) harbored in these products and characterization of their molecular and probiotic properties. These information are essential for authentic identification of beneficial strains and probiotic product development. To address these problems, this project was designed with objectives of molecular and probiotic characterization of lactobacilli isolated from traditionally prepared curd (*dahi*) and later preparation of curd using potential and promising strains. For this, total 64 samples were collected from four districts belonging to two geographical regions of Nepal. Of the 193 LAB obtained, 120 isolates (68%) were found to be rod shaped presumptive lactobacilli while 73 isolates were found to be cocci (32%). The dominant lactobacilli were further characterized at phenotypic, biochemical and molecular levels. Phenotypic and biochemical characterization such as colony morphology, microscopy (grams staining, negative staining & motility test) and catalase test were performed for presumptive confirmation of the *Lactobacillus* species. The presumptive 120 isolates were further identified at species level by using Internal Transcribed Spacers (ITS) amplification, 16S rRNA gene sequence determination, and species-specific PCR assays. Based on molecular analyses, the 120 isolates were classified as belonging to ten different *Lactobacillus* species *viz. L. delbrueckii*, *L. paracasei*, *L. rhamnosus*, *L. fermentum*, *L. parabuchneri*, *L. helveticus*, *L. coryniformis*, *L. harbinensis*, *L. brevis* and *L. plantarum*. *Lactobacillus delbureckii* was identified up to subspecies as *L. delbrueckii* subsp. *bulgaricus* using species specific PCR while representative isolates of *L. paracasei* were also identified at subspecies level as *L. paracasei* subsp. *paracasei* using High Resolution Melt Curve Analysis. Intra-species genetic diversity analysis was studied for *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei*, *L. rhamnosus*, *L. fermentum* and *L. parabuchneri* using various markers. A high degree of intra-species genetic diversity was obtained for all *Lactobacillus* species, except for *L. rhamnosus* isolates, all of which produced a single typing profile.

Of the 120 isolates, 24 were further selected for the analysis of probiotic properties based on the intra-species genetic diversity study and the geo-climatic origin of the isolates. Probiotic properties such as gastrointestinal transit resistance (acid and bile tolerance), adhesion, antimicrobial activity and antibiotic sensitivity test were investigated. All the isolates were able to survive the gastrointestinal transit resistance at pH 3 and 0.3% bile (average viable count Log10 CFU/mL ranged from 4.95 to 7.85) while only 15 isolates were able to survive the gastrointestinal transit resistance at pH 2 and 0.3% bile (average viable count Log10 CFU/mL ranged from 1.69 to 7.66). Seventeen lactic isolates were found to be adhesive while seven were non-adhesive in Caco-2 cell monolayer (average number of adhesion in Log10 ranged from 0.69 to 2.94). The antimicrobial activity tested by paper disc method was found to show weak inhibition towards the enteric food borne pathogens. The antibiotic sensitivity test was performed according to European Food Safety Authority (EFSA) guidelines. Of the 24 isolates, four isolates *viz. L. paracasei* NAST-RHM82, *L. fermentum* NAST-GHL2, *L. helviticus* NAST-RHL103 and *L. corniformis* NAST-RHM94 were found to be safe when tested for nine different antibiotics.

Further, six lactic cultures namely *L. fermentum* NAST-GHM2, *L. rhamnosus* NAST-GHM25, *L. delbrueckii* subsp. *bulgaricus* NAST-RHL74, *L. paracasei* NAST-RHM82, *L. delbreuckii* subsp. *bulgaricus* NAST-RHL101 and *L. helveticus* NAST-RHL103 were selected based on the *in vitro* probiotic attributes study for assessment of antimicrobial activity against enteric pathogens *viz. Bacillus cereus* F 4810, *Escherichia coli*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* FRI 722 using agar well diffusion method. Of the six isolates, two strains *viz. L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 were further selected for partial purification and characterization of the antimicrobial compound using skimmed milk broth. The strength of the inhibition (both bacteriostatic & bactericidal) was higher in cell free supernatants (CFSs) derived from skimmed milk broth as compared to the MRS broth. The skimmed milk concentrate of the respective cultures were further purified by using methanol and later methanol concentrate was purified (concentrated) using acetone. The strength of the inhibition increased relatively following different purification steps. Antimicrobial activity of skimmed milk concentrate and its derivatives of *L. helveticus* NAST-RHL103 were found to be very high as compared to *L. paracasei* NAST-RHM82 against all the pathogens.

Following the partial purification, probiotic curd and sweet curd were prepared and the effects of pre-processing and post-processing contaminants (using above mentioned four pathogenic strains) were analyzed to know the efficiency of the lactic culture for future biopreservative agent. Product formation conditions such as incoculum size (1%) and incubation conditions (time, temperature) were optimized and the product was developed with optimum conditions. *Lactobacillus helveticus* NAST-RHL103 formed the product with 1% inoculums at 37 ºC in 8 hours while *L. paracasei* NAST-RHM82 formed the product with 1% inoculum at 37 ºC in 30 hours. Microbiological, chemical and organoleptic properties of both the products were analyzed and found to be of high quality. However, from the results, it has been confirmed that the product formed with *L. helveticus* NAST-RHL103 is superior due to its better probiotic attributes, antimicrobial activity and the short incubation time. The results obtained for pre-processing and post-processing contaminant effects towards the pathogens showed that the contaminants could not grow in the probiotic curd entered as pre-processing contaminants, however the post-processing contaminants decreased considerably after 24 hours of storage in refrigerator. In addition, sweet curd was prepared using *L. helveticus* NAST-RHL103. This culture is also able to ferment the milk containing up to 20% sugar and produce sweet curd with acceptable chemical and sensory properties. Because of the innate properties of the lactic culture; *L. helveticus* NAST-RHL103 can be a potential probiotic candidate for industrial applications. Besides probiotic properties, this culture may also be developed as the bio-preservative agent in functional foods to increase the shelf life and to phase out the indiscriminate use chemical preservatives.

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CHAPTER 3

3. MATERIALS AND METHODS

LIST OF ACRONYMS AND ABBREVIATIONS

AAD	Antibiotic Associated Diarrhoea
AFLP	Amplified fragment length polymorphism
APACHE	Acute Physiology and Chronic Health Evaluation
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU	Arbitrary unit
BHI	Brain Heart Infusion
BP	Base pair
CFSs	Cell free supernatants
CFU	Colony forming unit
CoA	Coenzyme A
DHAP	Dehydroacetone phosphate
DNA	De-oxy ribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene-diaminetetraacetic acid
EFSA	European food safety authority
EMP	Embden Meyerhof Pathway
EtBr	Ethidium bromide
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and agriculture organization

FDP	Fructose-1, 6-diphosphate
FOS	Fructo-oligosaccharides
GAP	Glyceraldehyde-3-phosphate
GC	Gas chromatography
GC	Guanine, a purine base, and cytosine a pyrimidine base
GIT	Gastrointestinal transit resistance
GLB	Gel loading buffer
GPS	Geographical positioning system
GRAS	Generally regarded as safe
HRMa	High Resolution Melt Curve analysis
IBD	Inflammatory bowel disease
IBS	Irritable Bowel Syndrome
IgA	Immunoglobulin A
IGS	Intragenic spacer
ITS	Internal transcribed sequence
LA	Lactic acid
LAB	Lactic acid bacteria
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
MRS	de Rogosa and Sharpe
MSNF	Milk solid not fat
NDOs	Non-digestible oligosaccharides
NSLAB	Non-starter lactic acid bacteria
NTSYS	Numerical taxonomic system

ºC	Degree Celsius
PB	Phosphate buffer
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPM	Rotation per minute
RSA	Ribosomal spacer analysis
RT PCR	Real-time polymerase chain reaction
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SEM	Scanning electron microscope
SM	Simple matching coefficient
SNF	Sold not fat
TA	Titratable acidity
TAE	Tris-acetate EDTA
Taq DNA	Polymerase DNA polymerase enzyme isolated from *Thermus aquaticus* bacteria
TD	Travellers' Diarrhoea
UK	United Kingdom
UPGMA	Unweighted pair group method of arithmetic averages
USA	United States of America
WHO	World health organization
ZOI	Zone of inhibition

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1.0. INTRODUCTION

1.1. Introduction

1.1.1. Background

Human beings and other higher life forms would have never arisen and could not have sustained till now without the microorganisms. The co-existence of microbes with higher forms of life and symbiotic relationship for several host related functions show the necessity of microbes. Over millions of years, the diversity and functions of microorganisms has continued to change so as to adapt in the new habitats. Microorganisms, especially bacteria, exist in a wide variety of environmental niche and are found as complex communities rather than single cellular planktonic cells. The population present in each community is continually modulated in order to adapt and survive. The identification and culturing of all bacterial species in a given niche is not yet possible, but advancements in the field of biotechnology and culture independent techniques have enabled researchers to monitor the evolutionary divergence that can be used to identify and classify the bacteria.

Fermenting fruits, vegetables, cereals and milk can bring many benefits to the people. It plays important roles in providing food safety, enhancing health and improving the nutrition and social well-being of millions of people around the world. The food processing sector is more focused on innovations that could provide these requisite functional attributes through traditional foods which are inherent with human civilization. Studies into traditional foods have established the multiple advantages with reference to nutritive values, therapeutic properties and sensory attributes (Roopashri, 2012). The early human civilization with no concept of microorganisms was remarkable not only in developing the products, but also safely maintaining the cultures though centuries. In all these fermented foods, the process of fermentation is mainly initiated by lactic acid bacteria (LAB), followed by yeast in a few of them (Farnworth, 2008; Arques et al., 2014; Fernandez et al., 2014).

In general fermented foods are characterized by the accumulation of metabolites of microbial activities, both primary and secondary, which contribute to the overall functionality of products. Lactic acid bacteria which are most important bacteria and likely to bring desirable changes by fermentation of different substrates such as sour dough bread, sorghum beer, all fermented milks, and most “pickled” (fermented)

vegetables. *Lactobacillus acidophilus, L. bulgaricus, L. plantarum, L. pentoaceticus, L. brevis* and *L. thermophilus* are examples of lactic acid producing bacteria involved in food fermentations (Roopashri, 2012). Among them, some are homofermentative (produce lactic acid only) while others are heterofermentative (produce lactic acid and other volatile compounds and small amounts of alcohol).

There are sufficient evidences from various research for the hypothesis that diet is instrumental in controlling and modulating various functions of the body so that good health and a disease-free life is made possible (Varadaraj et al., 1993). It is this hypothesis, which is the corner stone of the concept of 'functional foods'. A functional food is defined as a 'food that contains an adequate concentration of one or more combinations of components which affects functions in the body so as to have positive cellular or physiological effects'. Any food may be termed "functional", if it fulfills the requirements of (i) demonstrates an interaction with one or more functions of the body, (ii) there is some understandings of the underlying mechanism of action and (iii) the establishment of the effect in relevant biological systems (Cakir, 2003).

Knowing or unknowingly, the human race has been consuming fermented products from time immemorial. Their origin has been primarily milk-based which has been the basis for the many investigations or the probable health benefits of these traditional fermented foods (Barrett et al., 2004). Although, Metchnikoff had worked upon this concept in the beginning of the 19^{th} century as an alternative to modification of mal-functioning gastrointestinal tracts, unfortunately, it was neglected for a long period of time. It was only from early 1990s, that focused attention has increased in view of the wellness of substance of biological origin, rather than performing those of chemical origin. The word 'probiotic' which is derived from two Greek words meaning "for life" was not established by definition till 1965. Any probiotic strain selected should be capable of withstanding the many unit operations of food processing such as concentration, freezing, storage conditions, cooking conditions of temperature and pressure so that a final required count of between 10^6–10^8 CFU bacteria is maintained in the product (Otles, 2013).

1.1.2. Traditional dairy products and lactic acid bacteria

In many countries, the microbiological characteristics of traditional fermented milk products have been studied for past few decades, and original collections of lactic acid

bacteria have been constituted (Karki, 1986; Tamang and Sarkar, 1988; FAO, 1990; Yu et al., 2011; Bao et al., 2012). They represent an important tool both for preserving the rich microbial biodiversity that characterizes naturally fermented food and for obtaining new cultures for commercial applications. It is known that traditional fermented foods have unique and different microbial populations dependent on the production technology as well as on the environmental characteristics of the localities where they have been produced (Colombo et al., 2009). Moreover, many reports have shown that artisanal dairy products can represent interesting source for isolation of bacterial strains with useful probiotic traits (Taverniti and Guglielmetti, 2010; Heller, 2011).

Dairy products are an important part of daily food intake in the Himalayan country, Nepal. As a consequence of availability of different geo-climatic conditions and the diversity of ethnic groups in the country, a wide variety of traditional fermented milk based products are produced and consumed such as curd (*dahi*), mohi, ghiu, chhurpi and others. These products are prepared with milk from different animals that are reared in the different geographical regions for dairy production, such as cow and buffalo are reared in the Terai and Hilly regions (from 100 to 3000 m) whereas Yak and Chauri at high altitudes (> 3000 m) in the Himalayan region.

In recent years, there has been a growing interest in genotypic and phenotypic studies on wild microbial isolates from traditional dairy products produced mainly without the addition of any starter cultures (Cogan et al., 1997; Dahal et al., 2005). Increasing information on the microbial diversity present in traditional dairy products can help prevent the loss of microbial biodiversity and consequently the loss of wide range of milk based traditional dairy products by different methods whose typical features depend on local and regional traditions and on the indigenous microbial population present in raw milk and selected by the environment (Fortina et al., 2001). Additionally, these studies could be the basis for the selection of new strains to be used either for large scale commercial production of specific cultures, traditional curd itself or together with classical starter cultures for improving the existing dairy product manufacture. Indeed, the diversity of starters used in industrial dairy fermentation is low and there is an increasing demand for new strains, starter and Non-starter Lactic Acid Bacteria (LAB and NSLAB), that show advantageous effects on various dairy products (Jacobsen et al., 1999; Delgado et al., 2007).

1.1.3. Probiotic properties of lactobacilli obtained from dairy products

Traditional fermentation is the most convenient, economic and widely applied empirical method for food preservation and also for enhancement of organoleptic and nutritional quality of fresh foods (Fortina et al., 2001). Traditional fermented foods are produced by the indigenous biotechnological process where natural microbiota associated with food materials are involved during as well as after fermentation. Metchnikoff (1908) who is also the inventor of probiotics, proposed that the acid producing organism in fermented dairy products could prevent fouling in the large intestine and thus lead to a propagation of life span of the consumer, but later it has been claimed that only strains of human origin could ensure a beneficial attributes in humans (Saarela et al., 2000; Heller, 2011; Coeuret et al., 2003). However, labeling of probiotic products has always been controversial as they are originated from intestinal flora (Gardiner et al., 1998) and hence organisms originated from other sources such as dairy fermented foods could be a potential probiotic candidate (Coeuret et al., 2004).

Over the years, mankind exploited lactic acid bacteria (LAB) for the production of traditional fermented dairy products because of their ability to produce desirable changes in taste, flavor and texture, extending the shelf life of food products (Dave & Shah, 1996; Fernandez et al., 2014). The popularity of dairy products is growing due to its perceived health benefits. In addition, many studies have reported that fermented dairy products such as yoghurt, cheese and other fermented milks can be best substrates to deliver probiotic LAB (Salminen, 1999). The presence of LAB in intestinal epithelium of human gastrointestinal tract, and their traditional use in fermented foods including dairy products without any adverse health problems prove their safety (Pangallo et al., 2008).

Locally called *Dahi* (curd) is the most indigenous and popular milk product of Himalayan country Nepal. It is a yoghurt-like product prepared in different parts of the country and used as nutritional food, appetizer or dessert as well as for the preparation of other ethnic dairy fermented products such as Ghiu, Mohi and soft Chhurpi (Tamang, 2010). LAB play significant role in almost all the fermented foods as well as in curd (*dahi*). It comprises a wide range of genera and *Lactobacillus* is one of the important genera. More than 80 species of *Lactobacillus* are harbored in dairy products (Coeuret et al., 2003) and some of them are natural components of the

gastrointestinal microflora (Dellaglio & Felis, 2005). Research on *Lactobacillus* isolated from the naturally fermented dairy products showed a long history of safe use (Holzapfel & Schillinger, 2002).

The health beneficial effects of lactobacilli have been widely explored and include stabilization of the indigenous microbial population, protection against intestinal infection, improvement of lactose intolerance, enhancement of nutrients bioavailability, reduction of the serum cholesterol level and non-specific enhancement of immune systems (Matar et al., 2001; Santosa et al., 2006; Gaudana et al., 2010). Several lactic acid bacteria including lactobacilli are being studied for novel bio-therapeutic agents (Sullivan & Nord 2005; Reid et al., 2006). The presence of lactic acid bacteria in curd makes it a therapeutic agent as these bacteria convert lactose into galactose and glucose which is beneficial for lactose intolerant patients) and it also prevents various stomach and intestinal disorders like autointoxication. Its usage is increasing day by day due to its health benefits such as enhanced intestinal health, preventing constipation, dysentery, diarrhoea and gastrointestinal infections. It may effectively reduce blood cholesterol (Kaic & Antonic, 1996), coronary heart disease, breast and ovarian cancer and also increases the level of immunity (Yadav et al., 2005; Jain et al., 2007). *Dahi* (Curd) prepared by fermentation of milk (from different animals such as Cow, Buffalo, Chauri, Yak) with an inoculum of previously made curd, is used in most households in Nepal where it constitutes a significant part of the daily diet. The LAB that ferment the milk are likely to differ slightly in each household as there is no standardized starter culture used to prepare curd. Curd is believed to have probiotic properties (Dewan et al., 2007; Dahal et al., 2005; Saran et al., 2002). Some research findings and documentations of curd in the literature is available from South Asian region but no such systematic research in LAB and probiotic properties of curd have been carried out in Nepalese context so far.

1.1.4. Noble lactobacilli, probiotic curd and future biopreservative agent

Lactic acid bacteria perform fermentative as well as preservative action in the product. The lactic acid that is produced from the fermentation of lactose (into glucose and galactose) contributes to the sour taste of curd (*dahi*) or yogurt by decreasing pH and formation of the characteristics texture by acting on the milk proteins. When pH drops below pH 5, miscelles of hydrophobic protein (caseins) loses its tertiary structure due to the protonation of its amino acid residues and

denatured protein resembles by interacting with other hydrophobic molecules. This interaction of caseins creates a structure that allows for the semisolid texture of yogurt or curd (Fadela et al., 2009; Lin & Pai, 2009). One of the best strategies for promoting safety of foods and extending their shelf life without any side effects on consumer's health and also for maintaining nutritious and organoleptic characteristics of food products is via using bio-preservation with the emphasis on LAB (Castellano et al., 2008). The LAB produce different antimicrobial compounds such as organic acids, hydrogenperoxide, carbondioxide, diacetyl, low molecular weight antimicrobial substances (for example, Reuterin and Reutericyclin) and bacteriocins (Ammor et al., 2006; Fernandez et al., 2014; Nami et al., 2015). Bacteriocins are ribosomally synthesized peptides or proteins which reveal antagonistic effects against other species particularly closely related to producer cell (Settanni & Corsetti, 2008). Bacteriocins produed from lactic acid bacteria have some advantages that nominate them as good choice for biopreservation. These bacteriocins are generally recognized as safe (GRAS) (Deegan et al., 2006) as they have no antagonistic effects on eukaryotic cells excepting cytolysin, produced by *Enterococcus faecalis* (Heng et al., 2007).

Since a long history, lactic acid bacteria have been playing a key role in preserving foods from spoilage microorganisms. They are commonly used in food fermentation, may produce several metabolites with beneficial health effects and, thus are generally recognized as safe (Fernandez et al., 2014). They are also one of the major bacterial groups present in milk with potential to produce antimicrobial compounds (Arques et al., 2014). Few of them have already been recognized as probiotics (Deegan et al., 2006). Besides starter culture in food, LAB have also been used for flavor and texture production in the food for long time (Caplice & Fitzgerald, 1999). Antagonistic action of these LAB, over harmful bacteria, makes many traditional and commercial foods microbiologically safe (Aguilar et al., 2011; Arques et al., 2014).

The use of chemical preservatives can be reduced considerably by the selection and addition of proper novel isolates of lactic cultures, enhancing nutrients and extending shelf life of food products (Bela et al., 2013). The lactic cultures obtained from various dairy products have been used in food preservation (Mirhosaini et al., 2006, 2010; Yateem et al., 2008; Ali, 2011). Lactobacilli, *Lactococcus lactis* and *Streptococcus thermophilus* are major LAB which inhibits food spoilage and

pathogenic bacteria thereby preserving the nutritive qualities of raw food material for an extended shelf life (O'Sullivan et al., 2002; Heller, 2011). Several metabolites and end products of LAB fermentation act as biopreservatives by reducing the risk of food borne diseases (Maciel et al., 2003) and increasing the shelf life of foods (Deegan et al., 2006). Thus, the presence of LAB may confer and enhance the desirable qualities leading to safety of products (Adams & Nicolaides, 1997). LAB were first isolated from fermented milk (Metchnikoff, 1908; Carr et al., 2002) and have since been found in food such as meat, milk products, vegetables, beverages and bakery products (Aukrust & Blom, 1992; Harris et al., 1992; Gobbetti & Corsetti 1997; Caplice & Fitzgerald 1999; Jay 2000; Lonvaud-Funel, 2001; O'Sullivan et al., 2002; Liu, 2003).

Bacillus cereus, Escherichia coli, Listeria monocytogenes and *Staphylococcus aureus* are frequent contaminants in milk products such as curd (*dahi*), cottage cheese, mawa, khoa, cream, butter and gulab jamun (Kumar & Sinha 1989; Rea et al., 1992; Oranusi et al., 2007). In case of milk products, the highest contamination was recorded in burfi (33%), followed by *dahi* (20%), gulab jamun (20%), Khua (13%) and ice cream (7%) (Kumar & Prasad, 2010). Different sources involved directly or indirectly during production of milk and its products such as dirty hands of workers, poor quality of milk, unhygienic conditions of the manufacturing unit and water supplied for washing the utensils are responsible for microbial contamination of milk products besides the post-manufacturing contamination (Tariq Masud et al., 1988; Elmahmood & Doughari, 2007). *Escherichia coli, B. cereus, L. monocytogenes* and *S. aureu* were reported to be the most common contaminant pathogenic bacteria for gastrointestinal infections, including food poisoning and food borne illness in Nepal (Joshi et al., 2003). Therefore, the use of lactobacilli having fermentative as well as preservative properties can overcome the problem pathogen contamination in the milk products.

It is important from an industrial point of view as any industry incorporating probiotic cultures should be able to cultivate and maintain the species and required strain(s) in the purest of forms and in very high quantities so that a sufficient count can be maintained. The strain so selected should not be viable to frequent mutations as this can change the characteristics, rendering strain addition useless, if not harmful (Dellaglio & Felis, 2005). On the other hand, it has to be amenable to molecular modification techniques so that some other desired properties may be incorporated to make the existent strain a superior one.

Owing to such varied health benefits and the age old practice of consuming these cultures through fermented foods has given it a good degree of safety, yet stringent safety and toxicity studies are being conducted, so that no aspect of the above mentioned properties remains unproven scientifically. This implies that the addition of the probiotic cultures should not impart any undesirable taste, odour or any other sensory attribute other than that required in the food product. If a culture fails to achieve this objective, then external additive addition may be necessitated which may change other product parameters and also decrease the effectiveness of the culture added.

1.2. Rationale

In the varied geo-climatic conditions found in the Himalayan country Nepal (ranging from low land Terai to high Himalaya), a wide range of dairy products originating from different animals such as Chauri, Cow, Buffalo and Yak are produced for domestic and commercial purposes. Also, very limited works so far have been carried out in LAB and Probiotics from dairy products of Nepal. Growing interest in Probiotics due to their human health benefits prompted us to undertake isolation, molecular and probiotic characterization of indigenous lactic cultures originating from traditionally prepared curd (*Dahi*).

All the lactobacilli obtained from curd collected from various geo-climatic conditions may not possess probiotic properties and hence it is necessary to screen and characterize for probiotic properties for future food, medical and industrial applications. Viability and survival of probiotic bacteria are important characteristics in order to provide health benefits. Besides colonization of the GIT, there are several factors including ability of the bacteria to tolerate acidic pH of the stomach and bile, binding capacity to intestinal cells and mucus, antimicrobial inhibition and antibiotic resistance profile are often the main criteria in order to be a putative probiotic candidate (van Belkum & Neiuwenhuis, 2007). However, a single probiotic candidate may not exhibit all the probiotic properties (Dellaglio & Felis, 2005). The beneficial microorganisms obtained from curd collected in Hilly and Himalayan regions of Nepal could be a potential source of biological materials for dairy industries. However, limited studies aiming at characterizing the diversity and probiotic properties of indigenous LAB of traditionally prepared *dahi* produced in different geo-climatic conditions of Nepal have been carried out so far.

The fermented milk products such as curd containing LAB are traditionally used every day as food in Nepal. The development of starter culture having fermentative as well as probiotic properties (pathogen protective) would hold a great promise for dairy industries. Hence present study was undertaken to examine antimicrobial properties of lactic cultures having some probiotic activities to develop probiotic curd (*dahi*) and sweet curd (*dahi*) in laboratory scale. Given the beneficial effects of probiotics, it is essential that a safe mode of their administration be established. Foods are an ideal mode of probiotic administration, as they are easily incorporated in them and also reach the site of action without any hassles.

Also, the concept of fermented foods, which is one of the easiest modes of probiotics incorporation, is well established and accepted. The great diversity in the traditional fermented food preparations gives ample opportunity for the use of many available cultures in the most suitable food matrices. Thus the available and novel foods that incorporate probiotics may be classified based on the substrate used for fermentation as milk based, cereal based, vegetable based and meat based. Characterizing indigenous lactic cultures from traditional fermented dairy such as curd (*Dahi*) and using them as probiotic culture by developing products with long shelf life, desired viable count having health promoting effect can contribute on a large scale to the human well being in the near future.

Research questions of this entire investigation are: 1) What kind of *Lactobacillus* species are found in curd? 2) Which are the dominant lactic acid bacteria present in traditionally prepared curd (*Dahi*) at different geo-climatic conditions of Nepal? 3) Does diversity exist in *Lactobacillus* isolates obtained from curd (*dahi*) prepared at different geographical location? 4) Does probiotic properties vary among *Lactobacillus* spp. Isolated from different locations? 5) Is it possible to develop a probiotic curd (*Dahi*) using a novel lactic culture having probiotic as well as fermentative properties?

1.3. Objectives

Under the present study, the isolation, molecular and probiotic characterization of lactobacilli was aimed from traditionally prepared curd (*dahi*) originating from different geo-climatic codifications in search of identifying novel probiotics with significant probiotic attributes for human consumption. In order to establish LAB as

potential probiotics, the isolates must be a known isolate (molecular identification is must), possess probiotic properties such as acid and bile tolerance, antimicrobial activity towards pathogens and adhesion ability to intestinal epithelial cells. Additionally, the lactic isolates should be safe for use with respect to transmission of antibiotic resistance genes. The milk product prepared with lactic culture with such properties could be an ideal product. Hence, to address these problems, following objectives have been set.

1.3.1. To isolate lactobacilli from traditionally prepared curd (*Dahi*) samples at various geo-climatic conditions of Nepal and to characterize them at phenotypic, biochemical and molecular level.

The specific objectives are:

1.3.1.1. Isolation and Biochemical Characterization of *Lactobacillus* spp. from curd samples.

1.3.1.2. Identification of the lactobacilli isolates at species level and study their intra species molecular diversity.

1.3.2. To characterize the probiotic properties of selected lactobacilli isolated traditionally prepared curd.

The specific objectives are to:

1.3.2.1. Evaluate gastrointestinal transit resistance.

1.3.2.2. Find ability to adhere Caco-2 monolayer.

1.3.2.3. Screen for antimicrobial activity against selected food borne pathogens.

1.3.2.4. Identify antibiotic resistance according to EFSA guidelines.

1.3.3. To prepare indigenous probiotic curd (*Dahi*) using the most potent lactobacilli isolates in laboratory scale.

The specific objectives are to:

1.3.3.1. Partially purify the antimicrobial substance of the indigenous lactic cultures (active against food borne pathogens).

1.3.3.2. Use indigenous novel lactic isolates for the preparation of Probiotic Curd

1.3.3.3. Evaluate the efficiency (action against pre-processing and post-processing contaminants) of lactic cultures for future biopreservative agents.

2.0. LITERATURE REVIEW

2.1.0. Microoganisms, Foods and Human Health

Human health is one of the main reasons behind food choices and this has led to a diverse range of food formulations with specific functionalities that provide better health and wellness. Among several health disorders, the most common health disorders associated with the diet pattern is that of gastrointestinal (GI) disorders. Such GI disorders can be prevented to a certain extent through routine consumption of foods with specific functionality. Hence, the concept of functional foods evolved as the role of food in the maintenance of human health and well-being and has received increased scientific and commercial interest (Swinbanks & O'Brien, 1993). For the preparation of functional food products, lactic acid bacteria (LAB) and Bifidobacteria are widely used. These organisms have been termed as 'Probiotic bacteria' which impart certain specific health promoting attributes through oral feeding (Marks, 2004). Simultaneous with probiotics, the other term 'prebiotics' are known to be non-digestible food ingredients (higher polysaccharides such as GOS, FOS, inulin) which beneficially affect the host by selectively stimulating growth and/or activity of selected group of bacterial genera (stimulation of the probiotic microroganisms) that are normal inhabitants of colon (Gibson & Roberfroid, 1995).

One of the essential criteria to confer health benefits on the host is that probiotic microbes must be able to grow in the human intestine and therefore, should possess the ability to survive the passage through Gastro Intestinal Tract (GIT), and this involves exposure to acidic environment in the stomach and bile in the small intestine (Kearney et al., 2008). Most of the probiotic strains are natural inhabitants of the human intestine and are 'Generally Regarded As Safe' (GRAS) along with acid and bile tolerance and ability to adhere to gut epithelial cells (Lankaputhra & Shah, 1998; Dunne et al., 2001). Hence, the best designed route for the entry of these probiotic bacteria is the diet, both for animals and human beings (Farnworth, 2008). Fermented foods based on milk, cereals and legumes are among the most accepted and widely used food carriers for delivery of viable probiotic cultures (Kearney et al., 2008; Roopashri, 2012). Probiotic bacteria and their fermentation products appear to influence the human health by preventing the colonization of pathogenic microbes (Gmeiner et al., 2000; Fernandez et al., 2014). There has been substantial evidence for

the benefits of probiotics and prebiotics in lowering of (i) lactose intolerance through the activity of ß-galactosidase, (ii) antibiotic associated diarrhea, (iii) colon carcinogenesis, (iv) hypocholesterolemic effect and (v) gut mucosal dysfunction (Gallaher & Khil, 1999; Isolauri et al., 2001; Salminen et al., 2004; Schmid et al., 2005; Casci et al., 2007).

Several research studies have established the ability of probiotic bacteria to inhibit pathogenic bacteria by the production of organic acids (such as lactic and acetic acids) during fermentation process leading to low the pH of the intestine and as a result inhibits the growth of undesirable bacteria (Goktepe, 2006; Varalakshmi et al., 2014). In addition to these beneficial health effects, researchers have also reported that the major end products of fermentation in humans are the Short Chain Fatty Acids (SCFA) like those of acetate, propionate, and butyrate (Cummings 1981, 1991). Besides, few other antimicrobial substances produced widely by lactic acid bacteria include hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins (Ouwehand & Vesterlund, 2004). Probiotic bacteria like LAB and bifidobacteria are also known to synthesize folate, vitamin B12 and vitamin K, which are vital components of the human diet and involved in the biosynthesis of nucleotides and cofactors in many metabolic reactions (Barrett et al., 2004).

2.2.0. Traditional Fermented Foods in Nepal

Fermentation is one of the oldest food preservation methods, which became popular with the dawn of civilization. As a result, the development of a variety of tastes, forms and other attributes have been coming up in the present society (Campbell-Platt, 1987). It appears that fermentation originated in the settlements of Indian subcontinent that predate the great Indus Valley civilization (Farnworth, 2008). Fermentation is a process of convertion of complex raw materials into simple one i.e. into a range of value added products by utilizing the growth phenomena of microorganisms and their activities on various substrates, wherein the knowledge of microorganisms is essential to understand the process of fermentation.

Traditional fermented foods and non-feremnted foods are common in Nepal. These have been the basis and are equally important for the food security, preservation, and cultural and ethical practices (Dahal et al., 2005). Diversified ethnic communities, culture and heritage have inherited various methods of food preparation and

consumption. Nepalese people are skilled at preparing and preserving locally available foods for their food security for a longer period of time. The traditional fermented foods are specific to certain geographic reasons and also to particular communities. Also, most of the traditional technologies are passed from generations as trade secrets in families of certain communities. Sometimes this kind of practice is protected by tradition. Despite the primitive technologies, they have played a major role in raising economic status of Nepalese people. However, complete scientific information on these various food products, their traditional principles, production and preparation methods, and mode of consumption of these products are lacking. Presently, a number of fermented foods (such milk, cereal and fruit & vegetables based) and beverages have evolved over the years (Farnworth, 2008).

2.2.1. Milk Based Products

Fermentation of milk, either knowingly or unknowingly has occurred since early times resulting in various fermented milk products. Fermented milk products are known for their taste, nutritive value and therapeutic properties (Fernandez et al., 2014). The nature of these products has differed from region to region depending on the indigenous microflora, which in turn depends upon the surrounding environmental factors (Tamime & Robinsorn, 1998). The most popular traditional fermented products of Indian subcontinent are Curd (*Dahi*), Mohi, Durkha, Chhurpi, Lassi, and others. The use of desired microorganisms as in the case of controlled fermentation would greatly enhance the chances of obtaining products with uniform and consistent quality products of acceptable attributes (Rati Rao et al., 2006).

Curd (*Dahi*)

Curd (*Dahi*) has been popular since antiquity which is widely consumed all over Nepal and in the neighboring countries such as India, Bangladesh, Pakistan and others because of cultural, ethnic, health beneficial value. It is consumed as plain, sugared or salted products in different countries. Varieties of curd available in the market are those of sweet, sour and flavored curd. The sweetened and concentrated form of *dahi*, known as *mishti doi* i.e. sweet *dahi* is popular in some parts of India such as Bengal; (Early, 1998; Raju & Pal, 2009). Although *dahi* and yogurt have many similarities, some differences exist between them. The starter organisms used for the preparation of yogurt are thermophilic lactic acid streptococci and thermophilic lactobacilli in

equal proportions, while *dahi* is prepared by using mixed culture of *Streptococcus spp.* (now *Lactococcus*) such as *Streptococcus thermophilus, Streptococcus citrophilus* and *Lactobacillus* species such as *L. bulgaricus, L. plantarum* etc.) (Mahanta, 1984).

Dahi is considered as a sign of love which is one of the components (out of five) in panchamrit (symbol of purity). Panchamrit is a religious symbol of Hindus (Internet visit 2014, http://alavelu.com/h-culture.html). *'Dahi* is a Nepali word for curd or yogurt. It is generally prepared from boiled cow or buffalo milk by inoculating with a small quantity of starter *Dahi* (back sloping technique) containing natural microflora or lactic acid bacteria (Koirala et. al., 2014). Different types of Dahi are prepared in Nepal. However, Jujudhou *Dahi* is the most famous sweetened and flavored *Dahi* produced in Bhaktapur and Kathmandu districts (Karki, 1986). The conversion of milk into *Dahi* is an important intermediary step in the manufacture of *Nauni* (indigenous dairy product equivalent to butter) and ghee (butter-oil).

Commonly two types of curd are produced (Kharel et al., 2009). They are: (i) Sweet and mildly sour variety with the pleasant flavor, and (ii) Sour variety with a distinct acid flavor. Sweet Curd is prepared using (singly or in combination) *Streptococcus lactis, S. diacetylactis,* and *S. cremoris* as the lactic culture. In Sour Curd preparation, additional cultures (singly or in combination) of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are used.

A good quality of curd (*dahi*) is of firm and uniform consistency with a sweet aroma and fresh acid taste. The surface is smooth and glossy and usually a cut surface is trim, free from cracks and gas bubbles. The nutritional and therapeutic values of curd are rated as high (Varadaraj et al., 1993) when compared to that of the milk used for its preparation. *Dahi* is easy to digest and has been found to have certain health benefits (Sinha & Sinha, 2000). Laxminarayana et al. (1952) reported average composition of *Dahi* prepared from whole milk is as follows: water 85-88%, fat 5-8%, protein 3.2-3.4%, lactose 4.6-5.2%, ash 0.7-0.75%, lactic acid 0.5-1.1%, calcium 0.12-0.14% and phosphorous 0.09-0.11%.

Besides commonly associated lactic acid bacterial strains *Lactococcus lactis* ssp*, lactic, Lc. lactis* ssp. *cremoris, Lc. lactis* ssp. *diacetylactic, Leuconostoc cremoris, Lactobacillus delbrueckii* ssp. *bulgaricus, Lb. acidophilus* and *Lb helveticus,* selection

of a good starter culture is important to obtain a good flavor with desirable characteristics. Use of both mesophilic and thermmophilic starters in various combinations has been reported (Baisya & Bose, 1975; Sharma & Jain, 1975). Addition of probiotic cultures such as *Lb. acidophilus* and *Bifidobacterium bifidum* along with the regular lactic culture of *Dahi* preparation increases the therapeutic nutritional value of Curd (Vijayendra, 1994). *Dahi* harbors potential source of lactic acid bacteria and species of *Lactobacillu*s are reported to be active against food borne pathogenic and spoilage bacteria such as *Staphylococcus aureus, Bacillus cereus, Bacillus licheniformis, Bacillus subtilis, Escherichia coli, Klebsiella* sp. and *Pseudomonas* sp. (Balasubramanyam & Varadaraj, 1994). Further, a strain of *Lb. delbrueckii* ssp. *bulgaricus* producing a heat stable bacteriocin with broad antimicrobial activity and a potential for use as a food biopreservative was isolated from fermented foods (Balasubramanyam, 1996).

As reported by Kharel et al. (2009) *Dahi* can also been classified into two groups and additional subgroups on the basis of intended use i.e.

I. For churning to produce *Nauni* and deshi butter.
II. For direct consumption
    a) Whole milk Curd and Skim milk Curd
    b) Sweet (mild) Curd, Sour Curd and Sweetened Curd

In Nepal, mostly whole milk curd is prepared, both for the production of *Nauni* (butter) and direct consumption. In the rural areas the milk may be boiled for longer period of time. The volume may be reduced to one third of its original volume and spontaneously fermented in *Theki* (a wooden vessel used for curd fermentation). Fermentation is carried out overnight at room temperature. Commercial production of sweet *dahi* is also under practice in urban areas of Nepal.

Juju Dhau is a popular set-type of yogurt produced only in Bhaktapur district by Newar community. *Juju Dhau* [(juju means '*King*' and dhau means '*Dahi*') in Newari dialect] has a peculiar taste and appearance. Due to its unique sensory property *Juju Dhau* is widely consumed as side dish in religious ceremonies, festivals and carnivals. Production of *Juju Dhau* has remained confined to domestic or cottage scale preparations for a long time (for over 400 years) in Bhaktapur of Nepal (Dahal et al., 2005). *Juju Dhau* is prepared preferably from buffalo milk. Because it is concentrated

for a fairly long time in Karahi (milk boiling iron vessel), the product is rich in fat and milk solid not fat (MSNF). Basically, there are two types of *Juju Dhau*, *viz.* (i) plain, and (ii) sweet. The plain type is made without addition of sugar while the sweet type is prepared by addition of 5% sugar on fresh milk basis.

Method of Curd (Dahi) preparation

There are different conventional methods for the preparation of *Dahi*, comprising the use of starter cultures, even though this is not a routine practice. In most cases, *Dahi* is traditionally made at household level, without starter cultures but using a portion of previously produced *Dahi* or Mohi or cream as inoculum. Usually, after heating or boiling, the milk is cooled to 30-40 $^{\circ}$C and then transferred to a wooden (locally named Theki) vessel where it is left overnight at room temperature (25-30 $^{\circ}$C). Fermentation is carried out spontaneously by natural microbiota of the milk, along with the microorganisms that persist on the surface of vessels and in the processing environment. In the Terai region and some Hilly regions, naturally red earthenware pots (clay pot locally called "maato ko kataaro") are more common used. These pots have a porous surface, so moisture is absorbed by the container especially when the *Dahi* tends to exude some whey and also gives a "muddy" flavor and a thicker texture. This vessel is wrapped in cloth or hay, put on the top of sawdust, or in a straw box to maintain the suitable temperature for the souring and coagulation processes. This step is the most difficult to achieve in many regions of Nepal at high altitudes (>3500 m), with low temperatures and consequent problems related to slow acidification and delay in coagulation. In Nepal, the traditional *Dahi* can also be obtained by a semi-continuous method. Boiled and cooled milk, inoculated with indigenous natural starter culture (in many cases, previously made *Dahi*), is put in the container, which is covered with clothes and kept warm. The next day, a further quantity of cooled boiled milk is added and the fermentation process is continued. This topping up with cooled boiled milk is repeated daily until the container is full. After the last incubation, the *Dahi* may either be consumed or churned to obtain butter or other dairy products.

The traditional method of curd (*dahi*) production is limited to small scale and is produced either in consumer's household or sweet-maker's shop (in urban areas). During winter season, the curd setting vessel (*Theki*) is kept over or near the fireplace to maintain the warmth needed for fermentation. It is also usual to wrap *Theki* with

woolen cloth for the same. In case of small scale commercial production method, instead of *Theki* a circular earthenware pot is used for curdling using specific mesophilic and thermophilic cultures. For this kind of curd preparation, a good quality of milk is boiled and cooled (spontaneous colling to body temperature) and previous day's curd or cultured butter milk or fermented whey is inoculated and incubated overnight preferably near the fireplace to maintin the warmth needed for the fermentation. After incubation, a firm coagulum called curd (*dahi*) is formed which is ready for consumption and is stored in refrigerator until use (Kharel et al., 2009).

In spite of the fact that curd and other dairy products play significant roles in human health, they are most perishable products and their contamination leads to various health hazards (Singh & Prakash, 2008). This problem can be solved by using a technique known as; biopreservation. It is the use of natural or controlled microbiota or antimicrobials as a way of preserving food and extending its shelf life (Baust & Baust, 2006). The increase in resistance of food spoilage microorganisms to current preservatives, the consumer's demand for safe and minimally processed foods, and the hazard associated with the use of high doses of chemical preservatives have led to the need for finding safer alternatives in food preservation. Consumers have also become more critical about the use of synthetic additives to preserve food or enhance characteristics such as colour and flavor (Bruhn, 2000). This has lead to a growing trend towards use of minimally processed foods. As a consequence, bacterial antagonism has received considerable attention in food preservation (Aguilar et al., 2011; Varalakshmi et al., 2014). Beneficial bacteria (such as probiotics) or fermentation products produced by these bacteria are used in biopreservation to control spoilage and render pathogens inactive in food (Drosinos et al., 2005).

2.3.0. Nutritional value of Nepalese Traditional Foods

Traditional foods, both fermented and non-fermented have been the basis and are equally important for food security, preservation, and cultural and ethical practices (Roopashri & Varadaraj, 2011; Roopashri, 2012). More than 80% of the people of Nepal are involved in the agricultural profession; most of the food based traditional technologies are passed on as trade secrets in families of certain communities, a practice protected by tradition (Karki, 1986; Dahal et al., 2005). Although the technologies are primitive, they have played a major role in the economic status of Nepali people. However, complete scientific information on these various food

products, their traditional ethics, production and preparation methods, and mode of consumption of these products are lacking.

Among the diverse range of traditional foods of Nepal, the most popular and widely consumed ones are those based on milk, cereals and legumes fermentation (FAO, 1990). The scientific knowledge basis of nutritional benefits of few traditional fermented products of Nepal is documented but much information is still lacking (Dahal et al., 2005). The complexity linked with the type of fermentation process, product preparation parameters and final profile gives ample opportunities to bring into focus the importance of nutritional constituents in various fermented products of Nepalese origin (Kharel et al., 2009).

Milk and milk products are main sources of protein, fat, lactose and minerals (Kharel et al., 2009). Traditional butter and ghee are the main source of energy. Chhurpi which is solid hard casein contains 81 % protein and 11 % fat. The normal traditional butter may contain on average 18-25 % moisture, 75.5 to 85.5 % fat, 1-1.5 % non fatty acids and solids and 0.2 to 0.5 % oleic acid (Kharel et al., 2009). The composition may differ according to the manufacturing process. *Dahi* and lassi are also of great importance in the diet as well as beneficial lactic acid bacteria (FAO, 1990).

Dahi is one of the main traditional fermented milk product consumed all over the country as a part of food or desert. Also Lassi is drunk and is mainly used in villages. Lassi contains more water than dahi. Curd and lassi both contain fat, protein, lactose, ash calcium, phosphorous which highlights that it has great nutritional value. Besides these there are several traditional fermented milk products with good nutritional values in different parts of Nepal. Koirala et al. (2014) reported that *dahi* from different geographical locations contains diversity of lactic cultures and these indigenous cultures are usually safe and could be used as probiotics in future.

Traditional foods have been a very important part of the lives of people all over the world, including Nepal. These products are rich in energy, vitamins, and other nutrients; organoleptically desirable; and add value to the raw ingredients (Dahal et al., 2005). However, complete scientific information on the various aspects of traditional foods is still lacking in Nepal. A complete scientific investigation on "*Dahi"*, one of the major traditional fermented foods consumed all the months

throughout the country, is must amid the isolation, purification, and identification of dominant desirable flora that are responsible for the unique quality of these products (Dahal et al., 2005; Kharel et al., 2009).

The scientific advancement with regard to the understanding of role of probiotics in the daily diet has proven beneficial to the health of the population (Dave & Shah, 1996; Salminen et al., 1996). Careful evaluation, selection and incorporation of the probiotic strains into fermented foods through the defined starters would improve the product further with better health benefits. Moreover, the fact that most of the alcoholic beverages and dairy products are consumed as such without further processing makes these foods the ideal choice of substrate for probiotic incorporation. The products also contain high amount of lactics and are known to produce bacteriocin that can act against foodborne pathogens (Varadaraj et al., 1993). Pure starter cultures with probiotic strains would eliminate pathogenic and toxin-producing microorganisms from the fermented product and ensure the health benefits to the consumer (Varadaraj & Ranganathan, 1984).

Most of the traditional fermented foods and beverages of other countries have been well investigated and documented. Statistical data on production, consumption, socio-economy, microbiology, biochemistry, nutritional profile, and optimized production methods of the traditional fermented foods of those countries are available. In Nepal, most of the traditional foods and beverages are yet to be investigated. Few studies on the selected foods like Gundruk, Masyaura, Kinema, and Poko have been reported so far (Dahal et al., 2005).

Some important aspects involved in the different traditional foods are isolation, purification, and identification of the dominant microorganism. Due to the diversified dietary cultures of various ethnic groups and wide variations in geo-climatic conditions of Nepal, study on the microorganisms associated in fermented foods may contribute significantly in food processing and value addition. The diversity of microorganisms in food ecosystems has not been sufficiently assessed, and they may contain a number of strains that could be of scientific interest or may have potential industrial application. Very few traditional fermented as well as non-fermented foods are being commercialized. However, Nepal still has a long way to go in value addition to compensate the food scarcity or the address the issue of food security.

2.4.0. Lactic Acid Bacteria

2.4.1. Historical background of lactic acid bacteria

Lactic acid fermentation has a very old history. Fermentation technique has been continewously used in various parts of the world by several ethnic communites to improve the storage qualities and retain the nutritive value of perishable foods such as milk, vegetables, meat, fish and cereals (Axelsson, 2004). Microorganisms such as lactic acid bacteria play important role in preserving foods by carrying out fermentation. In develoved countries, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk, and yogurt. The production and biotechnological uses of dairy starter cultures has become popular in food and health sectors in this century (Roopashri, 2012).

The group 'lactic acid bacteria' concept was created for bacteria causing fermentation and coagulation of milk, and is defined as those which produce lactic acid from lactose. Orla-Jensen (1919) used the family name Lactobacteriaceae to a physiological group of bacteria producing lactic acid alone or acetic acid, lactic acid, alcohol and carbon dioxide. Nowdays, lactic acid bacteria are regarded as synonymous with the family Lactobactericeae.

From the days of Russian scientist Metchnikoff (1908), lactic acid bacteria have also been associated with beneficial health effects. Nowadays, an increasing number of health foods and so called functional foods including pharmaceutical preparations are being promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Most of these strains, however, have not been thoroughly studied, and consequently the claims are not well substantiated (Matar et al., 2001). Moreover, health benefits are judged mainly using subjective criteria and the specific bacterial strains used in the studies are often poorly identified so the informations available about beneficial effects of lactic acid bacteria may not be reliable. There is clear need for comprehensive scientific study of the effect LAB strains on human health for the production of fermented foods and health supplements.

Lactic acid bacteria are Gram-positive, catalase negative, devoid of cytochromes, non-spore forming and non-flagellated rods or cocci, which produce lactic acid as the major end product during the fermentation of carbohydrates (Axelsson, 2004). The rods or cocci are present in varied forms like single, pairs, chains and tetrads.

Typically they are non-motile and do not reduce nitrate. Usually, LAB are found to be associated with habitats rich in nutrients such as milk, meat, beverages and vegetables products. Besides nutritional based habitats, some are also members of the normal flora of the mouth, intestine and vagina of mammals (Axelsson, 2004). They are divided into four genera *Streptococcus, Leuconostoc, Pediococcus, and Lactobacillus*. Recent taxonomic revisions suggest that lactic acid bacteria grouped could be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus*. Originally, bifidobacteria were included in the genus *Lactobacillus* and the organism was referred to as *Laactobacillus bifidus* (Axelsson, 2004).

The classification of lactic acid bacteria into genera is mainly based on morphology, mode of glucose fermentation, growth at different temperatures, and configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (van Belkum & Neiuwenhuis, 2007). Additional characteristics such as fatty acid composition and motility are used as basis of classification for some newly described genera of LAB (Ljungh & Wadstrom, 2009).

As mentioned above, lactic acid bacteria was a term used synonymously with “milk souring organisms’. Important progress in the systematics of LAB was made when the similarity between milk souring bacteria and other LAB from other sources was recognized (Axelsson, 1993). *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus* (documented in Bergey’s Manual of Systematic Bacteriology) are the genera that fit mainly with respect to general desctiption of the typical LAB (Kandler & Weiss, 1986). Basically *Lactobacillus*, *Leuconostoc*, and *Pediococcus* have remained unchanged excluding some rod-shaped lactic acid bacteria such as the genus Carnobacterium which was previously included in the genera *Lactobacillus* (Collins et al., 1987).

2.4.2. Identification and Classification at genus level

The general basis for the classification of lactic acid bacteria into different genera has mainly remained unchanged since the work of Orla-Jensen despite of their morphology is which is regarded as questionable key character in bacterial taxonomy (Woese, 1987). But morphology is still very important features for defining lactic acid bacteria. Lactic acid bacteria can be divided into rods (*Lactobacillus* and

Carnobacterium) and cocci (all other remaining genera). Glucose fermentation) with optimum conditions i.e., non limiting concentrations of glucose, growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability is one of the important characteristic used for differentiating lactic acid bacteria (Desai, 2008). Based on glucose fermentation, LAB can be divided into: 1) homofermentative (convert glucose almost quantitatively to lactic acid) and 2) heterofermentative (ferment glucose to lactic acid, ethanol/acetic acid, and CO_2) (Sharpe, 1979). In fact, a test for gas production from glucose Gas production during glucose utilization will further distinguish into different groups (Sharpe, 1979). Exluding *Leuconostocs* and a subgroup of *Lactobacillus* (which are heterofermentative) and all other lactic acid bacteria are homofermentative.

Culture physiology study (i.e. growth characteristics) at different temperature, pH, salt concentrations are userful to discriminate different genera of LAB (Table 2.1). Some of the cocci can be distinguished on the basis of growth at certain temperatures. Enterococci grow at 10 $^{\circ}$C and 45 $^{\circ}$C, lactococci and vagococci at 10 $^{\circ}$C, but not at 45 $^{\circ}$C. Streptococci do not grow at 10 $^{\circ}$C, while growth at 45 $^{\circ}$C is independent of the species (Axelsson, 1993). Salt tolerance (upto 6.5%) may also be used to distinguish among Enterococci, lactococci/vagococci, and streptococci can also be distinguished based on salt tolerance (upto 6.5%), even if different responses can be observed among streptococci (Mundt, 1986). The genera *Tetragenococcus* can be confirmed by extreme salt tolerance upto 18% Acid and alkali torance are are very useful characteristics (for differentiationg LAB as well as for determining probiotic properties). Growth at high pH of 9.6 can be confined as enterococci with few exceptions. Production of different isomers of lactic acid during fermentation of glucose can also be used to distinguish between Leuconostoc (produce only D-lactic acid) and most heterofermentative lactobacilli (forms DL-lactic acid) (Axelsson, 1993).

2.4.2.1. Hexose fermentation

Hexose (e.g., glucose) can be utilized in two ways by lactic acid bacteria (Axelsson, 2004) (Figure 2.1). Glycolysis (Embden-Meyerhof pathway), used by all lactic acid bacteria except leuconostocs and group C lactobacilli, is characterized by the formation of fructose-1-6-diphosphate (FDP), which is split by an FDP adolase into dehydroxyacetonephosphate (DHAP) and glyceraldehydes-3-phosphate (GAP). GAP

Table 2.1. Differential characteristics of Lactic acid bacteria. Aadopted from Axelsson (2004).

Rods						Cocci				
Character	Cornob.	Lactob.	Aeroc.	Enteroc.	Lactoc. Vagoc.	Leucon. Oenoc.	Pedioc.	Streptoc.	Tetragenoc.	Weissella[b]
Tetrad formation	_	_	+	_	_	_	+	_	+	_
CO_2 from glucose[c]	_[e]	±	_	_	_	+	_	_	_	+
Growth at 10 °C	+	±	+	+	+	+	±	_	+	+
Grwoth at 45 °C	_	±	_	+	_	_	±	±	_	_
Growth at 6.5% NaCl	ND[f]	±	+	+	_	±	±	_	+	±
Growth at 18% NaCl	_	_	_	_	_	_	_	_	+	_
Grwoth at pH 4.4	ND	±	_	+	±	±	+	_	_	±
Growth at pH 9.6	_	_	+	+	_	_	_	_	+	_
Lactic acid[d]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

[a] +, positive, -, negative, ±, response varies between species; ND, not detected.

[b] Weissella strains may also be rod-shaped.
[c] Test for homo- or heterofermentation of glucose, negative and positive denotes homofermentative and heterofermentative, respectively.
[d] configuration of lactic acid produced from glucose.
[e] Small amount of CO2 can be produced, depending on media.
[f] No growth in 8% NaCl has been detected.
[g] Production of D-, L- or DL- lactic acid varies among species.

(and DHAP via GAP) is then converted to pyruvate in a metabolic sequence including substrate level phosphorylation at two sites. Under normal conditions, i.e. excess sugar and limited access to oxygen; pyruvate is reduced to lactic acid by a NAD+ dependent lactate dehydrogenase (nLDH), thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end product, and the metabolic process is referred to as homolactic fermentation.

Another main fermentation pathway has had several designations, such as the pentose phosphate pathway, the pentose phosphoketolase pathway, the hexose monophosphate shunt, and used by Kandler & Weiss (1986) in Bergey's Manual, the phosphogluconate pathway. It is characterized by initial dehydrogenation steps with the formation of 6-phosphogloconate, followed by decarobxylation. The remaining pentose-5-phosphate is splited by phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same way as for the glycolytic pathway resulting in lactic acid formation. When no additional electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetyldehyde. Since this metabolism leads to significant amounts of other end products (CO_2, ethanol) in addition to lactic acid, it is referred to as heterolactic fermentation. Both fermentation pathways are shown in Figure 2.1.

2.5.0. Genus *Lactobacillus*

2.5.1. Historical background of the Genus *Lactobacillus*

Lactobacillus is the largest genus included in lactic acid bacteria. This genus is heterogenous consisting of species having different phenotypic, biochemical, and physiological properties (Ljungh & Wadstrom, 2009). The heterogeneity in the genus can be detected by mol % G+C content of the DNA of species. The range of mol % G+C content in *Lactobacillus* is 32-53% which is double the limit usually accepted for a single genus (Schleifer & Stackebrandt, 1983). This genus was defined by heterogeneity and is essentially rod-shaped lactic acid bacteria which can be compared by an arrangement with the entire coccoid lactic acid bacteria included in other genus. Summary of the characters used to distinguish among the three groups and some of the more well-known species included in each group (Table 2.2). physiological basis for the division is (generally) the presence or absence of the key

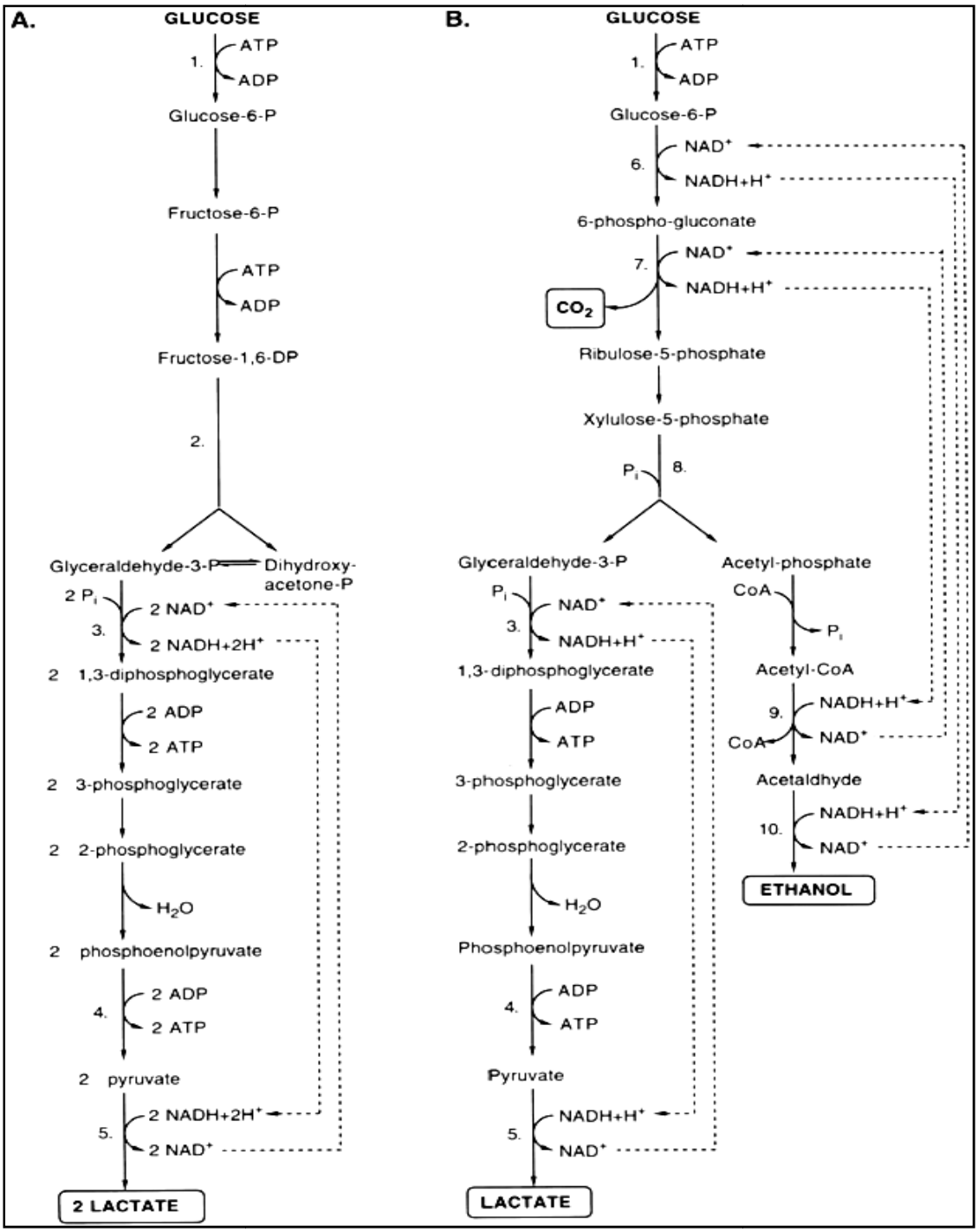

Figure 2.1. Major fermentation pathways of glucose: (A) Homolactic fermentation (glycolysis, Embden-Meyerhof pathways); (B) Heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-diphosphate aldolase; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-phosphate dehydrogenase; 7. 6-Phosphogluconate dehydrogenase; 8. Phosphoketolase; 9. Acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase (Axelsson, 1993).

enzymes of homo-and heterofermentative sugar metabolism, fructose-1, 6-diphosphate aldolase, and phosphoketolase, respectively (Kandler, 1983, 1984; Kandler & Weiss, 1986). Lactobacilli are found everywhere (with respect to their suitable nutritional environment) and many species have been used in food applications. They are Gram-positive, non-spore-forming, rods or coccobacilli with a G+C content of DNA usually <50 mol%. Lactic acid bacteria due to their higher molecular percentage of G+C contents in DNA and are placed in the Actinomycete branch while other lactic acid bacteria are replaced in the *Clostridium* branch (Table 2.4). The comparision of G+C contents in DNA of several genera of lactic acid bacteria and phylogenetic relationship of lactic acid bacteria according to G+C mol% present in DNA are presented in Table 2.3.

Table 2.2. Arrangement of the genus *Lactobacillus* based on the presence or absence of key enzymes furing fermentation (Kandler & Weiss, 1986).

Character	Group I obligately homofermentative	Group II facultatively heterofermentative	Group III obligately heterofermentative
Pentose fermentation	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
FDP aldolase present	+	+	-
Phosphoketolase	-	+[b]	+
	L. acidophilus	*L. casei*	*L. brevis*
	L. delbrueckii	*L. cirvatis*	*L. buchneri*
	L. helveticus	*L. plantarum*	*L. fermentum*
	L. salivarius	*L. sake*	*L. reuteri*

a. When fermented.

b. Inducible by pentoses.

Table 2.3. DNA base composition of different lactic acid bacteria (Salminen & von Wright, 1998).

Genus	G + C % range
Lactobacillus	34.7-50.8
Streptococcus	33-44
Leuconostoc	39-42
Bifidobacterium	57.2-64.5

Lactobacilli are ever-present where carbohydrate substrates are available and thus are found in a different habitats such as mucosal membranes of humans and animals (mainly in oral cavity, intestine, and vagina) and on plant materials and fermented foods (Hammes et al., 1991; Pot et al., 1994). Lactobacilli are strict fermentative, aero-tolerant to anaerobic, aciduric or acidophilic having many nutritional requirements (e.g. for carbohydrates, amino acids, peptides, fatty acid, esters, salts, nucleic acid derivatives, vitamins) for their growth and metabolism. They do not synthesize porphyrinoids and thus, are devoid of heme-dependent activities (Axelsson, 2004).

Table 2.4. Phylogenetic relationship of lactic acid bacteria based on the mol percent of G+C content in DNA (Salminen & von Wright, 1998).

Branch		
Mol % of G+C content in DNA	Clostridium	Actinomycete
	<50	>50
	Lactobacillus *Lactococcus* *Enterococcus* *Leuconostoc* *Pediococcus* *Streptococcus* *Staphylococcus aureus* *Bacillus subtillis*	*Bifidobacterium* *Propionibacterium* *Microbacterium* *Corneybacterium* *Brevibacterium* *Atophobium*

2.5.2. Grouping of *Lactobacillus*

The primary interest of Orla-Jensen's early description of lactic acid bacteria was aimed to identify these bacteria for their use in dairy industry in relevance to the study of those bacteria found in Danish 'dairy cheese' (Axelsson, 2004). Orla-Jensen recognized 10 species at that time. This number increased slowly only to 15 and 25 species, in the 7th and 8th editions of Bergey's Manual. Finally 44 species have been mentioned in the latest 9th edition of Bergey's Manual. The number of species is still increasing due to rapidly emerging of new taxonomic methods that allows precise discrimination and identification of strains (Axelsson, 2004).

Kandler & Weiss, 1986 grouped lactobacilli based on biochemical-physiological criteria neglecting the classical criteria of Orla-Jensen such as morphology and growth temperature since many of recently described species did not fit into Orla-Jensen's classification. End products derived from the fermentation of pentoses are not included in the description of new species although the enzymes of the pentose phosphate pathway may be present (permitting a homofermentative metabolism of pentose) in lactobacilli. However, traditional description is justified with regards to hexose utilization. But some facultatively heterofermentative species may produce acetate, ethanol and formate instead of lactate from pyruvate under low substrate concentration and strictly anaerobic conditions so the definitions have to be used according to their limitations (Axelsson, 2004).

Lactobacilli may undergo homofermentative or heterofermentative by during glucose fermentation. They may produce more than 85% lactic acid in homofermentation process while different metabolites such as lactic acid, carbon dioxide, ethanol or acetic acid may be produced during heterofermentation procecss. Higher amounts of acetate may be produced at the expense of lactate or ethanol in the presence of oxygen or other oxidants. Further, Hammes & Vogel (1995) grouped 56 species of lactobacilli which are divided into three metabolic groups based on their patterns of sugar fermentation and important physiological properties (Table 2.5).

Group A: The obligatory homofermentative lactobacilli are included in this group. Lactic acid is produced by hexose fermentation via EMP pathway while pentose or gluconate are not fermentated.

Group B: Facultative heterofermentative lactobacilli have been grouped in this grouping where hexoses are fermented to lactic acid via EMP pathway. Pentoses (and often floconate) are also fermented since the organisms possess aldolase and phosphoketolase.

Group C: In this group obligatory heterofermentative lactobacilli have been grouped. These lactobacilli follow the phosphogluconate pathway to produce lactate, acetic acid (ethanol) and CO_2 by fermenting hexoses. Also, pentose entering in this pathway and may be fermented.

Table 2.5. Grouping of different species of lactobacilli based on their patterns of sugar fermentation and important physiological properties.

Fermentation type	Species
Obligate homofermeters	*Lb. anamalis, Lb. aviarius, Lb. ruminis, Lb. salivarius, Lb. sharpeae, Lb. yamanashiensis (Lb. mali)* *P. damnosus, P. dextrinicus, P. parvulus*
Facultative heterofermenters	*Lb. agilis, Lb. alimentarius, Lb. bifermentans, Lb. casei, Lb. coryniformis, Lb. curvatus, Lb. graminis, Lb. homohiochii, Lb. paracasei, Lb. pentosus, Lb. plantarum, Lb. rhamnosus, Lb. sake* *P. acidialactici, P. pentosaceus*
Obligate heterofermeters	*Lb. brevis, Lb. buchneri, Lb. fermentum, Lb. fructivorans, Lb. hilgardii, Lb. kefir, Lb. oris, Lb. parabuchneri, Lb. reuteri, Lb. sanfrancisco, Lb. suebicus, Lb. vaccinostercus, Lb. vaginalis*

2.5.3. The taxonomy of the genus *Lactobacillus*

The genus *Lactobacillus* comprises a large group of lactic acid bacteria, which are all Gram-positive non-sporing cocci, coccobacilli or rods, having a DNA G+C content of less than 50% G+C and they lack catalase activity and need a fermentable carbohydrate for their growth. The lactic acid bacteria in the broad sense comprise genera such as *Aerococcus, Alloiococcus, Atopobium, Carnobacterium, Enterococccus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus,*

Paralactobacillus, Pedicococcus, Streptococcus, Tetragenococcus, Vagococcus and *Weissella*. The genus *Bifidobacterium, Gardenerella*, *Scrdovia* and *Parascardovia* are often also included in this collection, although phylogenetically they belong to the Actinobacteria subdivision (PHYLUM 3) of the Gram-positive Eubacteria (the Firmicutes), comprising also *Propionibacterium, Brevibacterium* and the microbacteria. The latter taxa are only very distantly related to the genuine lactic acid bacteria (Ljungh and Wadstrom, 2009).

Garrity et al., 2004 reported that the genus *Lactobacillus* belongs to the phylum *Firmicutes*. *Lactobacillaceae* family includes the main family in the order *Lactobacillales* which belongs to the class Bacilli. From the other members of the family mentioned above, the genera *Paralactobacillus* and *Pediococcus* are most noteworthy since species of these genera tend to intermingle phylogenetically with the variety of species of the genus *Lactobacillus* (Axelsson, 2004).

The phylogenetic classification was mainly based on the results of 16S rRNA sequence analysis (Garrity et al., 2004), and may not necessarily reflect the metabolic diversity. Next to the 16S rRNA sequencing technique is the species concept (Stackebrandt et al., 2002). However, the species concept remains subject of animated debates among taxonomists (Rossello-Mora & Amann, 2001; Rossello-Mora, 2003; Gevers et al., 2005) and it remains the formal unit of bacterial classification. Species concept is as 'a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics', and is differentiable by a discriminative phenotypic property (Rossello-Mora & Amann, 2001). These phylogenetic concepts need phenotypic characteristics which will discriminate a possible new species from its closest phylogenetic neighbors. A species can further be divided when phyenotypic variation is considerable and later a species may be further subdivided into subspecies based on this phenotypic variation and subspecies can but need not be supported by genetic determinants (Rossello-Mora & Amann, 2001).

Practically, two main genotypic criteria are used to define a species: strains with a total DNA similarity of 70% or higher (relative binding in a hybrid DNA reassociation experiment) and a difference in the melting temperature (ΔT_m) equal to or lower than 5 $^{\circ}$C, will be considered to belong to a single species In addition the 16S rRNA gene sequence similarity should not differ more than 3% (Axelsson, 2004).

Ljungh & Wadstrom (2009) reported that similar procedures based on DNA-DNA hybridization are still considered the gold standard technique for the delineation of bacterial species. Since this technique is laborious, it is very unpractical for the 113 species of the genus *Lactobaillus*. Thus, the closer phylogenetic species are often identified by a (partial) 16S rRNA sequencing, which after comparison with the large collection of 16S rRNA sequences available in public databases (such as GenBank), allows the identification of the most closely related species for which DNA-DNA hybridization will need to be set up.

Ljungh & Wadstrom (2009) summarized the several reasons given by Stackebrandt & Goebel (1994) for several reasons; 16S rRNA gene sequencing can never be used as the sole method for species delineation as listed below.

1. Single representative strain per species used for the 16S rRNA analysis might be the first limit which lacks the possibility to position a new isolate in the biological diversity of the species considered.
2. Also, the use of partial sequence is making the result of 16S rRNA sequences less reliable for the analysis.
3. Reference sequences might have many sequencing which will also influence the final tree
4. Alignment of sequences is essential to calculate sequence similarity which is highly subjective task. It relys not only on a wealth of algorithms, but often manual editing is necessary to improve the result obtained. Critical are the ease, one allows the software to create gaps, and the cost defined to extend these gaps.
5. In case of the the pairwise calculation of the sequence similarities either gaps can be included or not and phylogenetic corrections can be applied or not.
6. The choice of cluster algorithm and also the selection of reference sequences included can affect the shape of the final tree.
7. It was concluded that a conserved taxonomic marker, the 16S rRNA is not really suitable to study small difference between closely related species.

Because of these reasons, 16S rRNA sequencing will be useful to frame a new isolate in a well known phylogenetic scheme, but may not solve the identification or classification problem that exist in the taxonomy. Van Damme et al. (1996a) suggested bringing a variety of information sources for a more reliable identification or classification.

Identification and classification are very important since lactic acid bacteria have been used widely in food applications (Van Demme et al., 1996a). This discussion regarding evolutionary deductions should automatically reflect on nomenclatural designation (Dellaglio et al., 2004b) is very relevant one. Nomenclature is essential for proper food labeling and will allow producers to communicate in a formal way about the bacteria they add to foods. Safety aspects, for example have also been linked to species definition (EFSA, 2008). Therefore it is very important to support the automatic link between evolution and speciation (de Quieroz & Gauthier, 1992; Woese, 1998; Cantino 1999) but its automatic translation into nomenclatural designations can be questioned for practical and other reasons.

2.5.4. The taxonomic description of the phylogenetic groups

According to Schleifer & Ludwig (1995), Felis & Dellaglio (2005) and Salveti et al. (2012), 15 groups have been reocognized. These fifteen groups are: 1) *Lactobacillus delbreuckii* Group (27 member of species), 2) *Lactobacillus salivarious* Group (25 member of species), 3) *Lactobacillus ruteri* Grop (15 member of species), 4) *Lactobacillus buchneri* Group (12 member species), 5) *Lactobacillus alimentarius* (11 member of species), 6) *Lactobacillus brevis* Group (10 member of species), 7) *Lactobacillus collinoides* Group (5 member of species), 8) *Lactobacillus fructivorans* Group (5 member of species), 9) *Lactobacillus plantarum* Group (5 member of species), 10) *Lactobacillus sakei* Group (4 member of species), 11) *Lactobacillus casei* Group (3 member of species), 12) *Lactobacillus coryniformis* Group (3 member of species), 13) *Lactobacillus manihotivorans* Group (3 member of species), 14) *Lactobacillus prolens* Group (3 member of species), 15) *Lactobacillus vaccinostercus* Group (3 member of species).

2.5.5. Taxonomic techniques used with lactobacilli

The bacterial lassification systems tend to be dynamic and as a result the *Lactobacillus* taxonomy based on biochemical and physiological criteria differs considerably with the schemes revealed by molecular methods (Stiles & Holzapfel, 1997).

2.5.5.1. Morphological techniques

Lactobacilli are rod shaped and therefore are difficult to recognize by simple morphological tests. Moreover, growth conditions and growth stage of the bacterial

cells may seriously affect their morphology and many heterofermentative lactobacilli may exhibit a coccobacillus morphohlogy, which is difficult to discriminate, for example leuconostocs. The presence of endospores or inclusion bodies, the number and type of flagella, and the cell size can also be used as the morphological techniques to describe lactobacilli (Stiles & Holzapfel, 1997).

2.5.5.2. Phenotypic techniques

For species description phenotypic characters are important and also have practical importance. For example, discrimination between *Lactobacillus* and *Carnobacterium*, can easily be achieved since the later cannot grow at pH 4.5 or on acetate agar but can grow at pH 9.0 (Hammes et al., 1991). But simple physiological tests are not sufficient for discriminating the 113 species of *Lactobacillus*, and hence should be supplemented with additional analyses of fermentation products (Hammes et al., 1991) and genotypic data.

There are number of disadvantages when the fermentation pattern is used for identification and taxonomic classification. For example, inter-laboratory variation, strain to strain variation and long processing times, which generally result in low reliability of the identification and classification obtained. In order to improve reproducibility, highly standardized, commercially available systems can be used, such as API 20 STREP, API 50CH (BioMeriecux, France), Distabs (Rosco, Denmark) or BIOLOG GP MicroPlate (BIOLOG Inc., USA). When a database has to be prepared, e.g. for identification purposes, these systems are preferred, as they often also offer relevant reference databases (Stiles & Holzapfel, 1997).

Immunological techniques have been used in discriminating LAB of various species or strains, for example, fructose-1, 6-biphosphate aldolase (London & Kline, 1973; London et al., 1975; London & Chase, 1976), malic enzymes, and glyceraldehydes-3phosphate dehydrogenase (London & Chace, 1983) have been used as evolutionary probes. Phenotypic tests also include, for example, the registration of the electrophoretic mobility of lactic acid dehydrogenases or the electrophoretic separation of whole cell proteins using (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; SDS-PAGE). The first technique (electrophoretic mobility of lactic acid dehydrogenases) has only historical importance and was used in the *L. acidophilus* group (Fujisawa et al., 1992). SDS-PAGE, however, is still used today, as

it is reliable for most lactic acid bacteria at the species and subspecies level (Schleifer & Stackebrandt, 1983; Dicks & van vuuren, 1987; Pot et al., 1996; Dicks, 1995; Van Damme et al., 1996b). The major disadvantage of the technique is the fact that it yields only discriminative information on the species level, requesting a certain degree of pre-identification. This problem has been overcome by the creation of a database of digitized and normalized protein patterns for a wide selection of reference strains (Vandamme et al., 1996a). The instability of several phenotypic properties of lactic acid bacteria can probably be linked to the presence of plasmids. This phenomenon has hampered the use of phenotypic tests in the early taxonomy of the lactic acid bacteria (Stiles & Holzapfel, 1997).

2.5.5.2.1. Cell wall composition

The detection of the presence or absence of meso-diaminopimelic acid in the cell wall was one of the key parameters in the earlier identification of lactobacilli (Kandler & Weiss, 1986; Hammes et al., 1991; Hammes & Hertel, 2003) which involves a relatively simple thin layer plate chromatographic procedure, which can be done on a large number of strains (Kandler & Weiss, 1986).

In *Lactobacillus* species, peptidoglycan types differ in the amino acid sequence of the peptide moiety and the cross-linkage type. For many species these differences are stable on the species level. The Lys-D-Asp type is the predominant type within the genus *Lactobacillus.* This might be useful to differentiate the genus *Weissella* from other lactic acid bacteria (Bjorkroth & Holzapfel, 2003) and *L. ruteri* from *L. fermentum* (Kandler & Weiss, 1986). The analysis, however, requires the preparation of purified cell walls and is therefore time consuming.

2.5.5.2.2. Serology

Serological technique is used to characterize some lactobacilli. The sevel serological groups, labeled A-G, have been described (Sharpe, 1979, 1981; Kandler & Weiss, 1986). However, unlike for streptococci, these studies did not contribute significantly to the identification and the classification of lactobacilli.

2.5.5.2.3. Chemotaxonomic markers

Lactoabcillus are found to have some chemotaxonomic markers but their taxonomic role is very limited. Meanquinones (Mk), predominantly with eight and nine isoprenene units (Mk-8 and Mk-9), have been found in *Lactobacillus yamanashiensis*

(now *Lactobacillus mali*) only (Collins & Jones, 1981). Fatty acid methyl ester (FAME) analysis has been used in lactic acid bacteria (Schleifer et al., 1985; Collins & Widdel, 1986, Collins et al., 1989a; Schmitt et al., 1989; Shaw & Harding, 1989; Pompei et al., 1991; Bjorkroth & Holzapfel, 2003). Sensitivity of the method to growth conditions particularly the growth temperature is the major disadvantage of FAME analysis.

2.5.5.3. Genotypic techniques

The classification of LAB, described above, is largely based on phenotypic and biochemical characters. In practice, in the routine identification of isolates, these characteristics may not be enough to definitely assign a strain to a particular species. Today, with the availability of rapid and automated DNA sequencing technology, direct sequencing of the 16S rRNA gene has emerged as the most powerful and relatively easy one-step method for classification of bacteria. The determination of 16S rRNA sequences for the elucidation of the phylogeny of the LAB during 1990 initiated a rapid development of DNA probes for identification of these bacteria (Stiles & Holzapfel, 1997).

The PCR technology is becoming more and more useful in bacterial systematics and genetics (the identification and classification purposes). With this technique it is possible to amplify a gene or a part of a gene from a very limited number of cells for subsequent DNA sequencing. One of the targets for such amplifications is obviously rRNA genes, and this method has replaced the reverse transcriptase technique for collecting rRNA sequence data for phylogenetic analysis. With automated sequencing systems and convenient direct PCR sequencing methods, it has become an easy task to determine the 16S rRNA sequence from any bacterium in a short time. PCR can also be used in combination with probing techniques (Salminen et al., 2004) or actually can replace them since the oligonucleotide probes designed from 16S rRNA sequencing also can be used in PCR applications.

2.5.5.3.1. DNA-DNA hybridizations and DNA base content

Wayne et al. (1987) mentioned the importance of DNA-DNA hybridization technique particularly in the species delineation. This technique has been applied extensively in the study of the taxonomy of lactobacilli (Simonds et al., 1971; Dellaglio et al., 1973, 1973, 1975; Vescovo et al., 1979; Johnson et al., 1980; Lauer et al., 1980; Dellaglio &

Torriani, 1986). The hybridizations are often limited to the type strains of a small set of closely related species, often revealed after (partial) 16S rRNA sequencing (e.g. *Lactobacillus concavus*: Tong & Dong, 2005). Problems arise with DNA-DNA hybridization when strains are phenotypically identical but reassociation values around or below 70% limit are obtained (Van Damme et al., 1996a).

Also, the DNA base content is part of every species definition and for lactic acid bacteria it is generally below 50%. Some *Lactobacillus* species, however, have values up to 55%. In practice, the G + C content should not vary more than 5% within a species and 10% within a genus (Schleifer & Stackebrandt, 1983). For *Lactobacillus* this limit is clearly exceeded (Axelsson, 2004), indicating the necessity of subdivision.

2.5.5.3.2. Comparative analysis of 16S/23S rRNA sequences

Comparative analysis of 16S/23S rRNA sequencing is one of the most powerful tools for finding phylogenetic relationships among bacteria (Ennahar et al., 2003). The universal presence and the conserved nature, 16S/23S rRNA has excellent properties as molecular clock and was shown to be extremely reliable to study phylogeny (Ljungh and Wadstrom, 2009). As a result, large database for sequence comparison have been possible, which fortified the usefulness of the technique for phylogeny reconstruction. This 16S rRNA encoding gene region is relatively small molecule and it can be sequenced directly from PCR amplicons using universal 16S primers. Even though public databases contain relatively large numbers of partial 16S rRNA sequences, mostly 1500 base pairs full 16S rRNA sequences are obtained (Figure 2.2). Once sequences are obtained they can be aligned and similarity can be calculated using various distance based and character based algorithms. Sequence alignment can be performed using a variety of algorithms and settings, implemented in a variety of computer programmes (such as BioEdit Sequence Editor for assembly and alignment). Phylogenetic relationship obtained (in terms of phylogenetic tree) may slightly vary but bootstrap analysis can be carried out to test overall stability of individual branches of the tree. Another way to reconstruct phylogeny is based on parsimony, which means that evolution is assumed to have reached current situation by the shortest possible route. Although this principle can be questioned, it has provided a stable basis for phylogenetic analysis over the years (Ennahar et al., 2003; Ljungh & Wadstrom, 2009).

Most species descriptions of the lactic acid bacteria include a 16S rRNA gene sequence (partial or full) for re-evaluation of the species definition in bacteriology based on the Ad Hoc Committee report. Being a conserved housekeeping gene, 16S rRNA gene is useful as a phylogenetic marker to reveal relationships between more distantly related genera of LAB or it can also poin out the misidentifications that occurs at the genus level (Martinez Murcia & Collins, 1990).

Historically, early 16S rRNA sequencing based contributions for the lactic acid bacteria were made by Yang & Woese (1989); Collins et al. (1989a, 1990, 1991); Martinez Murcia & Collins, (1990); Williams et al. (1990); Wallbanks et al. (1990); Bentley et al. (1991). As a major result, one should conclude that the genetic description of various species of Lactobacillus does not fit with the phylogenetic structure (Collins et al., 1991). The most striking conclusions highlighted by Pot (2007) are: (i) the heterogeneity of the lactobacilli, (ii) the rather close relationships between some lactobacilli and some pediococci, (iii) the separate position of the carnobacteria, more distantly related from the lactobacilli and more closely grouped with the enterococci and with *Vagococcus*.

Obligately heterofermentative as well as the obligately homofermentateive lactobacilli are scattered over various evolutionary branches. A number of branches, however, show a common type of carbohydrate fermentation, which could be used for reclassification, supported by evolutionary relationships (Hammes et al., 1991).

However, as stated earlier, one of the disadvantages of the 16S rRNA sequencing technique is that the molecule is too conserved to clear discrimination at the species and subspecies levels (De Parasis & Roth, 1990; Weisburg et al., 1991; Fox et al., 1992; Martinez Murcia et al., 1992; Stackebrandt & Goebel, 1994). Species of the lactic acid bacteria have been found to comprise identical 16S sequences (Yoon et al., 2000; Bjorkroth et al., 2002; Leisner et al., 2002). Some of the *Lactobacillus* species have been named while for other species there are sufficient phenotypic and genotypic differences to maintain a separate species status. G + C content, 16S rRNA similarity values can only be used as an excluding criteria, and other genotypic methods, such as DNA-DNA hybridizations, but also multilocus sequence analysis, may need to be used (Ljungh and Wadstrom, 2009) for identification purpose.

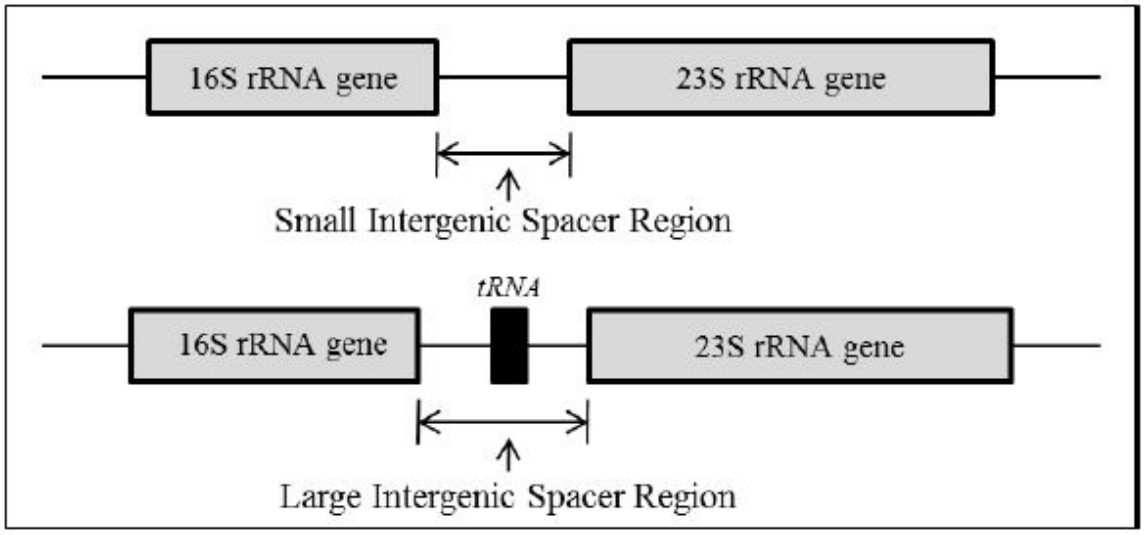

Figure 2.2. Schematic representation of 16S-23S rRNA gene intergenic region of bacteria.

The 16S rRNA gene sequence analysis is widely accepted technique for phylogenetic analysis, 16S rRNA gene (1500bp) represents only small portion of the whole genome of bacteria (Ennahar et al., 2003). In certain cases, complementary information is needed to discriminate the strains of a given species otherwise sequence varation between the 16S and 23S rRNA gene and length variation will be helpful for discrimination of the strais (Figure 2.2). This intergenic spacer region is about 200 bases in length if tRNA genes are abscent (small spacer sequence) and hypervariable in case of lactobacilli (Kim et al., 2003; Cachat & Priest, 2005). ITS is a simple way to identify the bacteria at species level. Tannock et al. (2002) showed the identification of lactobacilli at species level using intergenic region sequence analysis of isolate form human feces, rodent gastrointestinal samples and procine gastrointestinal contents. According to (Fortina et al., 2003) it can be used as a qualitative technique to confirm isolate as lactobacilli by simply comparing the electrophoretic mobility pattern of PCR amplified products on agarose gel, with the same of any standard lactobacilli strain. In fact, the sequencing of whole bacterial genome is the most useful tool to identify and characterize bacteria including probiotics. But genome sequencing is a laborious and relatively expensive technique hence it is not widely accepted. However, whole genome sequencing has been very useful to understand the underlying molecular mechanisms behind the probiotic properties and other important properties of dairy strains (Fortina et al., 2003).

2.5.5.3.3. Sequencing of housekeeping genes

For the species definition in Bacteriology, sequencing of housekeeping genes has been a promising tool for several phylogenetic studies (Stackebrandt et al., 2002). The typical constitutive genes are known as housekeeping genes which are required for the maintenance of basic cellular function. These housekeeping genes are expressed in all cells of an organism under normal and patho-physiological conditions (Eisenberg & Levanon, 2003; Kon Butte, 2001; Zhu, 2008). Also it was suggested to use a Multi Locus Sequence Typing (MLST) approach along with the sequencing of at least five house keeping genes located in diverse chromosomal loci which are widely distributed among taxa. The MLST technique has been very useful for taxa such as *Neisseria*, while for other taxa such as *Helicobacter* it was not very conclusive (Ljungh and Wadstrom, 2009; Internet visity, 2014. http://pubmlst.org/). For streptococci and enterococci traditional MLST techniques have been purposed (Enright & Spratt, 1998; Enright et al., 2001; Homan et al., 2002; King et al., 2002; Ljungh and Wadstrom, 2009; Internet visit, 2014. http://efacium.mlst.net/). Recently, Ramachandran et al. (2013) reported that MLST technique is useful for characterization and strain discrimination of *Lactobacillus* species as compared to PCR-RFLP which clusters strains into the species but lack strain level discrimination and RAPD-PCR which shows highly discriminatory profiles but lack reproducibility.

In *Lactobacillus*, sequencing of a more limited number of selected genes has proven to be quite promising. The a 38 kilodalton protein essential for the repair and maintenance of DNA (*recA*), 60 kDa Chaperonin protein subunits (*cpn60*), EF-tu elongation facture (*tuf*) and surface layer protein (*slp*) genes have been used in case of *Lactobacillus* (Felis et al., 2001; Torriani et al., 2001; Dellaglio et al., 2004b; Bringel et al., 2005; Cachat & Priest, 2005). In addition, phenylalanyl-tRNA synthase alpha subunit (*pheS*) and the RNA polymerase alpha subunit (*rpoA*) (Vancanneyt et al., 2006; Naser et al., 2007) have also been used in *Lactobacillus*. In *Enterococcus*, the alpha subunit of ATP synthase (*atpA*), *rpoA* and *pheS* have been used successfully (Dahlhof et al., 2000; Naser et al., 2005) and *gyrA, gyrB, soda* and *parC* genes in *Streptococcus* (Kawamura et al., 2005). The most interesting aspect of these evolutionary studies is the comparison of the phylogenetic trees obtained with different housekeeping genes, combinations of housekeeping genes or the comparison

with trees obtained from 16S or 23S ribosomal RNA sequence (Ljungh and Wadstrom, 2009).

2.5.5.3.4. Genotyping of lactobacilli

Many molecular typing techniques revealing relationships at the subspecific or strain level have been used in lactobacilli. These techniques include DNA restriction fragment length polymorphism (RFLP) (Uaviz et al., 2004), ribotyping (Bjorkroth & Korkeala, 1997; Bjorkroth et al., 2002, 2003; Suzuki et al., 2004; Kostinek et al., 2005) or involving in the use of specific oligonucleotide probes or primers that have been designed to allow quick identification of various species (Andrighetto et al., 1998; Torriani et al., 1999; Lick et al., 2000).

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is one of the PCR based genotyping technique used in lactobacilli which has allowed the construction of a library of reference strains for identification (Giraffa et al., 1998; Ventura et al., 2001). Polymerase chain reaction (PCR)-based techniques have also been very successful. The target sequence can be a Repetitive Extragenic Palindromic (REP) sequence (ERIC-PCR, GTG5-PCR, Box-PCR) (De Urraza et al., 2000; Gevers et al., 2001; Masco et al., 2003; Kostinek et al., 2005; Svec et al., 2005a,b) or a random sequence (Random amplified Polymorphic DNA; RAPD) (Moschetti et al., 1997; Torriani, et al., 1999, Dellaglio et al., 2005; Valcheva et al., 2005). These techniques all have in common that they are easy to perform, without extensive laboratory equipment. The disadvantage using RAPD-PCR is the least reproducible and comparatively REP-PCR is more reproducible.

More reliable is the Amplified Fragment Length Polymorphism (AFLP) fingeriprinting technique, which also gives information at the species level. This technique has been used in lactic acid bacteria by Dellaglio et al. (2005); Valcheva et al. (2005); Vancanneyt et al. (2005). A fluorescent variant has been described by Vancanneyt et al. (2005). AFLP fingerprinting has also been used to trace the origin of LAB that are dominant during a fermentation process (Nokuthula et al., 2000).

In clinical microbiology, Pulsed Field Gel Electrophoresis (PFGE) technique is considered as the gold standard for bacterial typing and epidemiology. However, it has not been used extensively in LAB. Examples in lacatics can be found in Guopeng

& Holley (1999), Orrhage et al. (2000), Blaiotta et al. (2001); Somers et al. (2001); Coppla et al. (2003); Dalgaard et al. (2003).

2.5.6. The *Lactobacillus* Classification and Phylogeny

Various methods and principles can be used for the classification of bacteria. Phylogenetic principle is one of them and the most reliable and accepted one as it deals with evolutionary principle. The phylogenetic position of *Lactobacillus*, according to the Taxonomic outline of the Prokaryotes (Garrity et al., 2004), is within the phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae. Its closest neighbours are the genera *Leuconostoc, Oenococcus* and *Wissella* (Hammes & Hertel, 2003; Pot, 2007). Most species of the genus *Pediococcus* and *Paralactobacillus selangorensis*, however, fall within the phylogenetic group of the lactobacilli.

Colllins et al. (1991) originally discriminated three phylogenetic groups *viz.* 1) the *L. delbrueckii* group, 2) the *L. casei – Pediococcus* group and 3) the *Leuconostoc* group. Later, some *Lactobacillus* species were classified in the genera *Leuconostoc* and *Weissella* reducing the heterogeneity of genus *Lactobacillus* (Collins et al., 1993). The *L. delbrueckii* group was renamed as *L. acidophilus* group by Schleifer & Ludwig (1995) and the heterogenous *L. casei – Pediococcus* group was splited into smaller subgroups: the *Lactobacillus buchneri* group, the *L. casei* group, the L. *plantarum* group, the *L. reuteri* group and *L. salivarius* group. In the meantime, some species of the genus have also been transferred to genera as a *Carnobacterium* and *Atopobium*. It is very likely that the genus will be further subdivided into multiple new genera, restricting the name *Lactobacillus* to the group of species around *L. delbrueckii*, the type species. As mentioned before, the taxonomic heterogeneity is nicely illustrated by DNA G + C content which range from 32 to 54 mol%, far too broad for a genus (Schleifer & Stackebrandt, 1983).

The number of genera to be defined or created could be a matter of long debate. The parameters and algorithms used for phylogenetic tree construction as well as the number of 16S rRNA sequences used, will heavily determine the result of the analysis. More diverse sequences will result in many more gaps in the aligned sequences and therefore give rise to different levels of similarity. Phylogenetic positions of species, represented by a single sequence, will therefore easily change

positions when included in a dendrogram representing the family Lactobacillaceae, the genus *Lactobacillus* or the subgroups *L. acidophilus*. Dellaglio & Felis (2005) also noted that the addition of novel species dramatically changes the phylogenetic structure of the genus, even in short time, which can also explain why in the taxonomic literature many different groupings and dendrograms will be encountered for the genus *Lactobacillus*. This phenomenon will clearly render the decision to create new genera for recognized phylogenetic groups rather difficult. The lack of clear phenotypic markers that helps to recognize these phylogenetic groups has rendered the task extremely difficult. It can be hoped that the gradual availability of other sequences than 16S rRNA sequences (e.g. protein coding sequences from the *recA, cpn60, tuf, slp, pheS* and *rpoA* genes) will help to delineate consensus groups that can be considered for the definition of new genera (Collins et al., 1993).

2.6.0. Probiotics

2.6.1. Introduction

The Gastro Intestinal (GI) microflora plays an important role in the health status of people and animals (Fuller, 1989). In past years, the primary function of the human gastrointestinal tract had been considered as digestion and absorption of nutrients and excretion of waste products. However, these days, it has been accepted that the gastrointestinal tract performs many other functions, which are essential for our well-being (Mombelli & Gismondo, 2000). The GI tract represents a much larger contact area with the environment, compared to the two square meter skin surface of our body (Van Dijk, 1997). The mucosal surface of the small intestine is increased by forming circular folds called intestinal villi and the formation of microvilli in the enterocyte resorptive luminal membrane. The resulting surface of the GI system is calculated to be 150-200 square meter. Therefore it provides enough space for the interaction related to the digestion and for adhesion to the mucosal wall. The intestinal tract of human is host to a vast ecology of microbes and harbors more than 500 identified species that can be cultured and many bacteria that cannot be cultured and properly identified (Ljungh & Wadstrom, 2009). These bacteria which are necessary for health have the potential to contribute to the development of diseases also by a variety of mechanisms (Pham et. al., 2008). Mucosa of the GI tract function as a barrier excluding and eliminating numerous antigens derived from the external environment. The peaceful co-existence of microbes with the host is referred to as host-microbe

cross-talk, implying a benefit of the microbial presence to the host. Perturbations in the intestinal epithelium can lead to an inflammatory response resulting directly from microbial products that alter underlying epithelium or allow bacterial and food antigens to stimulate the mucosal immune system. Interactions between intestinal microbes and the host are the subject of intensive ongoing research as these changes influence a variety of diseases. The intestinal microflora can be altered by the administration of antibiotics, prebiotics (i.e. dietary components that promote the growth and metabolic activity of beneficial bacteria), or administration of probiotics (i.e., beneficial bacteria) (Harish & Varghese, 2006).

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). The term probiotic was initially used in the 1960s and came from a Greek word meaning "for life". Although a relatively new word, the beneficial effects of certain foods containing live bacteria have been recognized for centuries. However, it was not until the early 20th century that investigators suggested gut flora could be altered with beneficial bacteria replacing harmful microbes, leading to the concept of probiotics (Senok et. al., 2005). Increasing clinical evidence supports some of the proposed health benefits related to the use of probiotics, particularly in managing certain diarrheal diseases. Microorganisms considered as probiotics are listed (Table 2.6). Probiotics, which are regulated as dietary supplements and foods, consist of yeast or bacteria. These days they are also available as capsules, tablets, packets, or powders and are contained in various fermented foods, most commonly yogurt or dairy drinks. Probiotics products may contain a single microorganism or a mixture of several species. The most widely used probiotics include lactic acid bacteria, specifically *Lactobacillus* and *Bifidobacterium* species. It is noteworthy that probiotic effects tend to be specific to a particular strain, so a health benefit attributed to one strain is not necessarily applicable to another strain, so there can be variation in probiotic properties even within one species. Therefore, generalizations about potential health benefits should not be made (Vanderhoof & Young, 2008).

The rationale for using probiotics involves restoring microbial balance. An appropriate balance of gut flora is generally maintained, however, antibiotics, immunosuppressive medications, surgery, and irradiation can cause an increase in the pathogenic bacteria and disrupt this homeostasis (Guarner & Malagelada, 2003).

Table 2.6. Microorganisms considered as probiotics (Ljungh & Wadstrom, 2009).

Lactobacillus spp.	*Enterococci*	*Bifidobacterium* spp.	Other organisms
L. acidophilus	*E. faceium*	*B. adolescentis*	*Bacillus cereus var.* toyoi
L. amylovorus	*E. faecalis*	*B. animalis*	*Escherichia coli* strain Nissle
L. crispatus		*B. bifidum*	*Saccharomyces cerevisiae (boulardii)*
L. delbrueckii ssp. *Bulgaricus*		*B. breve*	
L. fermentum		*B. infantis*	
L. gallinarum		*B. longum*	
L. gasseri			
L .helviticus			
L. johnsonii			
L. paracasei			
L. plantarum			
L. ruteri			
L. rhamnosus			
L. salivarius			

Probiotics may restore the microbial balance in the gastrointestinal tract. However, in order for probiotics to be successful, they must possess certain characteristics. Probiotic organisms must be able to withstand passage through the gastrointestinal tract (i.e., survive acid and bile environment), colonize and reproduce in the gut, attach and adhere to the intestinal epithelium, and stabilize the balance of the gut flora. Furthermore, probiotic strains must be safe and effective in humans, remain viable for the shelf life of the product, and do not have pathogenic properties (Goldin, 1998). Products containing more than one organism are particularly appealing for two reasons: colonization in some patients may occur with one strain and not another, and probiotic mixtures may be synergistic in suppressing pathogens (Gibson & Fuller, 2000).

2.6.2. The History and the Definition of Probiotics

The word ‘probiotic’ came from Greek language ‘pro bios’ which means ‘for life’ as opposed to ‘antibiotics’ which means ‘against life’. Probiotics history initiated with the history of man consuming fermented foods which is well known Greek and Romans history (Gismondo, et. al., 1999; Guarner, et. al., 2005). In 1908 a Russian researcher Ellie Metchnikoff, a Nobel Prize winner firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented dairy products which consists of rod shaped bacteria, the *Lactobacillus* spp. Therefore, these bacteria positively affect the gut microflora and decrease the microbial toxic activity (Cakir, 2003; Chuayana et. al., 2003). The term ‘probiotic’ was first used in 1965 by Lilly & Stillwell to describe substances which stimulate the growth of other microorganisms (Guarner & Malagelada, 2003). Subsequently, the word ‘probiotic’ was used with different meaning according to its mechanism and effects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined ‘probiotic’ as ‘substances and organisms which benefits to the intestinal microbial balance’. Later, the meaning of ‘Probiotic’ was improved by Fuller (1989) as a live microbial supplement which affects host’s health positively by improving its intestinal microbial balance. This definition was further broadened by Havenaar & Huis in’t Veld in 1992 including mono or mixed culture of live microorganisms which is applied for animal and human (Guarner, et. al., 2005; Sanders, 2003). In the following years, lots of researchers studied on probiotics and proposed many definitions which are listed below.

1. “Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” by Schaafsma (1996).
2. “A microbial dietary adjuvant that beneficially affects the host physiology by modulation of mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract” by Naidu et al. (1999).
3. “A live microbial food ingredient that is beneficial to health” by Salminen et al. (1998).
4. “A preparation of a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in

a compartment of the host and by that exert beneficial health effects in this host" by Schrezenmeir & de Vrese (2001).

5. "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" is accepted by FAO/WHO (2001) (Kalaenhammer 2000; Sanders 2003; Guarner et al., 2005).

The probiotic concept has broad applications in many fields relevant for human and animal health, and probiotics are also challenging for industrial applications (Guarner & Malagelada, 2003). Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have beneficial effects on health of the host (Fernandez et al., 2014). They can contain one or several species of probiotic bacteria. Most of the products for human consumption are produced in fermented milk and are given in powders or tablets. These capsules and tablets are not used for medical applications. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. Numerous studies suggest that probiotics have beneficial effects on microbial disorders of the gut, but it is really difficult to show the clinical effects of such products. The probiotic preparations have been applied in the treatment of traveler's diarrhoea, antibiotic associated diarrhoea and acute diarrhea which have showed that they have positive therapeutic effect (Quwehand, 1999; Gismondo et al., 1999; Cakir, 2003).

Human intestinal tract harbors hundreds of bacterial species which enormously is a complex ecosystem that includes both facultative anaerobic and anaerobic microroganims (Naidu et al., 1999). The numbers of genera is nearly steady, because each has its own growth niche (Fooks et al., 1999). The gut microflora composition is stable but some times can be affected by several factors such as age, diet, environment, stress and medication. To have a healthy intestine the balance of bacteria must be maintained but this is difficult as the lifestyles are changing. Lots of factors may change the balance away from potentially beneficial or health promoting bacteria like lactobacilli and bifidobacteria and favor potentially harmful or pathogenic microorganisms like clostridia, sulphate reducers, and bacterioides species (Jacobsen et al., 1999). It makes the host more susceptible to the illnesses. In this case the prevalence of the beneficial bacteria must be supported. Use of proboitics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks et al., 1999).

Probiotics are suggested as food to provide the balance of intestinal flora (Holzapfel et al., 1998). Probiotics are being used for a long time in food ingredients for human and also to feed the animals without any side effects (Arques et al., 2014). Probiotics are acceptable because of being naturally harbored in intestinal tract of healthy human and in foods (Chakir, 2003).

2.6.3. The Beneficial Effects of Probiotics on Human Health

A number of research studies have been conducted to search on the health benefits of fermented foods and probiotics. However, in most of these studies researchers did not use sufficient scientific tests and did not use well characterized and itentified microorganisms (Cakir, 2003). While a number of reported effects have been only partially established, some can be regarded as established and clinically well documented for specific strains (Elmer et al., 2012). The health related beneficial effects of probiotics are: 1) Managing lactose intolerance, 2) Improving immune system, 3) Prevention of colon cancer, 4) Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence), 5) Lowering blood pressure, 6) Reducing inflammation, 7) Reduction of allergic symptoms, 8) Beneficial effects on mineral metabolism, particularly bone density and stability, 9) Reduction of *Helicobacter pylori* infection, 10) Suppression of pathogenic microorganisms (antimicrobial effect), 11) Prevention of osteoporosis and 12) Prevention of urogenitial infections (Dugas et al., 1999; Schrezenmeir and De Vrese, 2001; Dunne et al., 2001; Cakir, 2003; Luongo et al., 2013). Some of these effects are reviewed in detail in following section.

2.6.3.1. Lactose Intolerance

In humans, particularly among non-Caucasians, lactose intolerance is prevalent after weaning. These lactose intolerant people cannot metabolize lactose due to the lack of essential enzyme β-galactosidase. When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence, cramping and diarrhea ensue (Lee & Salminen, 2008). If lactose passes through from the small intestine, it is fermented to acid and gas in the large intestine by the colonic microflora. Also, the presence of breath hydrogen is a signal for lactose maldigestion (Otles, 2013; Fernandez et al., 2014). The studies provide information that the addition of certain starter cultures to milk products, allows the lacatose intolerant people to consume those products without the usual rise of breath hydrogen or

associated symptoms (Scheinbach, 1998; Fooks et al., 1999). The beneficial effects of probiotics on lactose intolerance can be explained in two ways. One of them is lower lactose concentration in the fermented foods due to the high lactase activity of bacterial preparations used in the production. The other one is increased active lactase enzyme enters the small intestine with the fermented product or with the viable probiotic bacteria (Salminen et al., 2004; Mattar et al., 2012). When the yogurt is compared with milk, because the lactose is converted to lactic acid and the yogurts consist of bacterial β-galactosidase enzyme, it is suitable and beneficial to be consumed by lactose intolerant people. Furthermore, the LABs which are used to produce yogurt *viz. Lactobacillus bulgaricus* and *Streptococcus thermophilus* are not resistant to gastric acidity. Hence, products containing probiotic bacteria are more efficient for lactose intolerant human.

It is thought that the major factor that improves the digestibility by the hydrolysis of lactose is the bacterial enzyme β-galactosidase. Another factor is the slower gastric emptying of semi-solid milk products such as yogurt. So the β-galactosidase activity of probiotic strains and other lactic acid bacteria used in dairy products is very important. β-galactosidase activity of various probiotic organisms varies greately. Both the enzyme activity of probiotic strain and the activity left in the final product have to be considered for their use in lactose intolerant subjects (Salminen et. al., 2004).

2.6.3.2. Effects on Immune System

The effects of probiotics on the immune system are promising, however, the mechanisms involved are not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts (Mombelli & Gismondo, 2000; Luongo et al., 2013). Several researchers have studied on the effects of probiotics on immune system stimulation. Some *in vitro* and *in vivo* researches have been carried in mice and some in human (Figure 2.3). Data indicate that oral bacteriotherapy and feeding live bacteria in fermented milks supported the immune system against some pathogens. Probiotics affect the immune system in different ways such as producing cytokines, stimulating macrophages and increasing secretory IgA concentrations (Scheinbach, 1998; Dugas et al., 1999; Cakir 2003). Link-Amster et al. (1994) examined whether eating fermented milk containing *Lactobacillus acidophilus* La1 and Bifidobacteria could modulate the immune

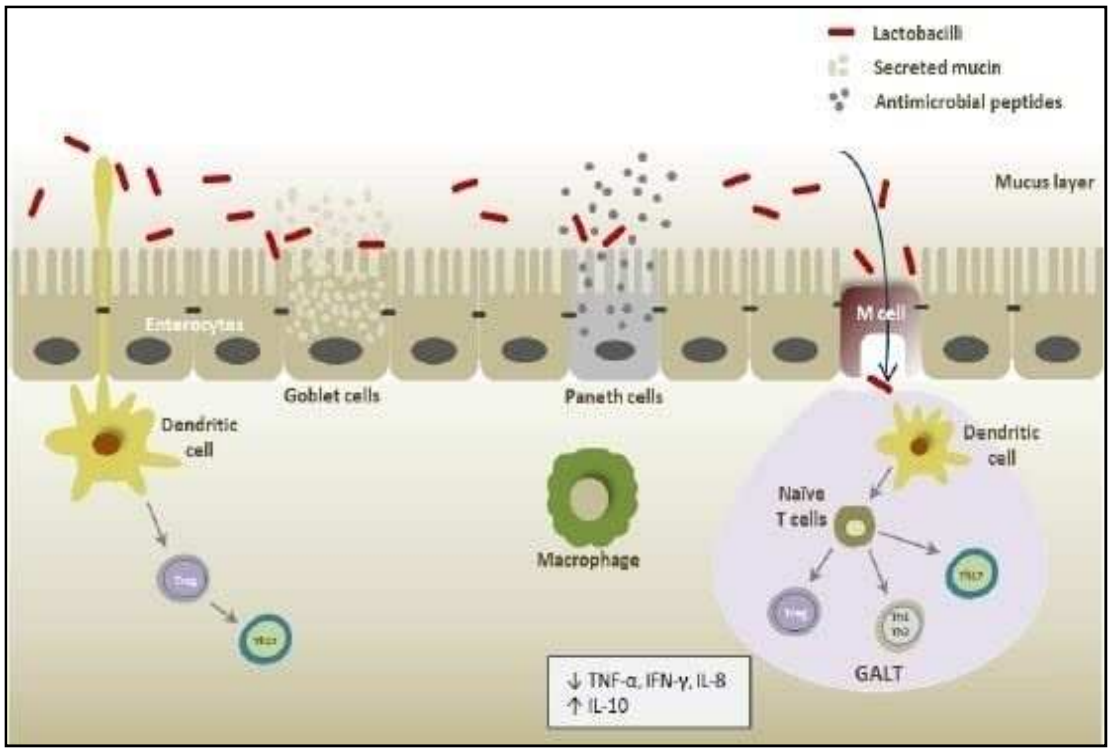

Figure 2.3. Schematic representation of lactobacilli mediated immune modulation in intestinal mucosa. Probiotic lactobacilli induces 1) the mucin secretion from globlet cells, 2) secretion of antimicrobial peptides induced from Paneth cells, and 3) translocation via M cell to the localized lymphoid tissues and interact with immune cells which in turn alter the cytokines and chemokines expression and also activate the native T cells.

response in human. Volunteers were given the test fermented milk over a period of three weeks during which attenuated *Salmonella typhi* Ty21a was administered to mimic an enteropathogenic infection. After three weeks, the specific serum IgA titre raised in the test group was > 4-fold and significantly higher ($p=0.04$) than in the control group which did not eat fermented foods but received *S. typhi* Ty21a. The total serum IgA was also found to be increased. These results showed that LAB which can survive in the GI can act as adjuvant to the humoral immune response (Quwehand et al., 1999). Perdigon et al. (1986) fed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations (Scheinbach, 1998). Also in a human trial Halpern et al., (1991) fed humans 450 g of yogurt every day for four months and at the end a significant increase is observed in the production of γ-interferon (Fooks et al., 1999) was observed. Also *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 derived extracts suppressed lymphocyte proliferation *in vitro* (Mattilla-Sandholm & Kauppila, 1998). Further evidence found for immunomodulation by these two strains in a children trial was in severe atopic

eczema resulting from food allergy. Children fed with *Lactobacillus rhamnosus* and *Bifidobacterium lactis* Bb-12 showed improvement in clinical symptoms of eczema compared to placebo group (Saarela et al., 2000).

2.6.3.3. Diarrhoea

Causes of Human diarrhoea may be diverse creating difficulties in the evaluation of the effects of probiotics on diarrhea (Otles, 2013; Arques et al., 2014). However, many evidences support that probiotics have beneficial effects on some types of diarrhoea. Diarrhoea is a major reason for children mortality worldwide and viral diarrhoea caused by rotavirus is most important one (Scheinbach, 1998). In the treatment of rotavirus diarrhoea, *Lactobacillus rhamnosus* GG is reported to be very effective (Otles, 2013; Fernandez et al., 2014). The best documented probiotic effect is shortening the duration of rotavirus diarrhoea using *Lactobacillus* GG (Guandalini, 2006). Also, *Lactobacillus acidophilus* LB1, *Bifidobacterium lactis* and *Lactobacillus reuteri* are reported to have beneficial effects on shortening the diarrhoea period (Salminen et. al., 2004). Another type of diarrhoea is traveler's diarrhoea (TD) which diarrhea affects the healthy human in developing countries and also in the developed countries of Europe. Probiotics have beneficial effects in preventing some forms of TD. Oksanen et al. (1990) evaluated the efficacy of *Lactobacillus* GG in preventing diarrhoea in 820 people travelling from Finland to Turkey while Black et al. (1989) performed a double-blind study by lyophilized bacteria (*L. acidophilus, L. bulgaricus, B. bifidum, S. thermophilus*) in 56 Danish tourists on a two week trip to Egypt, in both the cases, the occurrence of diarrhoea in the group receiving the lactic acid bacteria was 43% while it was 71% in placebo group (Gismondo et al., 1999).

Sometimes use of antibiotics causes mild and severe outbreaks of diarrhoea. The normal microflora may be suppressed during the antibiotic therapy and results in colonization by pathogenic strains. The changes of microflora may also encourage the resistant strains such as *Clostridium difficile,* which is the reason for the development of Antibiotic Associated Diarrhoea (AAD). Several clinical trials have used *Saccharomyces boulardii, Lactobacillus* spp. and *Bifidobacterium* spp. in AAD. Probiotic microorganisms which can restore and replace the the normal flora should be used (Hickson, 2011). Probioitcs should also be used in high risk patients such as old, hospitalized or immune suppressed. Studies have shown that *Clostridium difficile*

concentration was decreased in the presence of *Saccharomyces boularidii* (Gismondo et al., 1999).

2.6.3.4. Cancer

Increased consumption of satureaed fatty acids have been pointed out during epidemiological studies in the western world which increases the incidence of colon cancer (Otles, 2013). Bacterial enzymes (β-glucurornidase, nitroreductase and azoreductase) convert pre-carcinogens to active carcinogens in the colon (Lee & Salminen, 2008). It is thought that probiotics could reduce the risk of cancer by decreasing the enzyme activity. Although the exact mechanism for the anti tumor action of probiotics is not known, some suggestions have been proposed (Scheinbach, 1998; Fooks et al., 1999):

1. Suppressioin of carcinogen/pro-carcinogen by binding, blocking or removal using probiotics.
2. Inhibit the bacterial growth with enzyme that may convert the pro-carcinogens to carcinogens.
3. Change in intestinal pH which may alter microbial activity and bile solubility.
4. Removal of fecal mutagens by altering the colonic transit time.
5. Stimulation of the immune system.

The *in vitro* and *in vivo* evidences from animal and human studies have shown that probiotics have beneficial effects on suppression of cancer. Oral administration of lactic acid bacteria has been shown to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa in rats model (Herias et al., 2005). The consumption of lactobacilli by healthy volunteers has been demonstrated to reduce mutagenicity of urine and feces associated with the ingestion of carcinogens in cooked meat. However, in some epidemiological studies, an association between fermented dairy products and colorectal cancer has been revealed (Raman et al., 2013). The regular consumption of certain quantity of fermented dairy foods (such as fermented milk products) harboring bacterial species such as *Lactobacillus* or *Bifidobacterium* may be related to lower incidences of colon cancer (Rafter, 2003). A number of studies have shown that cancer predisposing factors (increment of enzyme activity triggering carcinogens, increament of pro-carcinogenic chemicals inside the colon or modification of certain bacterial genera and species) are altered positively by consumption of certain probiotics (Brady et al., 2000). Synbiotics (combination of

probiotic and prebiotic) has been found to exert a synergistic effect in improving colon carcinogenesis compared to when both were used individually (Liong, 2008).

2.6.3.5. Cholesterol Reduction

Based on several studies, scientists have proposed that probiotics contain cholesterol reduction effects (Ha et al., 2006; Ooi & Liong, 2010) though the exact mechanism with respect to cholesterol reduction has not been properly explained so far. However, there are two hypotheses have been proposed to explain the mechanism of cholesterol reduction. 1) Bacteria may bind or incorporate cholesterol directly into the cell membrane and 2) Bile salt hydrolysis enzymes deconjugate the bile salts which are more likely to be exerted resulting in cholesterol breakdown greater than before (Scheinbach, 1998; Prakash & Jones, 2004). Kaur et al. (2002) reported cholesterol reduction by 38% when it *Lactobacillus reuteri* CRL 1098 was given to mice for seven days at the rate of 10^4 cells per day. Also, 40% reduction in triglycerides and a 20% increament in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen and liver was noted by the consumption of the above mentioned dose of bacteria.

2.6.3.6. Pharmacology

Lactic acid bacteria produce lactic acid, acetic acid, and propionic acid, which lower the intestinal pH and suppress the growth of various pathogenic bacteria, thereby reestablishing the balance of the gut flora (Alvare-Olmos & Oberhelman, 2001; Doron & Gorbach, 2006). Another mechanism of bacterial interference involves the production of various substances, such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants, that are toxic to pathogenic microorganisms (Vanderhoof & Young, 2004; MacIntre & Cymet, 2005; Doron & Gorbach, 2006). One probiotic with this ability is *Lactobacillus* species strain GG, which has been shown to secrete a low molecular weight compound that inhibits a broad spectrum of gram positive, gram negative and anaerobic bacteria (Silva et al., 1987).

Saccharomyces boularidii, a non-pathogenic yeast, may have a role in *Clostridium difficile* infection by producing a protease that decreases the toxicity of *C. difficile* toxins A and B (Castagliuola et al., 1999). Probiotics also decrease colonization of pathogenic organisms in the urinary and intestinal tracts by competitively blocking their adhesion to the epithelium (MacIntre & Cymet, 2005). Lactobacilli have been

shown to inhibit the attachment of *Escherichia coli, Kelebsiella pneumonia,* and *Pseudomonas aeruginosa* to uroepithelial cells and intestinal epithelial cells (Mack et al., 1999). This inhibition may occur because lactobacilli cause steric hindrance and upregulate intestinal mucins, which are high molecular weight glycoproteins produced by epithelial cells resulting into the formation of protective barrier. In addition, lactobacilli strengthen the gut mucosal barrier by stabilizing tight junctions between epithelial cells and decreasing intestinal permeability (Doron & Gorbach, 2006).

Another proposed mechanism of action of probiotics involves immunomodulation. Animal studies have found that some probiotic strains augment the immune response by stimulating the phagocytic activity of lymphocytes and macrophages (Reid et al., 2003). Probiotics also increase immunoglobulin A (IgA) and stimulate cytokine production by mononuclear cells and it has been found that children with acute rotaviral diarrhoea who were given *Lactobacillus rhamnosus* strain GG (LGG) had an increased IgA, immunoglobulin G, and immunoglobulin M response, resulting in a shortened duration of gastroenteritis symptoms (Kaila et al., 1992; Reid et al., 2003; Scarpellini et al., 2008). There is numerous health effects associated with probiotic use. While some of these indications are well documented, probiotics are often used to treat conditions for which data regarding the efficacy of probiotics are lacking or conflicting (Senok et al., 2005; Vanderhoof & Young 2008).

2.6.3.7. Inflammatory bowel disease

Ulcerative colitis (UC), Crohn's disease, and pouchitis are the inflammatory diseases of the digestive tract. An imbalance of intestinal microflora, specifically high numbers of enteroadhesive and enterohemorrhagic *E. coli* with UC and reduced levels of bifidobacteria with Crohn's disease, may contribute to the inflammation seen with these diseases. Probiotics may improve the microbial balance of the indigenous flora. Although studies have been conflicting, probiotics seem to be an attractive option in the treatment and prevention of inflammatory bowel disease, providing an appealing alternative to the use of antibiotics (Santosa et al., 2006; Doron & Gorbach, 2006). Several studies examining the role of probiotics in UC have suggested that they can reduce or maintain disease remission. Three controlled trials involving probiotics were compared against *E. coli* by Nissle (1917) in UC and found that the two mesalamine therapies were similar in preventing disease relapse, suggesting that the probiotic was equivalent to standard therapy with mesalamine in maintaining

remission. Two of the studies had notable limitations i.e. diverse patient population and short study duration but the more recent study was methodologically better and confirmed the results of the other two studies. The particular non-pathogenic *E. coli* probiotic strain used in these three studies has been shown to colonize the intestine and antagonize the pathogenic bacteria seen with UC (Kruis et al., 2004; Rembacken et al., 1999). Another study investigated the use of *S. boulardii* in 25 patients who developed a mild to moderate clinical flare-up of UC while taking standard maintenance therapy with mesalamine (Guslandi et al., 2000) and because of different reasons, treatment with corticosteroids was not suitable for these patients. Clinical remission, confirmed endoscopically, was attained in 68% of patients after adding a four-week course of *S. boulardii* to mesalamine treatment. This study was limited by its small sample size, lack of a control group, and open-label design. Bibiloni et al. (2005) noted that a six-week course of VSL#3 was also effective in inducing remission or causing a response in 77% of patients with active mild-to-moderate UC that was unresponsive to conventional therapy. This open-label trial also lacked a control group and involved only 34 patients. Studies have also investigated the role of probiotics in maintaining and remission of Crohn's disease. Guslandi et al. (2000) noted that patients with inactive Crohn's disease had a significantly lower clinical relapse rate when receiving a six-month regimen of *S. boulardii* plus mesalamine versus treatment with mesalamine alone (6.25% versus 37.5%, $p = 0.04$), suggesting that the probiotic yeast may be beneficial in the maintenance treatment of Crohn's disease. In contrast, Marteau et al. (2006) found that a six-month regimen of *Lactobacillus johnsonii* LA1 was not effective in preventing endoscopic recurrence of Crohn's disease after surgical resection.

Various studies support the use of probiotics, particularly VSL#3, in reducing relapse rates and maintaining remission of Pouchitis. Pouchitis is a non-specific inflammation of the ileal reservoir, which is formed surgically after an ileal puch-anal anastomosis from a protocolectomy. It is characterized by increased stool frequency and abdominal cramping (Gionchetti et al., 2000; Gionchetti et al., 2003; Mimura et al., 2004). Although the etiology of pouchitis is unknown, it may be associated with decreased lactobacilli and bifidobacteria counts as well as increased concentrations of other bacteria (Gionchetti et al., 2000). In addition to modifying the endogenous flora, VSL#3 alters the immune response in pouchitis by raising tissue levels of the anti-

inflammatory cytokine interleuckin 10 and reducing tissue levels of tumor necrosis factor a, interferon g, and matrix metalloproteinase activity. In a randomized, double-blind, placebo-controlled trial involving 40 patients with chronic relapsing pouchitis, Gionchetti et al. (2006) found that VSL#3 was significantly more effective than placebo in maintaining remission after nine months. All 20 placebo-treated patients experienced a relapse within four months, while 17 of the 20 patients treated with VSL#3 remained in remission after nine months ($p < 0.001$). When the probiotic was discontinued at the study's end, these 17 patients also experienced relapse within four months. In addition, fecal concentrations of lactobacilli, bifidobacteria, and *S. thermophilus* increased significantly from baseline in patients treated with VSL#3 ($p < 0.001$). Mimura et al. (2004) confirmed the efficacy of VSL#3 in maintaining remission in patients with recurrent or refractory pouchitis. In this study, 36 patients whose pouchitis was in remission were randomized to receive VSL#3 or placebo for one year or until relapse. Similar to the previous study, 17 of the 20 patients treated with VSL#3 remained in remission at one year versus only 1 of 16 patients who received placebo ($p < 0.0001$). In addition to preventing relapses, Gionchetti et al. 2006, showed that the probiotic mixture VSL#3 was significantly more effective than placebo in preventing occurrence of pouchitis ($p < 0.05$) during the first year after pouch formation in this randomized, double blind, placebo-controlled study involving 40 patients. In contrast to those studies with encouraging results using VSL#3 in pouchitis, a three-month trial involving LGG did not show any benefit as primary therapy for ideal pouch inflammation (Kuisma et al., 2003). This trial did not show differences in the mean pouchitis disease activity index scores between treatment with LGG and placebo, and only 40% of patients who received the probiotic had LGG recovered in their fecal flora.

2.6.3.8. Irritable Bowel Syndrome (IBS)

IBS is characterized by abdominal pain, bloating, flatulence, and altered bowel habits. These symptoms may be due to bacterial overgrowth in the small intestine, causing increased fermentation activities and gas production (Wilhelm et al., 2008). Some studies suggested that probiotics may be beneficial in reducing bloating and flatulence associated with IBS. The probiotics used most frequently in the treatment of IBS include lactobacilli and bifidobacteria. In addition, a combination product (VSL#3, VSL Pharmaceuticals, Inc., Towson, MD) has reduced abdominal bloating and

flatulence. This preparation contains eight bacterial organisms in large numbers: three bifidobacteria (*Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*), four lactobacilli (*L. acidophilus*, *Lactobacillus casei*, *L. bulgaricus*, and *L. plantarum*), and *S. thermophilus*. A meta analysis involving 20 trials (n = 1404) found that probiotics (most commonly lactobacilli and bifidobacteria) improved global IBS symptoms (RRpooled, 0.77; 95% CI, 0.62-0.94) and reduced abdominal pain (RRpooled, 0.78; 95% CI, 0.69-0.88) compared with placebo (McFarland & Dublin, 2008). This meta analysis was not able to examine other types of individual IBS symptoms (e.g. bloating or distension, flatulence, stool frequency) or the effectiveness of specific probiotic strains due to insufficient data. A review of 14 clinical trials also revealed that probiotics (most commonly lactobacilli, bifidobacteria, and VSL#3) improved overall symptoms associated with IBS compared with placebo; however, the contributing studies had methodological limitations (Wilhelm et al., 2008). Although probiotics may be beneficial in treating IBS symptoms, limitations exist in interpreting trial results due to the lack of standardization of strains, dosages, treatment durations, and assessment of clinical outcomes. More data are needed before probiotics can be recommended as typical solution in the treatment of IBS.

2.6.3.9. Allergy

Some studies have found that probiotics have a beneficial effect on atopic eczema. A study conducted by Kalliomaki et al. (2003) in double-blind, randomized, placebo-controlled trial in which 159 pregnant women with a family history of atopic disease were given *Lactobacillus* GG or placebo daily for two to four weeks before their expected delivery date, followed by administration of the probiotic or placebo to the newborn infant for 6 months, of the total only 132 participants completed the trial. There was a 50% reduction in the frequency of atopic eczema during the first two years of the children's lives in those given probiotics compared with placebo [23% (15 of 64) versus 46% (31 of 68); RR, 0,51; 95% Ck, 0.32-0.84; p = 0.008). This cohort was reexamined after four years, and significantly fewer children who had previously received LGG were diagnosed with atopic eczema compared with placebo [26% (14 of 53) versus 46% (25 of 54); RR, 0.57; 95% Cl, 0.33-0.97], suggesting that the protective effect of this probiotic on atopic eczema in children at risk continues beyond infancy (Kallioaki et al., 2001). In one more randomized double-blind study,

27 infants (mean age, 4.6 months) with atopic eczema received formula supplemented with probiotics (either LGG or *Bifidobacterium lactis* Bb-12) or the same formula without probiotics (Isolauri et al., 2000). After 2 months, the Scoring Atopic Dermatitis index, revealed that the extent and severity of atopic eczema, was reduced significantly in the infants given probiotic supplemented formulas compared with those who did not receive probiotic supplementation ($p = 0.002$).

2.6.3.10. Genitourinary infections

Unusual vaginal microbiota may lead to the symptomatic infections, including vulvovaginal candidiasis (VVC). Lactobacilli, especially *Lactobacillus crispatus* and *Lactobacillus iners*, are the predominant vaginal microorganisms in healthy premenopausal women. When the normal vaginal microflora is disrupted, such as with use of broad spectrum antibiotics, overgrowth of *Candida albicans* may occur, causing VVC. Restoring the normal flora with lactobacilli may help treat this genital infection (Reid et al., 2003). A study involving 28 women with a history of recurrent VVC, suppositories containing LGG were given twice daily for seven days, all of the women reported an improvement in vaginal symptoms and reduced vaginal erythema and discharge (Hilton et al., 1997). Reid et al. (2001) investigated the ability of an orally administered solution containing *L. rhamnsosus* GR-1 and *L. fermentum* RC-14 to colonize the vagina in 10 women who were asymptomatic for infection but who had a history of recurrent urogenital infections, primarily recurrent VVC. The probiotic solution was administered twice daily for 14 days. Within one week, one or both of the *Lactobacillus* strains were recovered from the vaginas of all 10 women, and no VVC occurred during the study. Hilton et al. (1995) found that consumption of 8 oz of yogurt containing *L. acidophilus* daily for six months reduced vaginal colonization and infection by *Candida* species in crossover trial involving 33 women with recurrent VVC, 13 of whom completed the protocol. The mean number of candidal infections of the vagina and *Candida* colonization in the vagina and rectum were significantly lower in the women who consumed yogurt versus the control (0.38 versus 2.54, $p = 0.001$ and 0.84 versus 3.23, $p = 0.001$, respectively). However, these studies had important methodological limitations, including small sample sizes, inadequate controls, and lack of blinding. Two of the studies lacked detailed statistical analyses, one study had a high attrition rate and more than half of the women in one study had recently completed treatment with antifungal medications (Hilton et al.,

1995). Therefore, it is difficult to reliably conclude whether probiotics can prevent recurrent VVC.

2.6.3.11. Drug interactions

Since probiotics contain live microorganisms, concurrent administration of antibiotics could kill a large number of organisms, reducing the efficacy of the *Lactobacillus* and *Bifidobacterium* species. Patients should be instructed to separate administration of antibiotics from these bacteria derived probiotics by at least two hours (Natural Medicines, 2009). Similarly, *S. boulardii* might interact with antifungals, reducing the efficacy of this probiotic. According to the manufacturer, Florastor, which contains *S. boulardii*, should not be taken with any oral systemic antifungal products (Florastor, 2009). Probiotics should also be used cautiously in patients taking immunesuppressants, such as cyclosporine, tacrolimus, azathioprine, and chemotherapeutic agents, since probiotics could cause an infection or pathogenic colonization in immune suppressed patients.

2.6.3.12. Other Benefits and applications of probiotics

The action of microorganisms during the preparation of cultured foods or in the digestive tracts has been shown to improve the quantity, availability and digestibility of some dietary nutrients. The fermentations of food with lactic acid bacteria raise folic acid in yoghurt, bifidus milk and kefir (Guarner et al., 2005). Sour cream contains approximately twenty times more folic acid than milk. Lactic acid bacteria are known to release various enzymes into intestinal lumen that exert synergistic effects on digestion. The bacterial enzymatic hydrolysis may enhance the bio availability of protein and fat (Friend et al., 1984) and increase the production of free amino acids. Short chain fatty acids (SCFA) such as lactic acid, propionic acid and butyric acid are also produced by lactic acid bacteria, when absorbed these SCFAs contribute to the available energy pool of the host and may protect against the pathological changes into the colonic mucosa. Additional health benefits of probiotics are reviewed in the following sections.

2.6.3.12.1. Production of vitamin, hormones and synergistic ingredients

LAB produces small amounts of certain B vitamins, including folates and vitamin B_{12}. Microbial synthesis of vitamin K in the intestine appears to have nutritional significance in most animal species. Bifidobacteria, streptococci, and enterococci

have been shown to produce vitamin K (Bentely et al., 1982; Gugarner & Malagelada, 2003).

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, estrogens, growth hormone or cholesterol-lowering compounds, just to mention, a few is intriguing (Lee et al., 2008). The ability to produce *in situ* over a long period of time, drugs or hormones that are constantly required by individuals suffering from various diseases (such as diabetes and hypercholesteremia) is of particular interest.

2.6.3.12.2. Enhancement of mineral bioavailability

Mineral absorption requires an acidic medium, especially when the minerals are in the form of inorganic salts. Stomach acid is usually sufficient to dissolve mineral salts, but when stomach acid is inadequate mineral salts may not fully dissociate. Lactic acid bacteria aid mineral absorption via the production of acid microenvironments adjacent to the intestinal lining and by generating SCFA that donate protons necessary for mineral absorption. Animal studies have demonstrated that, LAB, especially in the presence of a probiotic growth factor like inulin, increase intestinal absorption of calcium, magnesium, potassium and zinc (Delzenne et al., 1995).

2.6.3.12.3. Active against pathogens

As discussed above, in the intestinal tract, a delicate balance constantly needs to be maintained between beneficial and pathogenic organisms. A variety of factors can shift the intestinal microfloral balance in favor of pathogens. These factors include antibiotics, immunosuppressant, stress, aging, poor diet, excessive alcohol intake, environmental pollutants and infections. Many studies have confirmed that probiotics promote a more favorable balance of intestinal microflora by reducing populations of harmful microorganisms. Probiotics accomplish this task primarily by producing substances toxic to pathogenic organisms such as lactic acid, acetic acid, formic acid, hydrogen peroxide, and bacteriocin (Rosenfeldt et al., 2003; Fernandez et al., 2014; Varalakshmi et al., 2014). In the long term, a re-established healthy balance may reduce the risk of a variety of chronic degenerative or immunologically mediated diseases (Hemiaiswara et al., 2013).

2.6.3.12.4. Promotion of digestion

Most lactic acid probiotic bacteria are capable of metabolizing a variety of carbohydrates, including lactose. Some LAB species also secrete proteolytic and lipolytic enzymes that facilitate digestion of proteins and fats (Otles, 2013). People who produce inadequate amount of stomach acid and cannot activate the proteolytic enzyme pepsin and individuals with pancreatic insufficiency in pancreatic proteases and lipases all benefit from dietary supplementation with probiotics. Enhanced protein digestion often benefits people with allergies due to increased gut permeability defects by reducing the ability of large proteins to cross the intestinal barrier, enter the bloodstream, and trigger immune responses (Rautava et al., 2005; Yehuda Ringel et al., 2012).

2.6.4. Mechanism of action of probiotics

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel et al., 1998). There are studies on how probitics work. So, many mechanisms from these studies have attempted to explain how probiotics could protect the host from the intestinal disorders. These mechanisms are summarized in Table 2.7.

Following mechanisms of actions have been reported by Fooks et al. (1999).

1. Production of inhibitory substances: Some inhibitory substances (to both gram positive and gram negative bacteria) such as organic acids, hydrogen peroxide and bacteriocins are produced.
2. Blocking of adhesion sites: Competetion occurs between probiotic and pathogenic bacteria for adhesion site where probiotic bacteria inhibits the pathogens by blocking adhesion sites in the intestinal epithelial surfaces.
3. Competition for nutrients: Probiotics inhibit the pathogens by consuming the nutrients which pathogens need for their growth and metabolism.
4. Stimulation of the immune system: One of the possible mechanisms may be stimulation of specific and non-specific immunity to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall component or cell layers may act as adjuvants and increase humoral immune response.

5. Degradation of toxin receptor: Research has shown that *S. boulardii* protects the host against *C. difficile* intestinal diseases by degrading toxin receptor on the intestinal mucosa. Suppression of toxin production, reduction of gut pH and attenuation of virulence may be other possible mechanisms.

Table 2.7. Examples of probiotics with proven mechanisms. Adopted from (Salminen, 1999; Rolfe, 2000; Cakir 2003).

Mechanism	Strain	Demonstrated effect	Reference
Immune	*B. longum*	Improvement of clinical appearance of chronic inflammation in patients, decreases in TNF-α and IL-1α	(Furrie et al., 2005)
Immune	BIFICO (3 bifidobacteria species)	Prevention of flare-ups of chronic ulcerative colitis, inactivation of NF-$_k$B and elevated expression of IL-10	(Cui et al., 2004)
Immune	*L. salivarius* ssp. *salivarius* CECT5713	Recovery of inflamed tissue in TNBS model of rat colitis, increase in TNF-α and iNOS (include NO synthase) expression	(Carroll et al., 2007)
Immune	*L. fermentum* *L. ruteri*	Improvement of histology in a TNBS model of rat colitis, decreased level of TNF-α and i-NOS expression	(Peran et al., 2007)
Immune	*L. casei* Shirota	Improvement in murine chronic inflammatory bowel disease, downregulation of pro-inflammatory cytokines such as IL-6 and INF-γ	(Matsumoto et al., 2005)
Immune	*L. casei* DN-114 001	Reduction in numbers of activated T lymphocytes in the lamina propria of Crohn's disease mucosa decrease of IL-6 and TNF-α	(Carol et al., 2006)
Immune	*L. plantarum* 299v	Decreased IL-I2, IFN-γ and IG2a at the mucosal level of specific pathogen free IL-10 KO mice. Decreased mesenteric lymph node IL-I2 and IFN-γ production as well as histologic colitis scores in the pretreatment of GF mice that were exposed to normal flora.	(Schultz et al., 2002)
Immune	*L. rhamnsosu* GG	Alleviating intestinal inflammation, decrease TNF-α. Specific inhibition of macrophages TNF-α production by a contact independent mechanism.	(Majamaa & E. Isolauri, 1997; Pena & J. Versalovic, 2003)
Immune	*L. salivarus* UCC188	Reduced production of proinflammatory cytokines in IL-10 KO mice injected subcutaneously with the probiotic strain	(Sheil et al., 2004)

Immune	*L. salivarus* UCC118	Reduction of *C. perfringes*, coliforms and enterococcus levels in IL-10 KO mice. Production of a peptide that inhibits a broad range of pathogens such as *Bacillus, Staphylococcus, Enterococcus, Listeria* and *Salmonella* species.	(O'Mahony et al., 2001) (Flynn et al., 2002)
Microbiota alteration	*L. ruteri*	Decreased concentration of colonic *Lactobacillus* species and increased concentration of mucosal adherent bacteria associated with colitis attenuation.	(Madsen et al., 1999)
Microbiota alteration	VSL#3	Delayed relapse into pouchitis after surgical resection in human patients. Decreased luminal pH in patients with UC that antagonizes pathogenic bacteria. Reduced counts of bifidobacteria and lactobacilli in pouchitis Delayed relapse into pouchitis after resection	(Gionchetti et al., 2003) (Venturi et al., 1999) (Gionchetti et al.,2003)
Microbiota alteration	*L. rhamnosus* GG, *L. rhamnosus* Lc705, P. *freudenreichii* ssp. *shermanii* JS and *B. breve* Bb99	Alleviating irritable bowel syndrome symptoms	(Kajander et al., 2006)
Unknown	*Bifidobacterium* and *L. plantarum*	Improvement of the disease activity index in an induced rat colitis model	(Osman et al., 2004)
Unknown	*Lactobacillus rhamnosus* GG	Improvement in the clinical status in children with mildly to moderately active stable Crohn's disease	(Gupta et al., 2000)
Unknown	*L. casei* Shirota	Improvement in the clinical condition of murin DSS model of ulcerative colitis	(Herias et al., 2005)

2.6.5. Selection Criteria for Probiotics

Surviability of probiotic strain is of great importance in the location where it is presumed to be active, for a longer period and possibly with better activity (Figure 2.4). Also, concern has to be taken that probiotic strain can proliferate and colonize in intestinal epethelium (Otles, 2013). Most likely only host-specific microbes are able to compete with the indigenous microflora and to colonise the niches. Besides, the probiotic strain must be tolerated by the host immune system and not provoke the formation of antibodies against the probiotic strain (Lee & Salminen, 2008). Also, host must be immuno-tolerant to the probiotic strains. Further, the probiotic strain

may act as an adjuvant and can stimulate immune system against pathogenic microorganisms. Thus a probiotic strain must be harmless to the host, there must be no local or general pathogenic, allergenic or mutagenic/carcinogenic reactions provoked by microrganismsitself, its fermentation products or its cell components after decrease of the bacteria (Otles, 2013). In order to maintain the favourable properties of probiotic microorganisms, the strain must be genetically stable. For the production of probiotics it is important that the microorganisms multiply rapidly and

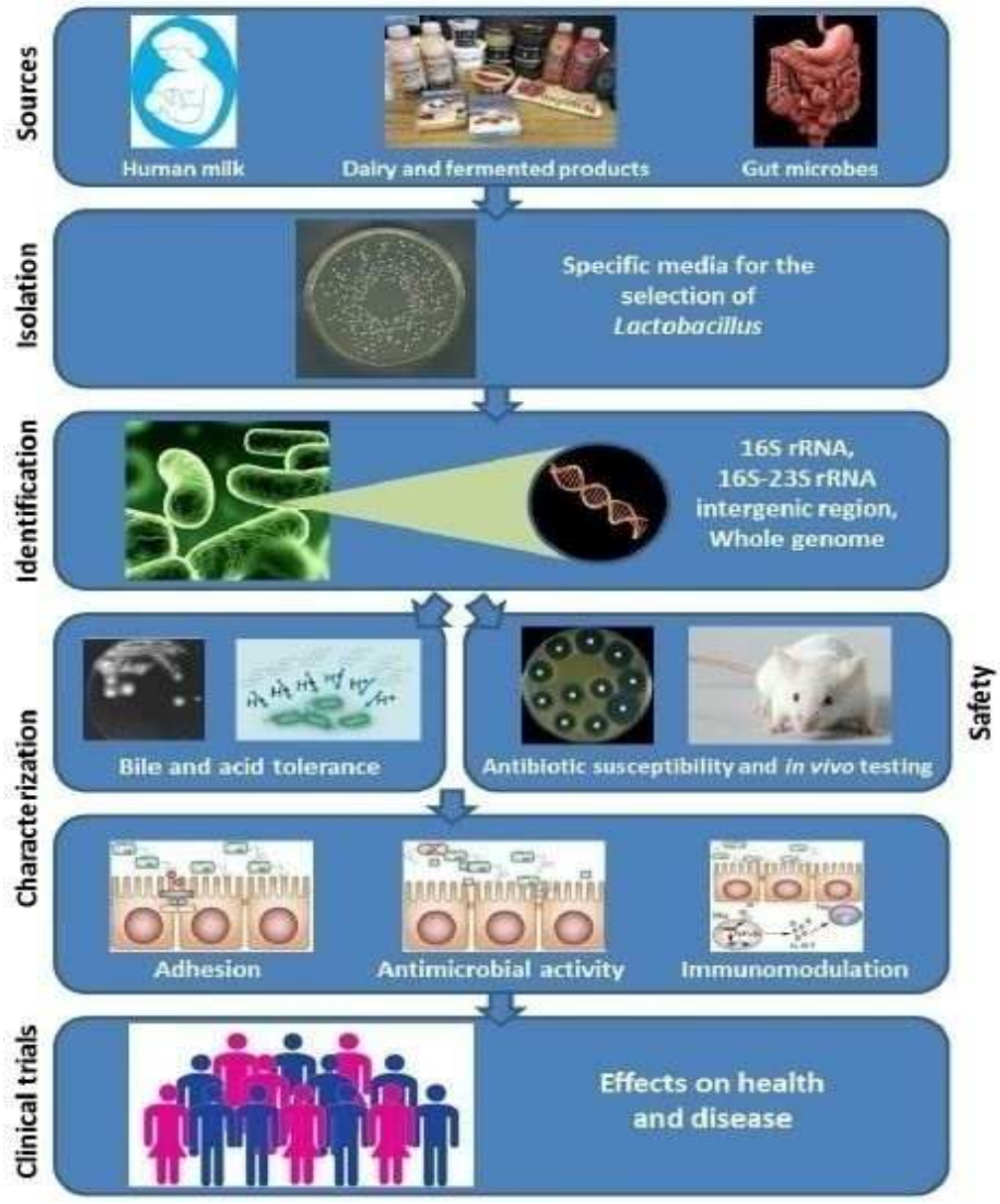

Figure 2.4. Flow chart indicating the various steps in order to isolate and characterize a novel probiotic strain.

Table 2.8. Selection criteria for Probiotics (Source: Quwehand et al., 1999; Cakir, 2003).

Probiotic Strain Properties	Criteria for selection
Human origin for human usage	Although the human probiotic *Saccharomyces boulardii* is not human origin, this criteria is important for species dependent health effects.
Acid and bile tolerance	Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surface	Important to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization.
Safe for food and clinical use	Identification and characterization of strains accurately, documentation of safety. No invasion and no degradation of intestinal mucus
Clinically validated and documented health effects	Minimum effective dosage has to be known for each particular strain and in different products. Placebo controlled, double-blinded and randomized studies have to be run.
Good technological properties	Survival in products if viable organisms are required, phase resistance, strain stability, cultivable in large scales, oxygen resistance, have no negative effects on product flavor.

densely on relatively cheaper nutrients and that they remain viable during processing and storage (Lee & Salminen, 2008). Besides the specific beneficial property, general requirements must be considered in developing new probiotics, but also for determining the scientific value of a claimed probiotic. A number of these requirements can be screened during *in vitro* experiments (Kechagia et al., 2013). It is advised of the drawing up of a decision-tree for the minimal requirements which can be tested *in vitro*, such as culture conditions and viability of the probiotic strains during processing and storage, sensitivity to low pH values, gastric juice, bile, pancreas, intestinal juice and intestinal or respiratory mucus, adherence to isolated cells or cell cultures and interactions with other (pathogenic) microorganisms (Lee & Salminen, 2008). If these *in vitro* experiments are successful, further research can be performed during *in vivo* experiments in animals or humans. In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a number of desirable properties. The selection criteria are briefly listed in Table 2.8.

However, a potential probiotic strains does not need to fulfill all these selection criteria (Quwehand et al., 1999).

According to Klaehammer & Kullen (1999), the selection criteria can be categorized into four basic groups. The appropriateness, technological suitability, competitiveness, performance and functionality are the assets to be considered. Strains that fulfill these criteria should be used in order to get and functional probiotic strains. Probiotics are chosen by using criteria in Table 2.8. Saarela et al., (2000) proposed the properties of probioitcs in three basic groups *viz.* safety aspects, aspects of functionality and technological aspects. Some major selection criteria will be discussed in following section.

2.6.5.1. Acid and bile tolererane

Bacteria used as probiotic strains join the food system with a journey to the lower intestinal tract via the mouth. In this food system, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. Then the journey proceeds to the stomach and enters the upper intestinal tract which contains bile. At this stage, strains should have the ability to resist the digestion process. It is reported that the total time at the first entrance to release from the stomach takes three hours (Lee & Salminen, 2008). Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile (Chou & Weimer 1999; Cakir 2003). To show probiotic efficiency, they should reach to the lower intestinal tract and maintain themselves there. Because of this desirable point the first criteria is looking for probiotic strains being resistant to acid and bile. Bile is synthesized in the liver from cholesterol and sent to the gall bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine bile salts undergo some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity (Lee & Salminen, 2008). Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli*, *Kelbsiella* spp., and *Enterococcus* spp. The deconjugated acid forms are more effective on gram positive bacteria (Dunne et al., 1999; Cakir 2003). *Lactobacillus acidophilus* is the most used probiotic strain in products like dairy products or capsules. Chou & Weimer (1999), tried to isolate acid and bile resistant variants of *L. acidophilus*. Probiotic strains were taken from American Type Culture Collection (ATCC) that had been isolated from different sources. Some of these strains were

found resistant to acid at pH 3.5 for 90 minutes at 37 °C. Also these strains were capable of growth in medium at pH 3.5 containing 0.2% mixed bile salts (Chou & Weimer, 1999). An investigation of probiotic potential of 47 selected strains of *Lactobacillus* spp. were examined for resistance to pH 2.5 and 0.3% oxgall (Jacobsen et al., 1999). They showed high resistance to bile salts and growth was delayed from 1 hour to more than 4 hours for 16 of these strains examined and except one, all of these strains survived for in condition mentioned above. The results obtained from *in vitro* experiments involving five *Lactobacillus* strains (*L. rhamnsosus* 19070-2, *L reuteri* DSM12246, *L. rhamnsosu* LGG, *L. delbrueckii* subsp. *lactis* CHCC2329 and *L. casei* subsp. *alactus* CHCC3137) were selected for *in vivo* studies (Jacobsen et al., 1999). For the selection of acid and bile resistant bifidobacteria, human fecal samples were screened and isolated strains from these samples were further examined for growth at pH 4.5 and pH 7.0 and oxgall (0.006% and 0.15%) (Chung et al., 1999). Then conditions were updated and isolated strains were examined for survival at pH 2.0, 3.0 and pH 7.0 with a final concentration of 0.0, 0.05% and 1.0% of oxgall. According to the results, the survival rate of the isolated *Bifidobacterium* strains was very similar at bile concentration of 0.5% and 1% for 12 hours exposure. Two selected *Bifidobacterium* strains, HJ 30 and SI 31, showed higher rates of survival.

In another study, a large culture collection of lactic acid bacteria of NZDRI (now Fontera Research Center) was screened to select strains to be used as probiotics. For this, over 200 strains including *Lactobacillus rhamnosus* GG and *Lactobaccillus acidophilus* LA-1 were used. The strains were analyzed for a series of pH between pH 1 and pH 3 and also for tolerance against bile at final concentrations of 0, 0.5 and 1% w/v. They were found to be tolerant for the conditions mentioned above. While the general survival patterns were similar, the strains from human origin showed higher tolerance. These strains were identified as *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN067, *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* HN019 (Prasad et al., 1998). More recently, Maragkoudakis et al. (2005) tested twenty nine *Lactobacillus* strains of dairy origin for *in vitro* probiotic potential. The resistance of bacteria was examined in pH between 1 and 3. Tolerance to bile salt was tested at 0.3% oxgall. All of the examined strains were resistant to pH 3 for 3 hours, but most of them lost their viability in 1 hour at pH 1. Also all of them were found to be tolerant in 0.3% bile salts concentration for 4 hours. For *in vivo*

testing the most suitable strains chosen were *L. casei* Shirota ACA-DC 6002, *L. plantarum* ACA-DC 146, *L. paracasei* subsp. *tolerans* ACA-DC 4037 (Maragkoudakis et al., 2005). Also, Martin et al., (2004) experimented on three *Lactobacillus* species isolated from human milk to test whether they may be potential probiotic strains. They were identified as two *Lactobacillus gasseri* strains and one *Lactobaillus fermentum* strain. Survival in low pH and in gastrointestinal environment was examined via their comparison with commercial probiotic strains, *L. rhamnosus* GG, *L. casei* imunitass and *L. johnsanii* La1. The strains especially *L. gasseri* showed that it can be used as a potential probiotic strain (Martin et al., 2004).

2.6.5.2. Adherence property of probiotic bacteria

Adhesion to the intestinal mucosa is one of the main criteria for probiotic strain selection. Selection of adhesive strains is performed *in vitro*. However, little is known, as to how well *in vitro* adhesion correlates with *in vivo* adhesion. Good adhesion to the intestinal mucosa is thought to be an important property. Adhesion can be specific or non specific and both may be the influencial to host and bacterial cells (Dixit & Kailasapathy, 2012). The original reason for screening of good adhesive candidate probiotic strains was the hypothesis that adherent strains would easily colonize the intestine, in particular in the small intestine, where flow rates are relatively high (Sanford et al., 1992). However, feeding studies have clearly shown that in general, probiotics do not colonize humans permanently. Once feeding ceases, the particular microorganisms can no longer be detected in the faeces after 1 or 2 weeks (Benno et al., 1996; Jacobsen et al., 1999; Tannock et al., 2000; Crittenden et al., 2002). In contrary, longer colonization of probiotic strains in the intestinal mucosa has also been reported (Alander et al., 1997). It is therefore desirable to refer to ‘transient colonization’ or ‘persistence’ of probiotics rather colonization (Arthur et al., 2003). The correlation between mucosal adhesion and transient colonization also appears to be less clear than expected (Arthur et al., 2003).

Several models have been employed to study the ability of putative probiotic strains to adhere to the intestinal epithelium. Studies have often been carried out with cellular lines obtained from human colon adenocarcinomas such as Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38), the latter being able to produce mucin (Hara et al., 1997; Kalliomaki et al., 2001; Chung et al., 2002; Vaughan et al., 2002; Bongaerts et al., 2005). Frequently, the adhesion ability of putative probiotics from different

collections has been extensively tested against mucus obtained from human (Mandalari et al., 2007; Crittenden et al., 2002; Boehm et al., 2005; Haarman et al., 2005) or animal origin (Van der Meulen et al., 2006).

Interestingly, some strains of *Bifidobacterium* adhere better to human mucus than to porcine mucus indicating that adhesion property is strain dependent (Haarman et al., 2006), because mucus from different origins (human, canine, possum, bird, and fish) did not modify the adhesion of probiotic strains. In addition, bacteria into the human mucus decreased with the age of the donor of the mucus sample, which could be one of the reasons for low bifidobacteria colonization in elderly subjects (Rinne et al., 2005). A good correlation between the human mucus model and the adhesion to Caco-2 has been demonstrated (Al-Tamimi et al., 2006), employing three *Lactobacillus* strains. Both methods are adequate for *in vitro* adhesion studies but some *ex vivo* models employing resected tissue of the intestinal mucosa from human or animals have also been shown to be useful (Wang et al., 2005; Fanaro et al., 2005). In the human intestinal mucus model proposed (Fanaro et al., 2005), the material is obtained from patients with colon cancer submitted to surgery. The healthy sections of resected tissue obtained from different sites of the colon are employed in these studies. In general, the strains tested showed higher adhesion to mucus than to colonic tissue and, depending on the strain, the location of the colonic tissue but not that of mucus, also influenced the adhesion properties of the probiotics tested. This is a good model for the assessment of the adhesion of LAB to GIT epithelium and to mucus.

The application of this screening method for selecting probiotics should be encouraged in the future. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium. Radiolabelling the microorganisms with an amino acid and then counting for adhering radioactivity in either ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods (Lee & Salminen, 2008). Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine.

2.6.5.3. Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer

Kullen, 1999; Dixit & Kailasapathy, 2012). Antimicrobial actions of lactic acid bacteria are determined by the production of some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide diacetyl, low molecular weight antimicrobial substances and bacteriocins (Cakir 2003; Quwehand & Vesterlund, 2004; Roopashri & Varadaraj, 2011). A number of reports have shown that different species of bacteria produce different antimicrobial substances. Examples are those of *Lactobacillus reuterii*, which is a member of normal microflora of humans and many other animals, produce a low molecular weight antimicrobial substances reuterin called Subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocins acidophilucin A (Quwehand & Vesterlund, 2004). Production of bacteriocins is highly dependent on the factors such as the species of microorganisms, ingredients and pH of the medium, incubation temperature and time. Nisin, produced by *L. lactis* subsp. *lactis* is the well known bacteriocin and it is allowed to use in food preparations (Cakir, 2003).

Lactobacilli and Bifidobacteria isolated from human ileum were assayed to investigate if they have antimicrobial activity against a rage of indicator microorganisms such as *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli, Lactobacillus, Streptococcus, Bifidobacterium* and *Lactococcus*. Antimicrobial activity of *Lactobacillus salivarus* UCC118 was counted against these bacteria. The study showed that *Lactobacillus salivarius* UCC118 is significantly capable of inhibiting *in vitro* growth of both of gram positive and gram negative bacteria such as *L. fermentum* KLD, *B. longum, B. bifidum, Bacillus subtilus, B. cereus, B. thuringiensis, E. faecalis, E. faecium* etc, although it is not effective against some of the species of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus*, etc. (Dunne et al., 1999). Some milk products were used to isolate potential probiotic bacteria and determination of their possible antimicrobial activities. *Staphylococcus marcescens* and *Candida albicans* were used as indicator microorganisms. The results, showed that, Yakult and Ski D Lite probiotics inhibited all of the test indicator microorganisms, NESLE yogurt probiotics were bactericidal for *S. aureus* and *P. aeruginosa* but inhibitory *for S. typhi*, Neslac probiotics killed *E.*

coli and *S. typhi* while they were inhibitory for *S. aureus* and *C. albicans*, GAIN probiotics inhibited *C. albicans* (Chuayana et al., 2003).

Eight lactic acid bacterial strains producing bacteriocins were isolated from Burkina Faso fermented milk and they were examined for the antimicrobial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP (Lee & Salminen, 2008). The lactic acid bacterial strains were identified as *Lactobacillus fermentum, Pediococcus* spp., *Lactococcus* spp., *Leuconostoc mesenteroides* subsp. *mesenteroides*. The diameters of inhibition zones were obtained between 8 mm and 12 mm. *Lactobacillus fermentum* (S1) gave the biggest zone of around 12 mm on *Enterococcus faecalis* while the smallest zone was obtained for *Leuconostoc mesenteroides* subsp. *mesenteroides* (S5) on the same strain *Enterococcus faecalis* (Savadogo et al., 2004). A research aiming at the production of bacteriocin by vaginal lactobacilli flora and characterization of this flora were carried out (Karaoğlu et al., 2003). First antimicrobial activity was assayed for 100 vaginal lactobacilli isolates. Six of them were determined for the production of bacteriocin. In this study, common human pathogens *Gardneralla vaginalis, Pseudomonos aeroginosa, Proteus vulgaris, Escherichia coli, Enterobacter cloacae, Streptococcus milleri, Staphylococcus aureus* and *Candida albicans* were used as indicator microorganisms. Six of the strains had bacteriocin activity against eight of fifteen different *Lactobacillus* species and also *S. milleri, P. vulgaris, P. aeroginosa, E. coli, E. cloacae* and *G. vaginalis*. But none of the isolated strains showed efficiency on test organisms *S. aureus* and *C. albicans*. Also some characteristics of bacteriocins were obtained from the research performed by Karaoğlu et al. (2003). In another research, potential probiotic lactobacilli strains (*L. reuteri, L. plantarum, L. mucosae, L. rossiae strains*) isolated from pig feces used as additives in pellet feeding, were examined according to their antibacterial activity against *Salmonella typhimurium* ATCC 27164, *E. coli, C. perfringens* 22G, *S. aureus* ATCC 25923, *B. megaterium* F6, *L. innocua* DSM 20649 and *B. hyodysenteriae* ATCC 27164 (De Angelis et al., 2007). Generally the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *B. hyodysenteriae* ATCC 27164. The study showed that, neutralization and treatment with catalase, affects the antibacterial activity to some extent (De Angelis et al., 2007). A similar study was conducted and in that study four *Lactobacillus* strains (*L. salivarus* CECT5713, *L.*

gasseri CECT5714, *L. gasseri* CECT 5715 and *L. fermentum* CECT5716) isolated from human milk were investigated to test whether they have antimicrobial potential and for comparison, *L. coryniformis* CECT5711 was used (Olivares et al., 2006). All of the strains showed antibacterial properties against pathogenic bacteria (*Salmonella choleraesuis* CECT4155, CECT409 and CECT443, *Esherichia coli* CECT439 and *E. coli* O157:H7 serover CECT4076, *Staphylococcus aureus* CECT4013 and CECT9776, *Listeria monocytogenes* Scott A and the spoilage strain *Clostridium tyrobutyricum* CECT4011). However, the antimicrobial properties of lactobacilli strains varied and *L. salivarus* CECT5713 revealed not only the best *in vitro* antibacterial activity, but also the highest protective effect against a *Salmonella* strain in the murine infection model (Olivares et al., 2006).

2.6.5.4. Other Properties required for Probiotics

In addition to the previously reviewed properties, other characteristics could also be tested to consider a strain as putative probiotic. From these screenings, it has been reported that some strains are able to modulate the immune system (Ziegler et al., 2007), produce antigenotoxic compounds, deconjugate bile salts (He et al., 2001; Resta-Lenert et al., 2006), and decrease cholesterol levels (Resta-Lenert et al., 2006).

2.6.6. Safety Aspects of Probiotics

There are evidences that probiotic strains used as commercial bacteria are safe to use in various applications. The safety of the probiotic products is appraised with the phenotypic and genotypic characteristics and the statistics of used microorganisms (Roopashri, 2012). However, with respect to the requisites of safety of probiotics, a set of general principles and practical criteria need to be generated to provide guidelines as to how any given potential probiotic microorganism can be tested and proven to have a low risk of inducing or being associated with the etiology of disease, versus conferring a significant health benefit when administered to humans (FAO/WHO, 2001). These guidelines should recognize that some species may require more vital assessment than others. In this respect, the evaluation of safety will require at least some studies to be performed in humans, and should address aspects of the proposed end use of the probiotic strain.

According to the information acquired to date, shows that lactobacilli have a long history of use as Probiotics without established risk to humans, and this remains the

best proof of their safety (Saxelin et al., 1996; Naidu et al., 1999). Also, no pathogenic or virulence properties have been found for lactobacilli, bifidobacteria or lactococci (Aguirre & Collins, 1993). Based on this it is said that under certain conditions, some *Lactobacillus* strains have been associated with adverse effects, such as rare cases of bacteremia (Saxelin et al., 1996). However, a recent epidemiological study of systematically collected lactobacilli, bacteremia case was reported which has shown that there is no increased incidence or frequency of bacteremia with increased usage of probiotic lactobacilli (Salminen et al., 2001). Also, some members of lactic acid bacteria, such as enterococci may possess virulence characteristics, for this and other reasons, Joint FAO/WHO (2001) recommended *Enterococcus* not be referred to as a probiotic for human use.

Safety aspects of probiotic bacteria include the following requirements *viz.* 1) strains for human use is preferred to be of human origin, 2) they are isolated from healthy human gastrointestinal tract, 3) they have to be non-pathogenic, 4) they should have no history of relationship with diseases like, infective endocarditis or gastrointestinal tract disorders, 5) they do not deconjugate bile salts, 6) they should not carry transmissible antibiotic resistance genes (Saarela et al., 2000).

2.6.7. Sources of Probiotic microbes

Probiotics are usually defined as microbial food supplements that when administered in adequate amounts exert beneficial effects on the host. Data supporting of health promoting effects of probiotics lactic acid bacterial strains is increasing day by day (Saarela et al., 2000). Recent scientific investigation has supported a role of probiotics as a part of a healthy diet for humans and animals and may be an avenue to provide a safe, cost effective, barrier against microbial infection (Parvez et al., 2006). On the industrial scale, a large number of dairy products are present on the market and are being promoted with health claims based on various characteristics (Succi et al., 2005). Strains of *Lactobacillus* (*Lb.*) *acidophilus, Lactobacillus paracasei,* and *Bifidobacterium* isolated from human or animal intestinal tracts have been the most extensively studied probiotics (Saito, 2004). They are increasingly incorporated into food as dietary adjuncts (Bernet et al., 1994; Patrignani et al., 2006). A number of requirements have been identified for strains to be effective probiotic microorganisms. They must simply be of human origin, and be able to survive through the gastrointestinal tract (Rolfe, 2000). Required characteristics include

resistance to gastric acid and physiological concentrations of bile and adherence to intestinal epithelial cells (Schillinger et al., 2005). Adherence of probiotic bacteria to intestinal mucosa is the first step in gut colonization. Gut colonization is important for a beneficial health effect as it prolongs the time the microorganism can influence the gastrointestinal immune system and microbiota of the host (Kirjavanainen et al., 1998; Forestier et al., 2001). Feeding methods have a significant influence on the relative proportions of bacteria that establish in the infant gut (Vernaza et al., 2006). Breast-fed infants develop a probiotic rich gut microflora with less pathogenic bacteria, compared with formula-fed individuals (Weizman et al., 2005). LAB and Bifidobacteria dominate the microbiota of the full term neonates especially when milk is a source of lactic acid bacteria of the infant gut (Martin et. al., 2003). LAB constitutes an integral part of the healthy gastrointestinal (GI) microecology and influence host metabolism (Gibson & Fuller, 2000).

2.6.8. Adverse effects and safety

Possibilitiesof having major and minor risk factors exists during probiotic treatment and its associated products (Lee & Salminen, 2008). Immunosuppression (including debilitated state or malignancy) and prematurity in infants are major risk factors while minor risk factors are the presence of a central venous catheter, impairment of the intestinal epithelial barrier (such as with diarrheal illness), cardiac vascular disease (*Lactobacillus* probiotics only), concurrent administration with broad spectrum antibiotics to which the probiotic is resistant, and administration of probiotics via jejunostomy tube (this method of delivery could increase the number of viable probiotic organisms reaching the intestine by passing the acidic contents of the stomach). It is recommended that probiotics to be used cautiously in patients with one major risk factor or more than one minor risk factors (Otles, 2013). Also, it is important to remember that the overall risk of developing an infection from ingested probiotics is very low, particularly when used by generally healthy individuals. Lee et al. (2008) reported that LGG has been routinely added to dairy products since 1990 in Finland. Even though a substantial increase in the consumption of *Lactobacillus rhamnosus* GG containing products from 1995 through 2000, there was no significant change in the incidence of *Lactobacillus* associated bacteremia observed during the surveillance period of 1990-2000.

When considering the safety of probiotics, several factors should be taken into account *viz.*: 1) the probiotic's abilitiy to cause disease (pathogenecity), 2) to produce damaging substances (virulence factors such as toxins), 3) to produce metabolic activities that might interfere with the body's normal functioning, 4) problems with quality control should also be considered. Most nonprobiotic drugs (such as prescription medications and counter drugs) cause some adverse effects, some of the quite serioius, but the drug has to be used anyway because its therapeutic benefits are judged to be greater than the risk from side effects. The same sort of balanced judgment should be used when considering the safety of probiotics (Wohlgemuth et al., 2010).

Potential risk of probiotics may raise becaue of: 1) Contamination, 2) Poor potency, 3) Unproven claims, 4) No strong federal oversight, 5) Adverse effects, 6) Infection. Probiotic preparations have been used for many decades in Europe and Asia, where people are quite used to taking them for many reasons. According to Elmer et al. (2012), over 200 billion doses of probiotic have been purchased, yet fewer than 30 cases of serious adverse effects were reported. This is remarkably good safety record. In United States the given descriptions are not scientific proof that the adverse effect is due to probiotics. Some cases are discussed in Table 2.9.

The study performed by Kalliomaki et al. (2003) found an increased risk of mortality when probiotics were used to prevent infectious complications in patients with predicted severe acute pancreatitis. These patients had acute pancreatitis and an elevated Acute Physiology and Chronic Health Evaluation (APACHE) II score, Imrie/modified Glasgow score, or C-reactive protein value, predicting a severe disease course and putting them at risk for developing infectious complications, including infected pancreatic necrosis. This multicenter, randomized, double blind, placebo-controlled trial involved 298 patients who received a multispecies probiotic preparation (*L. acidophilus, L casei, Lactobacillus salivarius, Lactobacillus lactis, B. fidum* and *B. lactis*) or placebo, administered twice daily for a maximum of 28 days. The study found that this combination of six probiotic strains did not decrease infectious complications in patients with predicted severe acute pancreatitis but rather was associated with significantly more deaths than was with placebo (24 versus 9, $p = 0.01$)

Table 2.9. Potential risk of Probiotics reported by various authors.

Probiotic product	Number of cases	Type of side effect and underlying health status	Strain causing disease identified as same strain as probiotic	Reference
Probiotic yogurt (supermarket brand, daily serving not given nor type of probiotic identified)	1	Fatal septicemia in 42 years old woman immunocompromised with recurrent *Clostridium difficile* disease and renal failure	*Lactobacillus rhamnosus* identified in blood infection	(McFarland et al., 1999)
Lactobacillus rhamnosus GG in probiotic dairy drink (1/2 liter per day, taken for 4 months)	1	Liver abscess in 74 years old woman with multiple medical conditions. Recovered with antibiotic treatment	*Lactobacillus rhamnosus* GG in milk strain causing liver abscess, identified as the same *Lactobacillus* in blood and probiotic not identified	(Silva et al., 1987)
Lactobacillus probiotic	2	*Lactobacillus* bacterimia in two children with short gut syndrome taking probiotic. Recovered with antibiotics	Only six had positive identification as S. *boulardii* in blood	(Goldin et al., 1992)
Saccharomyces boulardii probiotic capsules (doses varied from 500 to 2000 mg/day)	14 from 1991 to 2000	Fungemia (fever and yeast in the blood in all). All patients had been hospitalized with multiple conditions and central venous catheter		(Alander et al., 1999)

and an increased risk of bowel ischemia in the probiotic group compared with placebo (9 versus 0, p = 0.004). The authors stated that probiotics should not be routinely given to patients with predicted severe acute pancreatitis (Kalliomaki et al., 2003) and should be used cautiously in critically ill patients or those at risk for mesenteric ischemia. Drug interactions may arise since probiotics contain live microorganisms, concurrent administration of antibiotics could kill a large number of the organisms, reducing the efficacy of the *Lactobacillus Bifidobacterium* species. Patients should be instructed to separate administration of antibiotics from these bacteria derived probiotics by at least two hours. Probiotics should be used cautiously in patients taking immunosuppressants, such as cyclosporine, tacrolimus, azathioprine, and

chemotherapeutic agents, since probiotics could cause an infection or pathogenic colonization in immunocompromised patients (Otles, 2013).

2.6.9. Precautions and contraindications

Probiotics contains live microorganisms that is why some chances of occurring pathological infectioins always remains as a challengge, mostly in critically ill or severely immunocompromised patients. Probiotic strains of *Lactobacillus* have also been reported to cause bacteremia in patients with short-bowel syndrome, possibly due to altered gut integrity (Borriello et al., 2003; Elmer et al., 2012). Probiotic preparations using *Lactobacillus* in patients with a hypersensitivity to lactose or milk has also been controversial. Similarly, *Saccharomyces boulardii* based probiotics has been controversial in patients with a yeast allergy. No contraindications are listed for bifidobacteria or it is extremely rare, since most species are considered non-pathogenic and non-toxigenic (Borriello et al., 2003).

2.6.10. Limitations

The limitations of probiotics are discussed based on the view of Sheil et al. (2004), Medina et al., 2007, Lee & Salminen (2008) & Otles (2013). Probiotics are regulated as dietary supplements and not subjected to the same rigorous standard as medications. Probiotic products consist of the microorganisms so always a challenge exists with these products. As a result, consumers may find variying quantity and sometimes even different quality (ineffective or contaminated) of probiotics (may be yeast or bacteria). Several research studies regarding probiotics are limited with the number of samples and lack of control groups. Also, these studies should be interpreted based on their research methodology and the limitations. Because of different probiotic doses, strains, treatment, durations, and patient populations may have been used which could be the one of the factors for heterogenecity of the study. Since probiotic properties are strain specific, this may have important implications when interpreting meta-analysis, particularly if strain designations were not provided. Well designed research study in future with appropriate sample sinze, control groups and other consideration of other influencing factors (such as time, dose, strain etc) can address these limitations.

2.7.0. Prebiotics

Different factors such as age, drug therapy, diet, host physiology, peristalsis, local immunity and *in situ* bacterial metabolism have effets in the human gut microflora such as age, drug therapy, diet, host physiology, peristalsis, local immunity and *in situ* bacterial metabolism (Berg, 1986). However, diet is probably the most significant factor determining the type of gut flora that develops based on the type of foods, which provide the main nutrient source for colonic bacteria. This interactive development has led to the concept of prebiotics. The term 'Prebiotics' was proposed by Delzenne & Roberfroid (1994) for the non-digestible oligosaccharides (NDOs) used as food ingredients to modify the composition of endogenous gut microflora. The definition was developed by Gibson & Roberfroid (1995) who defined prebiotic as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health". This definition only reflected on microbial changes in the human colonic ecosystem. Therefore, later, a revised definition of a prebiotic was purposed as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al., 2005). Prebiotics are considered to stimulate selective bacterial groups such as bifidobacteria, lactobacilli and eubacteria, which reside in the colon and benefits human health (Roberfroid, 2000). Few major prebiotics are non-digestible oligosaccharides (NDO's, recently referred to as resistant oligosaccharides), which includes fructo-oligosaccharides (FOS), gluco-oligosaccharides, galacto-oligosaccharides, transgalacto-oligosaccharides, isomalto-oligosaccharides, xylo-oligosaccharides, soy bean oligosaccharides and also resistant starches, sugar alcohols and difructose anhydride. Inulin-type fructo-oligosaccharides have been the most investigated as prebiotics and is considered as typical 'bifidogenic factors' (Crittenden et al., 2001). The non-digestible oligosaccharides are complex carbohydrates which are resistant to hydrolysis by acid and enzyme present in the human gastrointestinal tract due to the chemical configuration. Therefore, prebiotics have also gained importance as functional food ingredients or products (Lee & Prosky, 1995).

Prebiotics concept is recent (started since 14 years) and has stimulated research in several areas including nutritional and medical sciences. According to Macfarlane et

al. (2006) the significance of prebiotics in human health could be attributed to different factors such as: 1) the growing belief towards the existence of a healthy or balanced gut microbiota, 2) the ability of prebiotics to alter the composition of microbiota towards deriving a more healthy profile. 3), as an alternative to probiotics, this can be difficult to handle in some foods. 4), prebiotics currently in use, especially inulin and its derivatives and galacto-oligosaccharides (GOS) are relatively easier to prepare or extract from plant sources. Certain established criteria are used to define prebiotics (Gibson et al., 2005). There criteria are: 1) resistant to gastric acid, hydrolytic enzymes and gastro intestinal absorption, 2) should be fermentable by the intestinal flora, 3) selective stimulation of intestinal flora and their growth which contribute to health and well-being.

2.8.0. Synbiotics

Synbiotics are the nutritional supplements with combined probiotics and prebiotics having synergistic effects on the host. Probiotics are not the only functional food ingredients developed to improve human health by modulating the intestinal microbiota. Prebiotic ingredients represent an alternative and potentially synergistic approach. These nondigestible carbohydrates pass through to the colon and these nondigestible carbohydrates will selectively stimulate the proliferation and/or activity of beneficial microorganisms within the intestinal microbiota (Gill et al., 2001). Hence, ingredients and food that contains both prebiotics and probiotics are called synbiiotics.

2.9.0. Interaction between prebiotics and microbiota

Prebiotics (as well as probiotics) have been regarded as functional food ingredients because of their putative beneficial effects. A functional food is a food that contains one or a combination of components that work together with physiological and later immunological functions in the body to improve them or to reduce the risk of associated diseases (Lee & Salminen, 2008). Many research bas been carried out to identify the possible mechanism of prebiotics influencing the different metabolic activities in the body that are useful for host physiology as well as for the reduction of risk or even the treatment of some pathologies in its early stages (Milner, 1994). Presently, there are only two food ingredients that fulfill the prebiotics criteria i.e., inulin type fructans and trans-galacto-oligosaccharides (Roberfroid, 2007). The

efficacy of prebiotics in promoting human health is strongly related to their chemical structure. In general, feeding of FOS leads to (i) an incerease in the population of *Bifidobacterium* spp. and *Lactobacillus* spp., (ii) increase in short-chain fatty acids (SCFA) levels, (iii) decrease the numbers of *Clostridium* spp., *Fusobacterium* spp., Bacteroides and (iv) lowers pH (Gibson et al., 1995; 1996; O'Sullivan, 1996; Fuller & Gibson, 1997). As a consequence of the metabolism of the FOS by fermentative bacteria, SCFA and lactic acid are produced. Both lead to the decrement of pH in the large intestine. This is beneficial for the organism as it constitutes an ideal medium for the development of the bifidogenic flora and at the same time, limits the development of bacteria which are considered pathogens (Rosenfeldt et al., 2002a). The underlying mechanisms of prebiotic induced alterations are not yet known. According to Seifert & Watzl (2008), extensive experimental data suggests that prebiotics induce their immunological effects by the following means: 1) selective increment/decrement in the specific bacteria responsible for the modulation of cytokines and production of antibody, 2) enhances the production and binding of SCFA to G-coupled protein receptors on leukocytes, 3) establishment of local and systematic contact with the immune system by partial absorption of prebiotics, 4) prebiotics interact with carbohydrate receptors on leukocytes leading to benefits.

2.10.0. Summary

This review highlighted the importance of microroganims, foods and human health in correlation to traditional foods, lactic acid bacteria (particularly lactobacilli) and probiotics. Traditional fermented foods are rich source of lactic acid bacteria particularly lactobacilli which are used as probitics. This review of literature indicates that several traditional foods of dairy, vegetable or cereals origin are found in Nepal. Lactic acid bacteria are friendly bacteria commonly found in traditional foods. These bacteria normally live in our digestive, urinary and gential systems without causing diseases.

Lactic acid bacteria are known for beneficial properties and those have been termed and used as "Probiotics". Probiotics have been extensively studied and explored commercially in many different products including traditional fermented foods throughout the world. Recent studies have suggested that probiotics have demonstrated beneficial effects to human and animal health. Much of the clinical probiotic research has been focused on infantile, antibiotic-related and traveller's

diarrhoea. The non-pathogenic organisms used as probiotics consist of a wide variety of species and subspecies, and the ability to adhere, colonise and modulate the human gastrointestinal system is not a universal property. *Lactobacillus* and *Bifidobacterium* are the main probiotic groups, however probiotic potential of yeasts have also been reported. Some of the identified probiotic strains exhibit anti-inflammatory, anti-allergic and other important properties. Besides, the consumption of dairy and non-dairy products stimulates the immunity in different ways.

3.0. MATERIALS AND METHODS

3.1. Isolation, biochemical and molecular characterization of *Lactobacillus* spp. from traditionally prepared curd (*dahi*) at different altitudes in Nepal.

3.1.1. Study site

Fermented milk product i.e. curd or *Dahi* were collected from four districts of Central Nepal two each from Himalayan and Hilly regions (Figure 1).

Figure 3.1. Map of Nepal showing sampling sites (districts with dark circles) of present study.

3.1.2. Field visit and sample collection

Samples were collected from four different districts *viz.* Gorkha (Manaslu Conservation Area) [Nepali date of collection: 2068.05.22 to 2068.06.09 Bikram Sambat (B.S.)], Rasuwa (Hill and Himalayan region) [2069.01.26 to 2069.02.01 B.S.], Bhaktapur [2068.08.02 to 2068.10.15 B.S.] and Lalitpur districts [2068.01.03 to 2069.01.20 B.S.] in a labeled sterile plastic bottles. All collected samples were transported to NAST Molecular Biotechnology Laboratory, Khumaltar, Lalitpur by preserving them in an Ice-Box. They were stored at 4 $^{\circ}$C until subsequent laboratory experiments. In addition, questionnaire survey on "Knowledge, attitudes and usage

patterns of processed and fermented dairy foods in Nepal" was conducted during field visit and sample collection (included in Appendix).

3.1.3. Isolation and enumeration

All collected samples were analyzed by the dilution pour plate method for the isolation of lactobacilli (Voeuret et al., 2003; Liew et al., 2005; Sahafiee et al., 2010). For this purpose, 10 grams of each sample were weighed aseptically and homogenized in 90 mL sterile normal saline (0.85% NaCl) and mixed well by using vortex (REMI CM 101, India). Then, serial dilutions of the homogenate were obtained. One mL aliquot of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} dilutions were used for the isolation of LAB. For initial isolation and enumeration of the LAB, de Man Rogosa and Sharpe (MRS) medium, and also M17 Agar medium, Lactobacillus bulgaricus Agar, Base and Bifidus Selective Medium (BSM) were used to obtain as many lactic acid bacterial isolates as possible. All the samples were examined in duplicates and also replicate plating was performed.

All the agar plates inoculated with different samples were incubated under aerobic conditions at 37 $^{\circ}$C (except M17 agar plates which was incubated at 30 $^{\circ}$C) for 24-72 hours. After incubation, colony forming units (CFU) ranging from 30 to 300 were selected for enumeration. After the colony counting, the numbers were expressed in logarithmic scales [Log10 (CFU/mL)]. Standard deviations and mean values were also calculated using MS excel 2007.

3.1.4. Morphological, microscopic and biochemical analysis

On the basis of colony morphology (colour, shape, size), isolates were observed in microscope as per negative staining and Gram staining methods. Gram positive rods were observed for catalase activity in 3% hydrogen peroxide solution following standard protocol. Morphological, microscopic and biochemical analysis were performed as follows.

3.1.4.1.Colony morphology

Isolates were further purified by streaking repeatedly on MRS agar plates, and the colony morphologies (colour, shape and size) were examined by naked eye.

3.1.4.2. Negative staining

All the catalase negative isolates were used for simple staining to differentiate between rods and cocci. An overnight culture of isolates grown in respective broth was used for this staining. A drop of Nigrosin stain (HiMedia, India) was placed in a clean glass slide and later thick smear was made with the help of loop. The smear was allowed for air dry and later observed microscopically (magnification of × 100 with immersion oil).

3.1.4.3. Gram staining

The Gram status of the isolates was determined by light microcopy after the Gram reaction (Collins et al., 2004). Cells from fresh cultures were used for Gram staining. For this, isolates were grown in MRS broth at 37 °C for 24 hours. A thin smear of bacteria was made on a clean glass slide, heat fixed and allowed to dry. Primary staining was carried out using Crystal Violet (CV) for one minute and excess stain was washed under tap water. Gram's iodine mordant was added on the smear for one minute and washed using decolourizing agent (95 % ethanol v/v) for six seconds. Finally, counter stain i.e. Safranin was applied for 30 seconds and excess Safranin was washed, allowed to dry and observed under light microscope (at magnification of × 1000 with immersion oil).

3.1.4.4. Scanning electron microscopy

Scanning electron microscopy was performed for ten representative isolates obtained after molecular characterization. An overnight grown cells were harvested, washed with sterile normal saline for three time, fixed using 0.2 % glutaraldehyde at 6 °C for 4 hours and washed with different percentage of ethanol in an increasing order (30%, 50%, 70%, 90%, 100% ethanol) (Acharya et al., 2005). After drying samples were stored in desiccators until use. Samples were spread on a clean glass cover slips and allowed to dry for three hours. Later, glass cover slips with samples were kept on double-sided conducting adhesive tape pasted on a metallic stub and subjected to gold covering and observed under scanning electron microscope (LEO 435 VP; LEO Electron Microscopy, Cambridge, U.K.) at 20 kV.

3.1.4.5. Catalase test

Catalase is an enzyme produced by many organisms and therefore the lack of catalase is a significant diagnostic characteristic. The enzyme breaks down hydrogen peroxide

into water and oxygen and gas bubbles are formed. The formation of gas bubbles therefore indicates the presence of catalase enzyme. LAB are known as catalase negative. Hence, in order to confirm catalase status of the isolates, catalase test was performed. For this purpose, overnight cultures of the isolates grown in respective agar plates were used. One drop of 3% hydrogen peroxide solution was placed on clean glass slide and the desired colony was transferred into the drop using sterile wooden stick (like tooth pick). Formation of effurbation indicated positive test (Aneja, 2005).

3.1.4.6. Motility test

Hanging-drop method (MacFaddin, 2000) was performed to determine the motility of the isolates. The slide was observed under a light microscope with × 40 magnification to check the motility of the bacteria.

3.1.5. Purification, stock culture maintenance and long term preservation

At least five individual colonies from each plate were picked using a sterile wooden stick and subcultured in respective broth medium and purified them by repetitive subculture in broth and agar medium. Pure cultures were preserved as frozen stocks in MRS broth medium containing 40 % (v/v) glycerol at - 40 $^{\circ}$C (Mehmood et al., 2009; Liu et al. (2004).

3.1.6. Molecular characterization of the isolates

3.1.6.1. DNA extraction and quantification

Genomic DNA for all PCR reactions was extracted from a 100 µL of an overnight culture diluted with 300 µL of TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0) as previously described (Mora et al., 2000). To this, 10 µL of lysozyme (from stock solution of 20 mg/mL) was added and incubated in water bath at 37 $^{\circ}$C. After 30 minutes, 12.5 µL Sodium Dodecyl Sulphate (SDS) (20%) and 10 µL Proteinase K (from a stock of 20 mg/mL) were added and incubated in water bath at 50 $^{\circ}$C for 15 min. To this, 300 µL of phenol was added and centrifuged (HER MLE Z233 M-2, BIO-RAD) at 15000 rpm for 5 minutes. After discarding the lower phase, 400 µL chloroform was added and centrifuged at 15000 rpm for 5 minutes. Then upper phase was transferred into a new sterile eppendorf tube and 40 µL sodium acetate (3 M) was added followed by the addition of 800 µL ethanol (95 %) and centrifuged at 15000 rpm for 20 minutes. Then upper ethanol layer was discarded and pellet was

washed with ice cold ethanol (70%) (300-400 µL) and centrifuged at 15000 rpm for 10 minutes. After discarding remaining ethanol, the DNA was dried in GYRO VAP (HOWE, Italy). The DNA was resuspended in 50 µL sterile distilled water.

The concentration and purity of the DNA samples were determined spectrophotometrically with a Take3 Micro-Volume Plate in the Econ BioTek Microplate Spectrophotometer (AHSI S.p.A., Bernareggio, Italy). After quantification, DNA samples were diluted with nuclease-free water to reach a concentration of 100 ng/µL and stored at - 20 °C.

3.1.6.2. Genetic identification and grouping of isolates

After DNA extraction and quantification, grouping (similar genus) of isolates was carried out based on internal transcribed spacers region (ITS) or Ribosomal Spacer Analysis (RSA). A PCR amplification of 120 lactobacilli isolates, with ITS primers was performed. The details of the primer and the thermal cycling parameters employed are shown in Table 3.1. PCR reactions were performed in 25 µL reaction volume containing 100 ng bacterial DNA, 2.5 µL 10 × reaction buffer of Dream Taq™ (Fermentas, Vilnius, Lithuania), 200 µM each of dNTPs, 0.5 mM $MgCl_2$, 0.5 µM each of primers, and 0.5 U Dream Taq™ DNA polymerase. Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf, Milan, Italy). Amplification products were electrophoresed in 2 % (w/v) agarose gel with 0.2 µg/mL of ethidium bromide in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and photographed using gel documentation system (Bio-Rad EZ Gel Doc Imager). A Gene-Ruler 100 bp plus (100 – 3000 bp) DNA ladder (Fermentas, Germany) was used as a size marker.

3.1.6.3. DNA sequence analysis of isolates and phylogenetic analysis

The 16S rRNA gene was amplified by PCR, using primers P0 (5’ GAAGAGTTTGATCCTGGCTCAG-3’) and P6 (5’-CTACGGCTACCTTGTTACGA-3’) (Edward et al., 1989). The PCR reactions were performed in PCR-Mastercycler 96 (Eppendorf, Germany) and PCR programme with 2 minutes at 94 °C, then 5 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C, 1 minute at 72 °C, followed by 30 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C, 1 minute at 72 °C, with a 7 minutes final extension at 72 °C. UltraClean PCR Clean-Up Kit (MoBio, Cabru s.a.s, Arcore, Italy) was used to purify PCR products prior to

sequencing. A 500 bp fragment of the 16S rRNA gene was sequenced for representative isolates of each cluster groups based on ITS/RSA profiling and amplification was performed using the primer (5'-AGAGTTTGATCCTGGCTCAG-3') position 8-27 of *Escherichia coli* (Lane, 1991). Thus obtained chromatogram data was analyzed using Chromas (Version 2.12) and the BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (Internet visit, 2015. http://www.ncbi.nlm.nih.gov/blast). The sequence information was imported into BioEdit Sequence Editor for assembly and alignment. The 16S rDNA sequences of curd isolates were compared to sequences from type lactobacilli strains held in GenBank and phylogenetic trees were constructed by the neighbor-joining method (Advanced www.Pylogeny.fr, 2015). *Bacillus subtilis* NCDO 1769 was used as an outgroup organism. The topologies of trees were evaluated by bootstrap analysis of the sequence data based on 100 random resamplings. (Ennahar et al., 2003). Nucleotide sequence accession numbers are given in annex.

The nucleotide sequences for the 16S rRNA fro Nepalese curd isolates were deposited with GenBank under accession number BankIt1665181 (for Seq1-Seq13) as KM009071, KM009072, KM009073, KM009074, KM009075, KM009076, KM009077, KM009078, KM009079, KM0090710, KM0090711, KM0090712 and KM0090713 (details given in Appendix 6).

3.1.6.4. Molecular identification of *Lactobacillus* at species and subspecies level

Many species-specific PCRs were performed for the identification of *Lactobacillus* at strains species and sub-species level. The primer sequences of the primers used to identify various species, subspecies, their corresponding specificities and the thermal cycling parameters employed are shown in Table 3.1. PCR reactions were performed in 25 µL reaction volume containing 100 ng bacterial DNA, 2.5 µL 10 × reaction buffer of Dream Taq™ (Fermentas, Vilnius, Lithuania), 200 µM each of dNTP, 0.5 mM MgCl2, 0.5 µM each of primer pair, and 0.5 U Dream Taq™ DNA polymerase. Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf, Germany). Amplification products were electrophoresed in 2 % (w/v) agarose gel with 0.2 µg/ml of ethidium bromide in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and photographed (Fortina et al., 2001). A Gene-Ruler 100 bp plus (100 – 3000 bp) DNA ladder mix (Fermentas, Germany) was used as a size marker.

An intraspecies-specific PCR was performed for identification of *L. delbrueckii* till sub-species level with the primers LB1 (5'-AAAAATGAAGTTGTTTAAAGTAGGTA-3') and LLB1 (5'-AAGTCTGTCCTCTGGCTGG-3') and the amplified DNA fragment was approximately 1065 bp long (Torriani et al., 1999).

3.1.6.5. Genetic typing and cluster analysis

Genetic fingerprinting was carried out by combined analysis of Repetitive Element PCR (rep-PCR) typing using primers (GTG)5 (5′-GTGGTGGTGGTGGTG-3′; annealing temperature T_a= 42 °C) and BOXAIR PCR (5′-CTACGGCAAGGCGACGCTGACG-3′; T_a= 48 °C) (Versalovic et al., 1994; De Urraza et al., 2000; Guglielmetti et al., 2008) and Random Amplified of Polymorphic DNA (RAPD)-PCR typing with primers M13 (5′-GAGGGTGGCGGTTCT-3′; T_a= 38 °C), AP02 (5'-AGTCAGCCAC-3'; T_a= 32 °C), OPI17 (5'-CGAGGGTGGTGATG-3'; T_a= 46 °C), OPI02 (5'-GCTCGGAGGAGAGG-3'; T_a= 48 °C) and 1254 (5'-CCGCAGCCAA-3'; T_a= 33 °C) (Torriani et al., 1999; Mora et al., 2000; Rossetti et al., 2005). PCR cycling conditions described by various authors and an amplification protocol of 35 cycles were used. The PCR products were separated by electrophoresis and photographed.

Genetic similarity among isolates was determined on the basis of presence or absence of bands amplified by different random primers and construction of Phenogram using the NTSYSpc software, version 2.11 (Applied Biostatics Inc., Port Jefferson, NY, USA), employing the SM, Dice and Jaccards' similarity coefficient. A Phenogram was deduced from a similarity matrix using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) clustering algorithm. The reliability of the cluster analysis was estimated by calculating the correlation coefficient value (r) for phenograms generated from simple matching Jaccards' and Dice coefficient.

3.1.6.6.Rapid genotyping of strains of *Lactobacillus casei* group of species by High Resolution Melt Curve analysis (HRMa)

Real time PCR was used for this purpose. *Lactobacillus casei* group of species includes three phylogenetically related species *viz. L. casei, L. paracasei and L. rhamnosus* (Blaiotta et al., 2008). The rapid discrimination of these closely related species (six representative curd isolates) was performed by high resolution melting

Table 3.1. PCR primers and conditions used for isolates identification.

Primer specificity and reference	Primer name and pair (5'to 3')	Thermal conditions	Amplicon size (bp)
L. delbrueckii [Torriani et al., 1999]	SS1; Fw: GTGCTGCAGAGAGAGTTTGATCCTGGCTCAG DB1; Rev: ACCTATCTCTAGGTGTAGCGCA	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 1 min at 57 ^{0}C, 1 min at 72 °C	1030
L. paracasei [Ward and Tammins, 1999]	Y2; Fw:CACCGAGATTCAACATGG PARA; Rev:CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 54 ^{0}C, 45 sec at 72 °C	290
L. rhamnosus [Ward & Tammins, 1999]	Y2; Fw:TGCATCTTGATTTAATTTTG RHAM; Rev:CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 54 ^{0}C, 45 sec at 72 ^{0}C	290
L. fermentum [Coton et al., 2008]	1391F;Fw:TGTACACACCGCCCGTC Lferm; Rev:TTTTCTTGATTTTATTAG	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 48 ^{0}C, 45 sec at 72 °C	460; 270
L. parabuchneri Coton et al., 2008]	1391F; Fw:TGTACACACCGCCCGTC Lparabuc; Rev:TGTTACTCCGGTCTGTGC	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 48 ^{0}C, 45 sec at 72 °C	330
L. helveticus [Fortina et al., 2001]	PeC_f; Fw:CTGTTTTCAATGTTGCAAGTC PeC_r; Rev:TTTGCCAGCATTAACAAGTCT PeN_f; Fw:CGCTGATTCTAAGTCAAGCT PeN_r; Rev:CGACTAAGAAGTGGAACATTA Tri_f; Fw:TCTTATTACGCAATGGACCAA Tri_r; Rev:AATACCGTTCTTGAGGTTAGA	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 58 ^{0}C, 1 min at 72 °C	918; 726; 524
L. brevis [Coton et al., 2008]	1391F;Fw: TGTACACACCGCCCGTC Lbrev; Rev:TAATGATGACCTTGCGGTC	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 48 ^{0}C, 45 sec at 72 °C	330
L. plantarum [Torriani et al., 2001]	planF; Fw:CCGTTTATGCGGAACACC pREV R; Rev:TCGGGATTACCAAACATCAC	2 min at 94 °C, 35 cycles of: 45 sec at 94 °C, 45 sec at 56^{0}C, 45 sec at 72 °C	318
Internal Transcribed Spacer region (ITS) [Jensen et al., 1993]	ITS-F; Fw: GAAGTCGTAACAAGG ITS-R; Rev: CAAGGCATCCACCGT	2 min at 94°C, 5 cycles of: 45 sec at 94^{0}C, 1 min at 55°C; 1 min at72°C and 30 cycles of: 45 sec at 94 °C; 45 s at 60 °C, 2 min at 72°C	

curve analysis (HRMa) for the DNA product obtained from Real Time Polymerase Chain Reaction (PCR). DNA samples of *L. paracasei* (five representative isolates from different geographical locations) and *L. rhamnosus* representative isolates from different geographical locations) together with other laboratory reference strains of *L. paracasei* subsp. *tolerance* and *L. casei* were diluted with nuclease-free water to reach a concentration of 5 ng μL^{-1} and stored at -20 °C. Quantitative real-time PCR reactions (qPCR) were carried out in a final volume of 15 µl containing 7.5 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories S.r.l., Segrate, Italy), 0.25 µM of each primer [GroHRM-F (5'-GTTTGATCGCGGCTATCTGA-3') and GroHRM-R (5'-CCTTGTTGMACGATTTCTTG-3')] and 15 ng of template DNA. Quantitative PCR (qPCR) experiments were performed in a CFX96 thermal cycler (Bio-RAD Laboratories) programmed with initial denaturation at 98 °C and 72 °C for 1second; a unique step of 65 °C for 10 seconds was finally performed. Each DNA sample was analyzed at least in duplicate. At the end of the amplification protocol, melting curve analysis of the amplicons were immediately determined by monitoring fluorescence from 65 to 95 °C, with temperature increments of 0.2 °C, (Koirala et al., 2015).

For HRMa, raw fluorescence data were exported from Bio-Rad CFX-Manager software and a single text (.txt) file was prepared for each strain by indicating data from 70 to 95 °C as depicted below:

Temperature (°C)	Fluorescence
70.00	8383.74
70.20	8348.91
70.40	8314.21
70.60	8279.45
…..	…..

The .txt documents were used as input files for software uAnalyze℠ v.1.8 (Dwight et al., 2012), available on line (Internet visit, 2015. https://www.dna.utah.edu/uv/uv.php), which was employed for the HRMa. The software parameters used for the normalization of raw fluorescence melt data were as follows: horizontal slider set at temperature 78 °C and 79.5 °C for the analysis of *L. casei* species, and 78 °C and 81 °C for the analysis of *L. paracasei* intra-species genotypes.

3.2. *In vitro* probiotic properties of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) of Nepal

3.2.1. Study samples

Lactobacilli isolates obtained from traditionally prepared curd or *dahi* collected at different geo-climatic conditions of Nepal were considered for the present study. These were selected based on the population distribution (genotyping) in different districts. Twenty four lactobacilli obtained from four districts of Nepal were selected for this study (Table 3.2.).

3.2.2. Strains, culture media and incubation conditions

Twenty four strains belonging to ten different *Lactobacillus* species originating from traditionally prepared curd or *dahi* samples collected from different geo-climatic conditions of Nepal were used for this study. The lactic cultures were; *L. delbrueckii* subsp. *bulgaricus* (n=6), *L. paracasei* (n=6), *L. fermentum* (n=3), *L. rhamnosus* (n=2), *L. parabuchneri* (n=2), *L. helveticus* (n=1), *L. brevis* (n=1), *L. harbinensis* (n=1), *L.*

Table 3.2. *Lactobacillus* isolates tested for *in vitro* probiotic properties.

Lactic culture used for *in vitro* probiotic test	Number of isolates form each geographical regions					
	Gorkha (HM)	Rasuwa (HL)	Rasuwa (HM)	Lalitpur (HL)	Bhaktapur (HL)	Total
Lactobacillus paracasei	2	-	2	2	-	6
Lactobacillus rhamnosus	1	-	-	-	1	2
Lactobacillus delbrueckii bulgaricus	-	3	-	1	2	6
Lactobacillus fermentum	1	1	-	-	1	3
Lactobacillus parabuchneri	1	-	-	-	1	2
Lactobacillus helveticus	-	1	-	-	-	1
Lactobacillus brevis	-	-	1	-	-	1
Lactobacillus coryniformis	-	-	1	-	-	1
Lactobacillus harbinensis	-	1	-	-	-	1
Lactobacillus plantarum	-	-	1	-	-	1
Total	5	6	5	3	5	24

plantarum (n=1), *L. coryniformis* (n=1) (Table 2). Two commercial strains *Lactobacillus rhamnosus* GG and *Lactobacillus casei* SHIROTA were also used as reference samples for conducting and optimizing experiments wherever needed. All the lactobacilli were cultured aerobically in MRS broth (Sigma and Difco, Germany) at 37 °C for 24-48 hours.

3.2.3. Gastro-intestinal transit resistance test

3.2.3.1. Resistance to pH 3 and 0.3% bile

To determine the transit tolerance through stimulated gastric juice, the method described by Charteris et al., (1998a) and Chou & Weimer, (1999) was used with slight modifications. Overnight culture broth (30 mL) of each test strain was centrifuged at 5000 rpm at 4 °C for 15 minutes. Supernatant was discarded and pellet was resuspended in 10 mL sterile peptone water (10 g/L peptone, 5 g/L sodium chloride, final pH 7.2±0.2 at 25 °C). From peptone water cell suspension, 100 µL was inoculated in 6 mL simulated gastric digestion model pH 3 (500 U/mL pepsin, 0.125 M NaCl, 0.045 M $NaHCO_3$, 0.007M KCl) and 6 mL control model (phosphate buffer pH 6.5-7.0) followed by gentle vortexing. From each model, 100 µL of sample was withdrawn for viable count at zero time (T_0 acid). Then the gastric model and control model inoculated with cultures were incubated at 37 °C. After 90 minutes, 100 µL of cell suspension was withdrawn from both models for viable count (T_{90} acid) and remaining volume of culture broth were centrifuged and supernatant were discarded. Then, to each individual pellet obtained, 5 mL of bile model (1g/L peptone, 3 g/L OxGall) and 5 mL of control (phosphate buffer pH 6.5) were added to respective tube models, mixed by using vortex. Again from each model, 100 µL of cell suspension was withdrawn for viable count at zero time (T_0 bile) and later incubated at 37 °C for 180 minutes. Then, 100 µL of culture broth from each model was withdrawn for viable count (T_{180} bile).

The culture broths withdrawn from gastric model/control and bile model/control at different intervals of time were serially diluted in normal saline (0.85 % NaCl) separately. Spread plate technique was used for viable count on MRS agar (pH 6.5) in replicates. All the plates were incubated at 37 °C for 24-48 hours. Colony forming unit per mL was noted for each diluents of both the models.

3.2.3.2.Resistance to pH 2 and 0.3% bile

Similar procedure as described above for determining gastrointestinal transit resistance at pH 3 and bile (0.3%) was followed for determining the survivability at pH 2 and bile (0.3%). But the composition of simulated gastric digestion model pH 2 was 500 U/mL pepsin, 1 g/L peptone and 0.1 M KCl and that of control model was phosphate buffer pH 6.5-7.0) and the rest remained same as resistance to pH 3 and 0.3% bile (as mentioned above).

3.2.4. Adherence properties

3.2.4.1. *In vitro* cultivation of epithelial cells

Caco-2 (heterogenous human epithelial colorectal adenocarcinoma cell line; ATCC 2102-CRL) cells were routinely grown in Dulbeco's Modified Eagle's Minimal Essential Medium (DMEM) supplemented with 25 mM glucose, 1.0 mM sodium pyruvate, 20% fetal calf serum and 1% non-essential amino acid and incubated at 37 °C in a atmosphere of 10% CO_2-90% air. Caco 2 cells were grown in 3 cm petri plates on microscope cover glasses. The culture medium was replaced every second day and monolayers were used in the adherence assay after 21 days of incubation (Guglielmetti et al., 2010).

3.2.4.2.Adhesion assay on Caco 2 cell line

To assay the adhesion properties of lactobacilli isolates, Jacobson et al., (1999) method was followed with slight modification. Caco 2 cells were grown in 3 cm petri plates on microscope cover glasses as described above. Cell monolayers were carefully washed with Phosphate Buffered Saline (PBS, pH 7.3) before bacterial cells were added. The bacterial cell concentration of an overnight culture was determined microscopically with a Neubauer-improved counting chamber (Marienfeld GmBH, Lauda-Konigshofen, Germany). Approximately 2×10^8 cells of each strain resuspended in PBS (pH 7.3) were incubated with a monolayer of Caco 2 cells at 37 °C. After 1 hour, all monolayers were washed three times with PBS to release unbound bacteria. Cells were then fixed with 3 mL of methanol and incubated for 8 minutes at room temperature. After removal of methanol with the help of pipette, cells were stained with 3 mL of Giemsa stain solution (1:20; Carlo Erba, Milan, Italy) and left for 30 minutes at room temperature. Cells were then washed until no colour was observed in washing solution and dried in an incubator at 30 °C for 1 hour.

Microscope cover glasses were then removed from the petri plate and examined microscopically (magnification of ×400). Adherent bacteria in 20 randomly selected microscopic fields were counted and average number of bacterial cells and also the nuclei were noted for result interpretation.

3.2.5. Antimicrobial activity

All selected isolates were screened for antimicrobial activity using paper disc diffusion method (Albano et al., 2007; Buntin et al., 2008; Abedi et al., 2013). Sterile paper disc (Whatman^{TM} Grade AA 9 mm DISC, UK) were dipped into three different preparations (an overnight grown culture broth of each lactobacilli, cell free supernatant and neutralized cell free supernatant) and then placed on solidified Brain Heart Infusion Agar (with 0.3% yeast extract, 1% glucose) seeded with 8 hours old culture of test pathogens (*Listeria monocytogenes, Salmonella enterica, Escherichia coli VE, Streptooccus pyogens* and *Pseudomonas aerugenosa*) obtained from laboratory collection. The plates were kept at 4 °C for 2 hours and then incubated at 37 °C for 24 hours. Afterward plates were observed for clear Zone of Inhibition (ZOI).

3.2.6. Antibiotic susceptibility testing

The inhibitory concentrations of nine antimicrobial agents were determined as Minimum Inhibitory Concentration (MIC) in microgram per milliliter (µg/mL), according to conventional broth microdilution protocols, in commercial 96-well microtiter plates (Guglielmetti et. al., 2010). Nine antibiotics *viz.* Ampicillin (0.25-16 µg/mL), Vancomycin (0.5-16 µg/mL), Gentamycin (1-128 µg/mL), Kanamycin (4-256 µg/mL), Streptomycin (2-256 µg/mL), Erythromycin (0.125-4 µg/mL), Clindamycin (0.125-4 µg/mL), Tetracycline (0.5-128 µg/mL) and Chloramphenicol (0.5-32 µg/mL) were used for antibiotic susceptibility testing. However, the concentrations of each antibiotics tested were different for different *Lactobacillus* species (EFSA, 2008).

For this, each test culture was grown in ISO-MRS broth at 37 °C for 24 hours. Culture broth was centrifuged at 14000 rpm for 1 minute, supernatant was discarded and the pellet was resuspended in phosphate buffer saline (pH 7.0) aseptically and the bacterial cell concentration was determined by means of a Neubauer-improved counting chamber (Marienfeld GmBH, Lauda-Konigshofen, Germany). The final

concentration of cell was maintained to 2×10^6 in 15 mL ISO-MRS broth. From this, 150 µL of cell suspension was added in wells of microtiter plate separately followed by 50 µL of each antibiotic in different concentration. ISO-MRS medium was used as negative control whereas 50 µL of ISO-MRS was used added with 150 µL of cell suspension as positive control. Precaution was taken for cross contamination during the addition of antibiotics and inoculation. The inoculated plate was incubated at 37 $^{\circ}$C for 24-48 hours. Results were noted by using spectrophotometer (BIORAD, Model 680, MICROPLATE READER, Germany) at 600 nm.

3.3. Preparation of Probiotic Curd (*Dahi*) and Sweet Curd (*Dahi*) using novel lactic cultures (*Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103) having probiotic properties.

3.3.1. Study strains of lactic cultures and Food borne pathogens

Indigenous lactic cultures obtained from traditionally prepared curd or *dahi* at different geo-climatic conditions of Nepal were used in this study. Initially, six cultures were considered for the preliminary selection (based on the *in vitro* probiotic properties) of which two cultures (based on antibiotic resistance profiling) *viz*. *Lactobacillus paracasei* NAST-RHM82 from Himalayan region of Rasuwa district and *Lactobacillus helveticus* NAST-RHL103 from Hilly region of Rasuwa district of Nepal were selected for the present study. From our previous study (mentioned previous chapter), these cultures have shown good *in vitro* probiotic properties (such as, gastrointestinal transit resistance and adhesion assets) and are safe for use as they are not resistant to various antibiotics tested according to EFSA guidelines.

3.3.2. Partial purification of antibacterial substance from indigenous lactic cultures

3.3.3.1. Bacterial strains and culture conditions

A working collection of six lactobacilli isolates originating from traditional fermented dairy product (*Dahi*) of Nepal were used in this study. The lactic cultures included in this study were 1) *Lactobacillus fermentum* NAST-GHM2, 2) *Lactobacillus rhamnosus* NAST-GHM25, 3) *Lactobacillus delbrueckii* subsp. *bulgaricus* NAST-RHL74, 4) *Lactobacillus paracasei* NAST-RHM82, 5) *Lactobacillus delbreuckii* subsp. *bulgaricus* NAST-RHL101, 6) *Lactobacillus helveticus* NAST-RHL103. These

lactobacilli were grown in de Man, Rogosa and sharpe (MRS) (HiMedia, India) at 37 ºC for 18-24 hours.

Pathogenic strains used in this study were *Bacillus cereus* F 4810, *Escherichia coli*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* FRI 722 obtained from laboratory collection. These indicator strains were maintained in broth and slant of brain heart infusion medium (HiMedia, India). The cultures were propagated successively twice in the repetitive broths, prior to their use in experimental trials.

3.3.3.2. Preliminary screening of Cell Free Supernatants (CFSs) derived from MRS broth culture

The ability of lactic cultures to produce antibacterial substances was determined using agar diffusion assay (Varadaraj & Ranganathn, 1984). Extracellular culture supernatants (ten times concentrate) of lactic isolates were used for screening against food borne pathogenic cultures of *B. cereus, E. coli, L. monocytogenes,* and *S. aureus*.

3.3.2.2.1. Preparation of CFSs from MRS broth culture

Each lactic culture was grown in MRS broth at 37 ºC for 36 hours. Cell Free Supernatants (CFSs) were obtained by centrifugation at 8000 rpm and 10 ºC for 20 minutes. The CFSs were concentrated ten times using Lyophilizer (SCANVAC Coolsafe 55-4 Pro, Denmark). After concentration, the lyophilized powder was resuspended in a phosphate buffer (pH 7) and later filter sterilized (MILLEX® GV Filter Unit, Durapore ® PVDF membrane, 0.22 µm). Thus prepared CFSs were ready for antimicrobial assay which was stored in freezer (- 20 ºC) until use.

3.3.2.2.2. Plate assay of CFSs derived from MRS broth (Bacteriostatic assay)

The agar well diffusion technique was used to determine the antimicrobial property of lactic isolates (Schillinger & Lucke, 1989; Varadaraj et al., 1993). An overnight culture of each indicator pathogenic strain was inoculated in a sterilized and molten (at 45 ºC) Brain Heart Infusion (BHI) agar medium (HiMedia, India). BHI inoculated with indicator strains (approximately 7.2 Log10 CFU/mL; Varadaraj & Ranganathan, 1984) was poured in a sterile Petri plate, mixed uniformly and allowed to solidify. The wells (10 mm) were made in the agar plate and filled with 100 µL of each of CFSs of lactic cultures derived from MRS broth and left for 30 minutes. Plates were kept in refrigerator for 3 hours and later incubated at 37 ºC for 18 hours. Results were noted for clear zone of inhibition (ZOI) in mm.

3.3.2.2.3. Plate assay of CFSs derived from MRS broth (Bactericidal assay)

Bactericidal assay was performed using *B. cereus* (Ogawa et al., 2001). The agar well diffusion technique was used as described above. An overnight culture of each indicator strain were inoculated in a sterilized and cooled (40-45 $^{\circ}$C) Brain Heart Infusion (BHI) agar (HiMedia, India). BHI inoculated with indicator strains were poured in a sterile Petri plates and mixed uniformly. After solidification, the wells (10 mm) were made and plates were incubated at 37 $^{\circ}$C for 12 hours for growth of pathogens. Later, each well was filled with 100 µL of CFSs and kept in refrigerator for 3 hours. Then the plates were left overnight at room temperature. Results were noted for clear zone of inhibition (ZOI) in mm.

3.3.2.2.4. Bactericidal assay of CFSs in broth

One mL of CFS derived from respective lactic cultures in MRS broth was taken in a sterile test tube (5 mL). An overnight grown culture of *B. cereus* (2×10^6 CFU/mL) was inoculated in all the tubes, incubated at 37 ºC for 24 hours and observation was made in terms of turbidity. Later 500 µL of aliquot was withdrawn from each tube and inoculated into new Brain Heart Broth (HiMedia, India) and incubated at 37 ºC for 24 hours. Results were observed for presence or absence of growth (Ogawa et al., 2001).

3.3.3.3. Assessment of antibacterial properties of cell free supernatants (CFSs) derived from skimmed milk broth culture

3.3.2.3.1. Media, lactic strains and culture conditions

Two lactic cultures *viz. Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL 103 were selected for profiling antibacterial activity. These two cultures were grown and maintained in skimmed milk broth. It was formulated by mixing 10 g skimmed milk powder, 0.5 g yeast extract, 0.5 g glucose and volume made to 100 mL using lukewarm distilled water followed by filtration through moistened cotton to remove some lumps of milk powder. Thus prepared skimmed milk broth was sterilized and used as medium for growth of lactic cultures to study antibacterial profile as well as maintaining of lactic cultures. The pathogenic strains used were *Bacillus cereus* F 4810*, Escherichia coli*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* FRI 722 obtained from laboratory collection.

3.3.2.3.2. Preparation of CFSs from skimmed milk culture broth

Two lactic cultures were grown individually in skimmed milk broth at 37 ºC for 36 hours. The CFSs were obtained by centrifugation at 8000 rpm at 10 ºC for 20 minutes. The CFSs were concentrated 10 times using Lyophilizer (SCANVAC Coolsafe 55-4 Pro, Denmark). After concentration (Table 3.3), the lyophilized powder was resuspended in a phosphate buffer (pH 7) and later filter sterilized (MILLEX® GV Filter Unit, Durapore ® PVDF membrane, 0.22 µm). Thus prepared CFSs were ready for antimicrobial assay and stored in freezer (at -40 ºC) until use. Each time same procedure was followed to obtain CFSs from skimmed milk.

Table 3.3. Concentration of cell free supernatant (CFSs) of skim milk broth of two lactic cultures viz. *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 at different volume.

Purification steps	Initial vol. (mL) of CFSs	concentrated vol. (mL)	withdrawn vol. (mL)	Left over vol. (mL)
Skim Milk CFSs	400	40	10	30
Methanol extraction	30	20	10	10
Methanol-Acetone extraction	10	8	0	8

3.3.2.3.3. Solvent extraction for purification of antimicrobial substance

3.3.2.3.3.a. Methanol Extraction of skimmed milk concentrate

Skimmed milk concentrate (20 ml) and ice cold methanol (60 mL) were mixed (in the ratio of 1:3) and uniformly dispersed at 5 ºC using Magnetic stirrer (2 MLH REMI MAGNETIC STIRRER, India). After 24 hours, supernatant was collected by centrifugation at 8000 rpm at 10 ºC for 20 minutes. Supernatant was concentrated to lowest volume (10 ml or half of the initial volume) using Rotary Evaporator (IKA® RV 10 digital). Methanol extract was filter sterilized (MILLEX® GV Filter Unit, Durapore ® PVDF membrane, 0.22 µm) and used for antibacterial profiling (Pulusani et al., 1979; Suma et al., 1998).

3.3.2.3.3.b. Acetone extraction of methanol concentrate

Methanol extracts (10 mL) and ice cold acetone (30 mL) were mixed and uniformly dispersed at 5 ºC using Magnetic stirrer (2 MLH REMI MAGNETIC STIRRER, India). After 24 hours, supernatant was collected by centrifugation at 8000 rpm at 10

ºC for 20 minutes. Supernatant was concentrated to lowest volume (5 mL or half of the initial volume) using Rotary Evaporator (IKA® RV 10 digital). Methanol-acetone extract was sterilized (MILLEX® GV Filter Unit, Durapore ® PVDF membrane, 0.22 µm) and used for antibacterial profiling (Pulusani et al., 1979; Suma et al., 1998).

3.3.2.3. Plate assay for antimicrobial activity of skimmed milk, methanol and methanol-acetone concentrate

Agar well diffusion technique was used for this assay. An overnight culture of each indicator strain was inoculated in sterilized and cooled (at 45 ºC) Brain Heart Infusion (BHI) agar. BHI inoculated with individual indicator strain was poured in a sterile petri plate, mixed uniformly and left for solidification. After solidification, wells (10 mm) were made and 100 µL of each lactic culture skimmed milk concentrate, methanol extract and methanol-acetone extract were poured in separate wells and left as such for 30 minutes. Plates were kept in refrigerator for 2 hours and later transferred to incubator at 37 ºC for 18 hours. Results were noted for clear ZOI in mm (Schillinger & Lucke, 1989; Varadaraj et al., 1993).

The sterile solution of 1% lactic acid in distilled water (pH 2.48), 1% lactic acid in phosphate buffer-pH7 (pH 4.18) and 10 µL lactic acid in 100 mL distilled water (pH 3.2) were used as negative control in above mentioned plate assay.

3.3.2.4. Protein estimation of CFSs and its derivatives

The protein content of skimmed milk concentrate, methanol concentrate and methanol-acetone concentrate were estimated using Bradford method (Bradford, 1976). A 100 µL of each sample was taken and distilled water was added to make up to 1.5 mL volume. To this, 1.5 mL of Bradford reagent was added and incubated in room temperature (RT) for 30 seconds for colour development. Then, light absorption at 595 nm was recorded on UV spectrophotometer (UV-1800, SHIMADZU, Japan). Reference solutions were prepared in identical manner as above, except that 100 µL of protein solution was replaced by distilled water.

3.3.2.5. Determination of Arbitrary Unit mL^{-1} (AU mL^{-1})

The antibacterial concentration of each sample (skimmed milk concentrate, methanol extract and methanol-acetone extract) was determined with the critical method of dilutions called AU mL^{-1} (Hernandez et al., 2005; Roopashri & Varadaraj, 2011). For this, all the samples were diluted to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 using phosphate

buffer (pH 7) and agar well diffusion assays were performed to calculate AU mL^{-1}. Antibacterial activity of the culture supernatants was calculated as the reciprocal of the highest dilution showing definite inhibition of indicator bacterial strains and expressed as arbitrary unit mL^{-1} (AU mL^{-1}).

3.3.2.6. Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined using skimmed milk concentrate, methanol extract and acetone-methanol extract against various pathogens mentioned above. For this, the entire sample was diluted to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 using phosphate buffer (pH 7) and one mL of each sample was used against 2×10^3 cells per mL. One mL of each extract diluents was transferred in sterile test tubes and each indicator strain was inoculated into respective diluent tubes of each extracts. All the inoculated tubes were incubated at 37 ºC for 24 hours. Results were observed based on the turbidity compared to positive (without any sample) and negative control (without pathogens). MIC was calculated with respect to arbitrary unit mL^{-1} (AU mL^{-1}) (Abdelbasset & Djamila, 2008).

3.3.2.7. Scanning electron microscopy (SEM)

Overnight grown pathogens (2×10^3 cells per mL) were treated with 1:1 dilution of methanol-acetone concentrate of both samples at room temperature for 18 hours. Cells were harvested, fixed using 0.2 % glutaraldehyde at 6 ºC for 4 hours and washed with different percentage of ethanol (30%, 50%, 70%, 90%, 100%) in an increasing concentration. After drying, samples were stored in desiccator until use. Samples were spread on a clean glass cover slips and allowed for drying. Later, glass cover slips with samples were kept on double-sided conducting adhesive tape pasted on a metallic stub and subjected to gold covering and observed under scanning electron microscope (LEO 435 VP; LEO Electron Microscopy, Cambridge, UK) at 20 kV (Acharya et al., 2005).

3.3.4. Product development using *Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103

3.3.4.1. Probiotic Curd (*Dahi*) preparation

3.3.4.1.1. Preparation of skimmed milk broth

Skimmed milk broth (10%) was prepared by mixing skimmed milk powder (100 g), yeast extract (5 g), and glucose (5 g) in distilled water (900 mL). For this, required quantity of skimmed milk powder was added into lukewarm distilled water with stirring (to avoid lumps formation) to dissolve it properly. Later, yeast extract and glucose were added and dissolved and final volume made up to 100 mL. Finally, the broth was filtered through moistened cotton, dispensed in 10 mL amount in test tubes or higher amounts either in glass beakers or conical flask depending on the experiment. Skimmed milk broth was sterilized for 15 minutes at 121 ºC.

3.3.4.1.2. Preparation of inoculums

Overnight grown cultures of *Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103 from MRS broth were separately inoculated in skimmed milk broth and incubated at 37 ºC for 24 hours. After successive subculture (at least two times) in skimmed milk broth, the lactic cultures were ready as starter culture for product development. Each time, lactic cultures were freshly grown for 18-24 hours prior to inoculation for the product development.

3.3.4.1.3. Media for Curd preparation

Commercially available homogenized milk was used as a source of medium for product development. Glass beakers (500 mL) and a measuring cylinder (100 mL) were covered with aluminum foil and sterilized by autoclaving. Then, milk (100 mL) were poured aseptically in pre-sterilized beakers (500 mL) with the sterile measuring cylinder (100 mL) and covered correctly with the aluminum foil. All the beakers with milk were autoclaved in a cooker for five minutes (after a first whistle). The milk medium was cooled to 40-45 ºC before inoculation.

3.3.4.1.4. Optimization of inoculum size, fermentation time, pH and titratable acidity for product development

Five sterile milk medium (100 mL each at 40-45 ºC) in glass beakers (500 mL) were used for optimization study. Of the five milk medium; two were inoculated with *L.*

paracasei NAST-RHM82 (1% & 2% v/v inoculums separately), two medium with *L. helveticus* NAST-RHL103 (1% & 2% v/v inoculums separately) and a beaker with a sterile milk medium was used as negative control. All the beakers were incubated at 37 ºC. The times for setting (curdling) were noted carefully in each case. After setting, products were transferred to the refrigerator for further analysis. The pH, titratable acidity and viable count were measured for all the products.

3.3.4.1.5. Preparation of product with optimized parameters

Two sterile milk medium (100 mL each at 40-45 ºC) in glass beaker (500 mL) were taken. Each medium were inoculated individually with one percent inoculum and incubated at 37 ºC. The product formed with *L. helveticus* NAST-RHL103 was set after 8 hours, so it was transferred to the refrigerator. Whereas the product formed with *L. paracasei* NAST-RHM82 was set after 30 hours and was transferred to the refrigerator. Thereafter in each case, chemical, organoleptic (sensory profiles) and microbiological properties of the products were assessed after 3 hours storage in refrigerator.

3.3.4.1.6. Analysis of the product properties

3.3.3.1.6.a. Chemical properties

Determination of pH

The pH of Curd samples was determined using a calibrated microprocessor based digital pH meter (Eutech, Cyberscan 1000, Singapore) (AOAC, 1990).

Titratable acidity

Titratable acidity of samples was measured by titrimetric method as described by Olubamiwa et al., (2007). A 0.5 mL of phenolphthalein (1% solution prepared in 95% alcohol) was added as an indicator in 10 mL of curd (1 mL sample plus 9 mL distilled water). The samples were then titrated with 0.1 N NaOH. All the determinations were carried out in triplicates and concurrent values were noted. The titratable acidity was calculated as lactic acid percentage (%) (Adesoji et al., 2010).

Titratable acidity = (mL NaOH × N NaOH × M.E. ×100)/volume of sample used (Rao et al., 1998)

Where, mL NaOH = volume of NaOH consumed during titration

N NaOH = Normality of NaOH

M.E. = molecular equivalent factor

Fat extraction

Fat extraction was carried out using Mojonnier Method (AOAC Intl. Methods 1989.05, 2006). Curd sample (10 g) was weighed accurately and transferred to extraction tube. To this ammonia (1.25 mL, sp. gr. 0.8974) was added and shaken thoroughly and incubated for 30 minutes. Then ethyl alcohol (10 mL) was added and mixed well. Thereafter, diethyl ether (25 mL, peroxide free) was added, closed with stopper cap and shaken vigorously for about half a minute. Then petroleum ether (25 mL, boiling range 40-60 ºC) was added and shaken again vigorously for about half a minute. It was allowed to stand until the upper clear ethereal layer was separated completely. When there is a tendency to form emulsion, a little alcohol was added to help separation of the layers. The clear ethereal layer was transferred into a round bottom flask. Previous step was repeated thrice and the clear ethereal layer was collected. The ethereal extract was added to the same container and evaporated completely using vacuum evaporator. The flask was dried in the hot air oven at 102 ± 2 ºC for 2 hours, cooled in a desiccator and weighed. The fat from the flask was washed out with petroleum ether carefully leaving any insoluble residue in the flask. The flask was heated again in the hot air oven for 30 minutes, cooled in a desicattor and weighed. The process was repeated until the difference between two successive weights did not exceed 1 milligram. Difference between duplicate determinations obtained simultaneously should not be more than 0.03 gm fat/100 gm product.

Fat percent w/w = (Weight of fat /weight of curd sample) ×100

Determination of fatty acids in* curd *samples by Gas Chromatography

The fat from the curd samples were extracted by Mojonnier method as described previously. The fatty acids present were converted into fatty acid methyl esters (FAME) by following procedure. Fat sample (200 mg) was transferred into a stopper capped glass centrifuge vial. Two mL of heptane or hexane and 0.1 mL of 1 N methanolic KOH were added. The vial was closed, shaken well for 30 seconds and centrifuged. Later two drops of upper layer were taken and diluted with two mL of hexane or heptanes. The final concentration of fatty acid methyl esters (FAME) in hexane was approximately 0.5%. FAME sample (0.2 µL) was injected into capillary

column of gas chromatography (GC-2010 Gas Chromatography, Shimadzu, Japan) (AOCS, 2003).

3.3.3.1.6.b. Organoleptic properties (Sensory profiling)

The curd samples were prepared and put in a refrigerator at 6 ºC for 3 hours. Then samples were coded as A, B, C and sensory analysis was carried out for three samples. The codes represented the stated samples below:

Sample A: Curd prepared using *Lactobacillus paracasei* NAST-RHM82

Sample B: Curd prepared using *Lactobacillus helveticus* NAST-RHL103

Sample C: Commercial Curd (*dahi*) in the local market, Mysore, India

Sensory analysis of Curd (A, B and C) samples were carried out by Quantitative Descriptive Analysis (QDA) which comprised of 15 cm scale anchoring at low (1.25 cm) or detection threshold and high (13.75 cm) or the saturation threshold. The intensity of each specific description was quantified on the structured scale. Suitable attributes specific to the curd (*dahi*) was identified in the preliminary session of evaluations. Panelists were suitably trained and oriented towards the sensory technique and the product to be evaluated. Care was taken to avoid interference from other sources or bias.

Evaluations were conducted under white fluorescent light, with the booth area maintained at temperature 22 ± 2 ºC and RH 50 ± 5 %. A suitable score card was developed using “Free-Choice Profiling” method selecting appropriate terminology. Samples were presented in petri plates coded with 3-digit random numbers, to the panelists. A glass of drinking water was also provided to cleanse the palate in between the samples.

Curd samples were subjected to instrumental colour measurement using colour measuring equipment (Colour Flex, EZ, Hunter Lab Association INC, Reston, USA). Analysis was carried out under D65 illuminant and 10 degree view angle.

3.3.3.1.6.c. Microbiological properties

The products were analyzed for viable counts of lactic count. A curd samples (10 mL) was serially diluted in 90 mL of sterile normal saline (0.85%). Second dilutions onwards were performed in one mL of sample and 9 mL of sterile normal saline up to desired number of dilutions. One mL of last four diluents tubes were used in

duplicates separately for pour plating using MRS agar. All the plates were incubated at 37 ºC for 48 to 72 hours and lactic count was enumerated through indirect counting using a colony counter (Digital Colony Counter, Sii SERWELL, Bangalore, India) followed by the dilution factor.

3.3.4.1.7. Pre-processing contaminant effect

In this experiment, starter culture and the indicator pathogen culture (four different pathogens in four different medium) were inoculated simultaneously to assay the antibacterial competence of the particular starter culture against respective pathogens. For this, two lactic cultures (*L. helveticus* NAST-RHL103 and *L. paracasei* NAST-RHM82) were used to make the products and the four different indicator pathogens *viz. Bacillus cereus, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*) were used as pre-processing contaminants.

For this, two sets of sterile milk medium [four sterile milk medium in each set (50 mL each at 40-45 ºC)] in glass beakers (250 mL) were prepared and inoculated with one percent inoculum of each lactic culture in both the sets separately. Simultaneously, 2×10^4 active cells of four respective pathogens were added in the milk medium inoculated with respective starter culture. After inoculation, all medium were incubated at 37 ºC. Soon after setting (8 hours for *L. helveticus* NAST-RHL103 product and 30 hours for *L. paracasei* NAST-RHM82 product), microbiological counting was carried out for individual pathogens in selective medium while lactic cultures were counted in MRS agar.

Pour plate technique was used for enumeration of lactic culture. For this, serial dilution was performed to the desired level and one mL of last four diluents were poured in a sterile petri plate and molten MRS agar (at 40-45 ºC) was poured, mixed uniformly and left for solidification. After solidification, all the plates were incubated at 37 ºC for 48-72 hours.

Spread plate technique was used for enumeration of pathogen strains. For this, serial dilution was performed up to the desired level and 0.1 mL desired diluents tube were transferred in respective selective agar plate and spread by using L-shaped rods. All the plates were incubated at 37 ºC for 24-48 hours. In all the cases, duplicate plating was performed and average of count was considered for data analysis. The entire selective media used in this study were of HiMedia, India. The medium used were

MYP Agar Base, MacConkey Agar, Listeria Selective Agar and Baird Parker Agar for *B. cereus, Escherichia coli, Listeria monocytogenes* and *S. aureus* respectively.

3.3.4.1.8. Post-processing contaminant effect

3.3.3.1.8.a. Post processing contaminant effect with product

Two lactic cultures (*L. helveticus* NAST-RHL103 and *L. paracasei* NAST-RHM82) were used to make products separately in four different beakers (250 mL) and soon after product formation four different pathogens (*Bacillus cereus, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*) were inoculated individually as post-processing contaminants, incubated at 6 ^{0}C up to 24 hours and analyzed the effect of lactic culture towards the pathogens. In each case (two separate lactic cultures as starter), four sterile milk medium (50 mL each at 40-45 ºC) in glass beakers (250 mL) were taken. All media in both the cases were inoculated separately with one percent inoculum of each lactic culture and incubated at 37 ºC. Soon after setting (incubation time 8 hours for *L. helveticus* NAST-RHL103 product and 30 hours for *L. paracasei* NAST-RHM82 product), products were inoculated separately with 2×10^4 active cells of four different pathogens in both the cases. Each product inoculated with separate pathogens was mixed properly with the help of sterile glass rods separately followed by incubation at 6 ºC. After 12 & 24 hours of incubation, each product was analyzed for individual pathogen count in respective selective medium.

Serial dilution was performed to desired level and spread plate technique was used for enumeration of pathogens. In each case, 0.1 mL of diluents from above desired dilution was transferred in respective selective agar plate and spread by using L-shaped rods. All the plates were incubated at 37 ºC for 24-48 hours. In all the cases, duplicate plating was performed and average of count was taken into consideration for data analysis.

3.3.3.1.8.b. Post-processing contaminant effect in addition to 10X skimmed milk concentrate in the product

Product was prepared using *L. helveticus* NAST-RHL103 and soon after product formation, four different pathogens (*Bacillus cereus, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus.*) were inoculated individually as post-processing contaminants and simultaneously one mL of 10X skimmed milk

concentrate of *L. helveticus* was added into each products, incubated at 6 ºC up to 24 hours and analyzed the effect of lactic culture towards the pathogens.

For this, four sterile milk medium (50 mL each at 40-45 ºC) in glass beakers (250 mL) were taken and were inoculated separately with one percent inoculums and incubated at 37 ºC for 8 hours. Soon after setting, each product was inoculated with 2×10^6 active cells of four different pathogens separately and simultaneously, one mL of skimmed milk concentrate (10X) of *L. helveticus* was added in all the beakers. Each product inoculated with separate pathogen and skimmed milk concentrate was mixed properly with the help of separate sterile glass rod and were incubated at 6 ºC. After 12 & 24 hours, each product was analyzed for individual pathogen in selective medium. Serial dilution was performed to desired level and spread plate technique was used for enumeration of pathogens. In each case, 0.1 mL of diluents from above desired diluents was transferred in respective selective agar plate and spread by using L-shaped rods. All the plates were incubated at 37 ºC for 24-48 hours. In all the cases, duplicate plating was performed and average of count was considered for data analysis.

3.3.4.2. Sweet Curd (*Dahi*) preparation

3.3.4.2.1. Preparation of cell suspension by stabilizing cells in high concentration glucose medium

Lactobacillus helveticus NAST-RHL103 was grown in four different MRS broths (10 mL) at 37 ºC for 18 hours. Cells were harvested, washed two times with sterile normal saline (0.85 %) and resuspended in 10 mL of sterile normal saline until use. All the cells from each broth were inoculated in MRS broth containing 10%, 15%, 20% & 25% glucose in (10 mL) and incubated in refrigerator. The culture broth tubes were mixed twice every day by using vortex (REMI CM 101, India). After 4 days, the cells were harvested by centrifugation and suspended in sterile normal saline at 6 ºC until use.

3.3.4.2.2. Media preparation for Sweet Curd

A 400 mL of homogenized milk was taken in a stainless steel vessel and boiled in a low flame with a continuous stirring till the volume was reduced to 50 % of initial volume. Then, milk was divided into four parts in a sterile beaker (250 mL) and covered with aluminium foil. Different amount of sugar was weighed to maintain

10%, 15%, 20% and 25% separately. The sugar was heated in low flame to melt and added to different beaker labeled with different concentration of glucose medium. Medium was sterilized by autoclaving for 10 minutes.

3.3.4.2.3. Preparation of *Sweet* Curd with optimized parameters

Concentrated milk media with different sugar concentrations (10%, 15%, 20% and 25% sugar) were cooled to ambient temperature after sterilization. All the cells of cell suspension obtained by culturing in respective sugar percentage in MRS medium (as mentioned above) were inoculated into respective sugar percentage of medium and incubated at 37 ºC for 16 hours. After setting, the products were transferred to refrigerator for analysis of chemical, sensory and microbiological properties.

3.3.4.2.4. Analysis of the product

3.3.3.2.4.a. Chemical properties

Determination of pH

The pH of Sweet curd samples was determined using a calibrated microprocessor based digital pH meter (Eutech, Cyberscan 1000, Singapore) (AOAC, 1990).

Determination of Titratable acidity

Titratable acidity of samples was measured by titrimetric method as described by Olubamiwa et al. (2007). Phenolphthalein (0.5 mL, 1% solution in 95% alcohol) was added as an indicator in 10 mL of curd (1 mL curd sample plus 9 mL distilled water). The samples were then titrated with 0.1 N NaOH. All the determinations were carried out in triplicates and concurrent values were noted. The titratable acidity was calculated as lactic acid percentage (%) (Adesoji et al., 2010):

Titratable acidity = (mL NaOH × N NaOH × M.E. ×100)/volume of sample used (Rao et al., 1998)

Where, mL NaOH = volume of NaOH consumed during titration

N NaOH = Normality of NaOH

M.E. = equivalent factor

Total sugar estimation

Total sugar estimation was carried out using modified phenol sulphuric acid method (Sturgeon, 1990). Sample or carbohydrate solution (0.5 mL) was taken in a test tube and phenol solution (0.3 mL of 5% phenol) and concentrated sulphuric acid (1.8 mL) were added and mixed properly. After allowing the test tubes to stand for 10 minutes, they were vortexed for 30 seconds (REMI CM 101, India) and allowed to remain for 20 minutes at room temperature for colour development. Then, light absorption at 490 nm was recorded on UV spectrophotometer (UV-1800, SHIMADZU, Japan). Two reference solutions were prepared in identical manner as above, except that 0.5 mL of carbohydrate solution was replaced by distilled water.

3.3.3.2.4.b. Organoleptic properties (Sensory profiling)

The Sweet curd samples were prepared and put in a refrigerator at 6 ºC for 3 hours. Then samples were coded as A, B, C and sensory analysis was carried out for three samples. The codes represented the stated samples below:

Sample A: Sweet Curd with 10 % glucose medium

Sample B: Sweet Curd with 15 % glucose medium

Sample C: Sweet Curd with 20 % glucose medium

3.3.3.2.4.c. Microbiological properties

The Sweet curd products were analyzed for viable counts of lactic cultures. 10 mL of curd samples was serially diluted in 90 mL of sterile normal saline (0.85%). Second dilutions onward were performed in one ml of sample and 9 mL of sterile distilled water up to desired number of dilutions in order to get isolated colonies. One mL of last four diluents was used in duplicates separately for pour plating using MRS agar. All the plates were incubated at 37 ºC for 48 to 72 hours and lactic count was established through indirect counting using a colony counter (Digital Colony Counter, Sii SERWELL, Bangalore, India) multiplied by the dilution factor.

4.0. RESULTS AND DISCUSSIONS

The present study was an attempt for phenotypic, genotypic and beneficial attributes characterization of lactobacilli strains isolated from traditionally prepared curd (*dahi*) obtained from various geo-climatic conditions of Nepal. Various microbiological and biochemical tools were used to isolate, screen and obtain lactic acid bacteria. Following phenotypic identification, study was focused on the dominant *Lactobacillus* species, where molecular tools were used to identify and study their genetic diversity based on geo-climatic conditions. In total, 120 *Lactobacillus* strains belonging to ten different strains were obtained. Of the 120 lactic cultures, 24 were further selected for *in vitro* probiotic attributes study. Probiotic attributes such as gastrointestinal transit resistance (at pH 3 & 0.3% bile and pH 2 & 0.3% bile), adhesion to Caco-2 cells, antimicrobial activity against enteric pathogens and antibiotic susceptibility testing was carried out. Further, two strains identified as *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 having good *in vitro* probiotic properties was further used to develop Probiotic curd (*dahi*) and Sweet curd (*dahi*). This section describes the stepwise experimental results obtained and an effort has been made to discuss the findings.

4.1. Results

4.1.1. Isolation, biochemical and molecular characterization *Lactobacillus* spp. from traditionally prepared curd (*dahi*) at different altitudes in Nepal

4.1.1.1. Geographical details of sample

Traditionally prepared curd samples were collected from four districts, two each from the Hilly and the Himalayan regions. The sampling details with geographical

Table 4.1. Sample collection details (altitude, latitude, longitude and temperature of the sample) of Gorkha District (Manaslu Conservation Area).

Sample	Altitude (m)	Latitude	Longitude	Temp. (°C)	Collected site
GHM1	3718	28°30'12.5"	84°48'32.1"	17.2	Kaaltal , Manaslu Conservation Area (MCA), Gorkha
GHM2	3704	28°30'8.1"	84°48'29.7"	17.2	
GHM3	3721	28°30'2.3"	84°48'26.7"	17.0	
GHM4	3769	28°29'58.7"	84°48'26.2"	17.0	
GHM5	3791	28°29'57.8"	84°48'27.6"	17.0	
GHM6	3627	28°33'36.5"	84°41'50.3"	17.1	
GHM8	3615	28°30'12.2"	84°46'41.1"	17.1	

positioning system (GPS) readings, altitude and the temperature of the sample at the time of collection are given in Table 4.1, 4.2, 4.3, 4.4. The samples collected in the sterile plastic container were preserved in ice-box and transported to Molecular Biotechnology Laboratory, NAST, Khumaltar, Lalitpur and preserved at 4 ºC until subsequent experiments.

Table 4.2. Sample collection details (altitude, latitude, longitude and temperature of the sample) of Lalitpur District.

Sample	Altitude m)	Latitude	Longitude	Temp. (°C)	Collected site
LHL1	1333	27°39'26.12"	85°19’41.10"	21.2	Khumaltar
LHL2	1332	27°39'26.44"	85°19’43.56"	22.2	
LHL3	1345	27°39'21.12"	85°19’18.90"	20.1	
LHL4	1349	27°39'18.54"	85°19’16.91"	20.4	

Table 4.3. Sample collection details (altitude, latitude, longitude and temperature of the sample) of Bhaktapur District.

Sample	Altitude (m)	Latitude	Longitude	Temp. (°C)	Collected site
BHL1	1313	27°39.956'	085°25.469'	20.9	Suryabinayak, Jagati, Adarsha Dattatraya, Chyamasignh, Kamalbinayak, Muldhoka, Mahalaxmi and Mahakali of Bhaktapur District
BHL2	1320	27°39.996'	085°26.007'	21.1	
BHL3	1316	27°40.001'	085°26.206'	20.9	
BHL4	1318	27°40.039'	085°25.009'	21.4	
BHL5	1324	27°40.410'	085°26.298'	20.9	
BHL6	1338	27°40.557'	085°26.226'	21.2	
BHL7	1342	27°40.605'	085°26.116'	20.2	
BHL8	1344	27°40.555'	085°25.908'	21.7	
BHL9	1343	27°40.567'	085°26.040'	20.5	

Table 4.4. Sample collection details (altitude, latitude, longitude and temperature of the sample) of Rasuwa District.

Sample	Altitude (m)	Latitude	Longitude	Temp. (°C)	Collected site
RHM1	3019	28°06'991"	85°21'864"	18.1	Sampe RHM1-RHM16 represents samples collected from Mukharka, Lauribinayak of Rasuwa District
RHM2	3020	28°06'993"	85°21'936"	17.9	
RHM3	3017	28°06'958"	85°21'975"	17.9	
RHM4	2966	28°07'0.8"	85°21'50.3"	17.8	
RHM5	2874	28°07'05.9"	85°21'06.0"	17.9	
RHM6	2953	28°07'11.0"	85°21'47."	18.0	
RHM7	2908	28°07'15.5"	85°21'35.5"	18.0	
RHM8	2862	28°07'22.6"	85°21'31.3"	17.8	
RHM9	2773	28°07'32.2"	85°21'31.9"	17.8	
RHM10	2716	28°07'41.3"	85°21'27.8"	17.9	
RHM11	2961	28°07'07.1"	85°21'58.1"	17.9	

RHM12	2971	28°07'06.3"	85°22'56"	18.7	
RHM13	2948	28°07'06.7"	85°22'06.1"	18.6	
RHM14	2959	28°07'07.1"	85°21'56.1"	17.9	
RHM15	3977	28°5'32,17"	85°23'7.28"	17.8	
RHM16	3693	28°6'9.43"	85°22'1.30"	17.8	
RHL17	1696	27°59'57.7"	85°13'35.6"	18.9	Sample RHL17 to RHL24 represents samples collected from Kalika Sthan, Janglang VDC, Rasuwa District
RHL18	1696	27°59'57.7"	85°13'35.6"	18.9	
RHL19	1731	27°59'56.9"	85°13'36.5"	19.8	
RHL20	1731	27°59'56.9"	85°13'36.5"	19.8	
RHL21	1730	27°59'57.2"	85°13'36.4"	19.8	
RHL22	1730	27°59'57.2"	85°13'36.4"	19.8	
RHL23	1717	27°59'57"	85°13'35.5"	19.7	
RHL24	1717	27°59'57"	85°13'35.5"	19.7	

4.1.1.2. Isolation, microscopic and biochemical characterization of LAB

Total mesophilic count of LAB on MRS agar medium is listed below in Table 4.5. All the curd samples collected were indigenous product (*Dahi*) produced by traditional method from different geographical regions. Colonies showing different morphological characteristics (colour, shape, size, etc.) were sub-cultured in MRS

Table 4.5. Details of sampling site, animal source of milk products and enumeration of viable LAB.

S.N.	Sampling Site	Region	Altitude (m)	Milk from Animal	No. of Samples (Replicate)	Range of LAB count (Log CFU/mL)	Mean of total samples (Log CFU/mL)	Total No. of LAB
1	Gorkha (MCA)	Himal	3615-3791	Cow	8	6.3 – 8.1	7.94±0.90	73
2	Rasuwa (>3000 m)	Himal	3017-3977	Chauri	4	6.9 – 8.2	7.85±0.72	23
3	Rasuwa (<3000 m)	Pahad (Hill)	1717-1731	Buffalo	7	7.2 – 9.4	7.88±0.35	20
4	Lalitpur	Pahad (Hill)	1330-1366	Buffalo	4	9.2 – 10.4	9.25±1.20	14
5	Bhaktapur	Pahad (Hill)	1342-1357	Cow & Buffalo	9	7.3 – 8.4	7.04±0.80	63
	Total				32			193

broth and pure cultures were developed on MRS agar medium (pH 5.5). Only Gram positive non motile bacilli and catalase negative isolates were considered for further study. All together 120 lactobacilli isolates were obtained from 64 curd samples (32 duplicate samples). Glycerol stock (40%) preparation of all 120 isolates was carried out and preserved at – 20 °C (Table 4.6).

Table 4.6. *Lactobacillus* isolates (gram positive rods).

S.N	District	*Lactobacillus* isolates	Remarks
1.	Gorkha	42	preserved at – 20 °C in glycerol stock in
2.	Rasuwa (Himalayan region)	14	
3.	Rasuwa (Hilly region)	14	
4.	Lalitpur	10	
5.	Bhaktapur	40	
	Total	120	

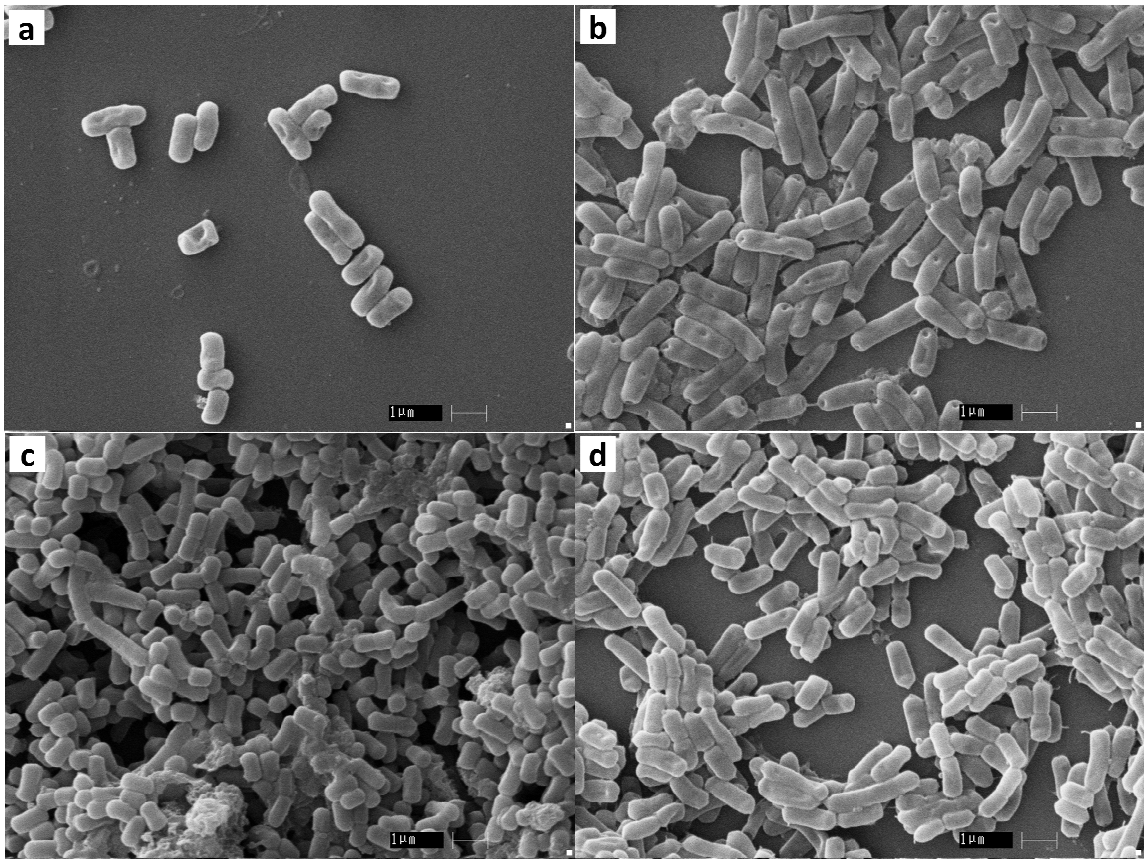

Figure 4.1. Scanning electron microscopy (40×10^3 × magnification): a) *L. fermentum,* b) *L. rhamnosus,* c) *L. parabuchneri*, d) *L. paracasei*.

The scanning microscopic photographs of representative *Lactobacillus* species (identified after molecular characterization) are shown below (Figure 4.1, 4.2 and 4.3) in which significant differences in the morphology can be observed despite of the

same genus. The morphological study could be useful for metabolism study with respect to the probiotic attributes (Varadaraj et al., 1993).

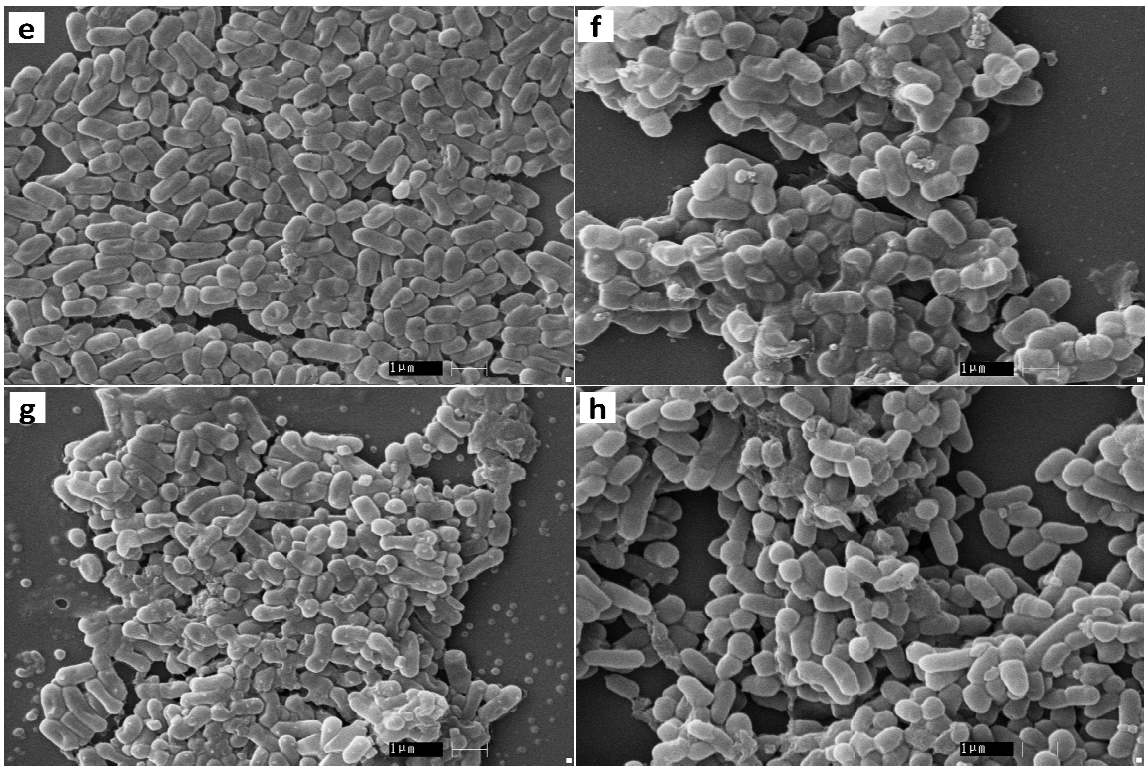

Figure 4.2. Scanning electron microscopy (40×10^3 × magnification): e) *L. brevis*, f) *L. plantarum* g) *L. coryniformis*, h) *L. harbinensis.*

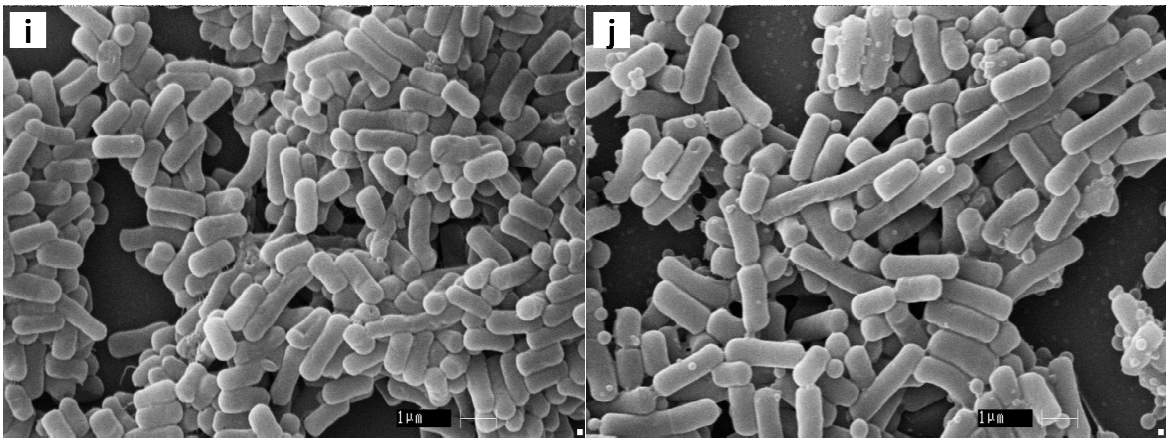

Figure 4.3. Scanning electron microscopy (40×10^3 × magnification): i) *L.delbru eckii* subsp. *bulgaricus*, j) *L. helveticus*.

4.1.1.3. Molecular characterization of the isolates

4.1.1.3.1. Genetic identification of the LAB isolates based on RSA profiling, DNA sequencing, phylogenetic analysis and species specific PCR

A total of 120 isolates were used for molecular analysis. A first grouping step was reached by PCR amplification of the 16-23S rRNA Spacer Region (RSA). Altogether ten different profiles characterized by three bands of about 300-550 bp molecular weight were obtained following electrophoresis of 120 isolates (Figure 4.4a).

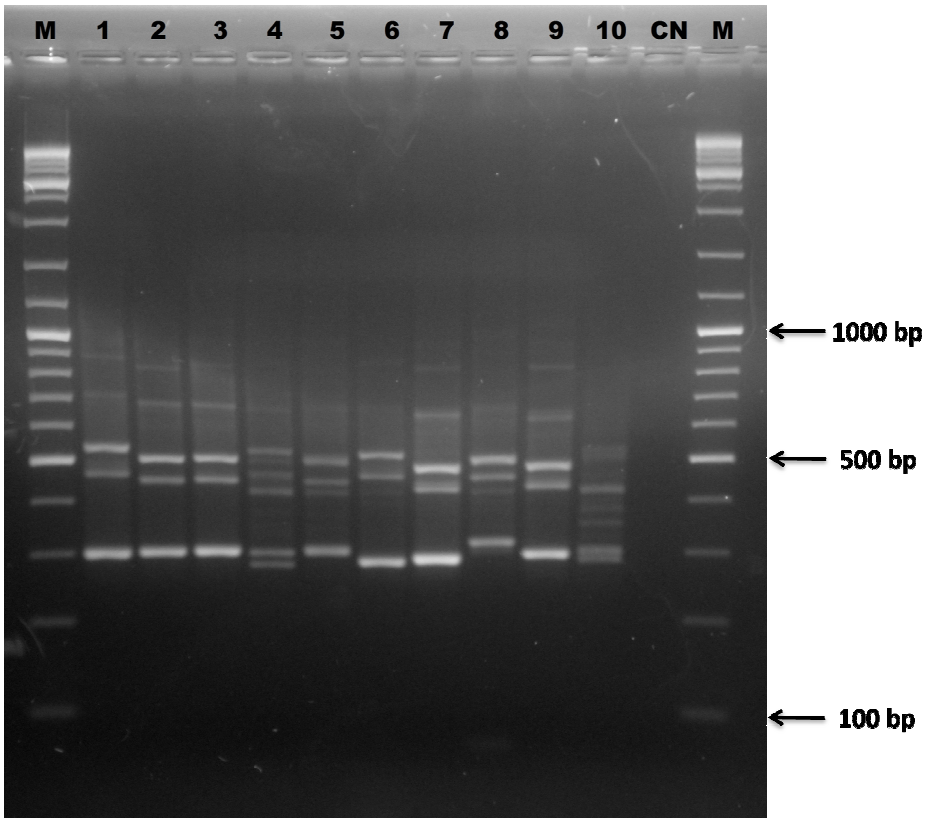

Figure 4.4a. ITS profiles obtained from representative lactobacilli isolates from traditionally prepared curd samples: Lane marked M is 100 bp ladder molecular weight marker and Lane 1-10 represents different *Lactobacillus* species; Lane 1 (*Lactobacillus delbrueckii*), Lane 2 (*Lactobacillus paracasei*), Lane 3 (*Lactobacillus rhamnosus*), Lane 4 (*Lactobacillus fermentum*), Lane 5 (*Lactobacillus parabuchneri*), Lane 6 (*Lactobacillus helveticus*), Lane 7 (*Lactobacillus brevis*), Lane 8 (*Lactobacillus harbinensis*), Lane 9 (*Lactobacillus coryniformis*) and Lane 10 (*Lactobacillus plantarum*).

Representative strains of these 10 clusters were subjected to 16S rRNA gene sequencing where 10 different species of LAB were identified. The sequence data obtained were compared with the GenBank sequences which allowed identification of all the representative isolates from the RSA migration profile (Figure 4.4a). In an

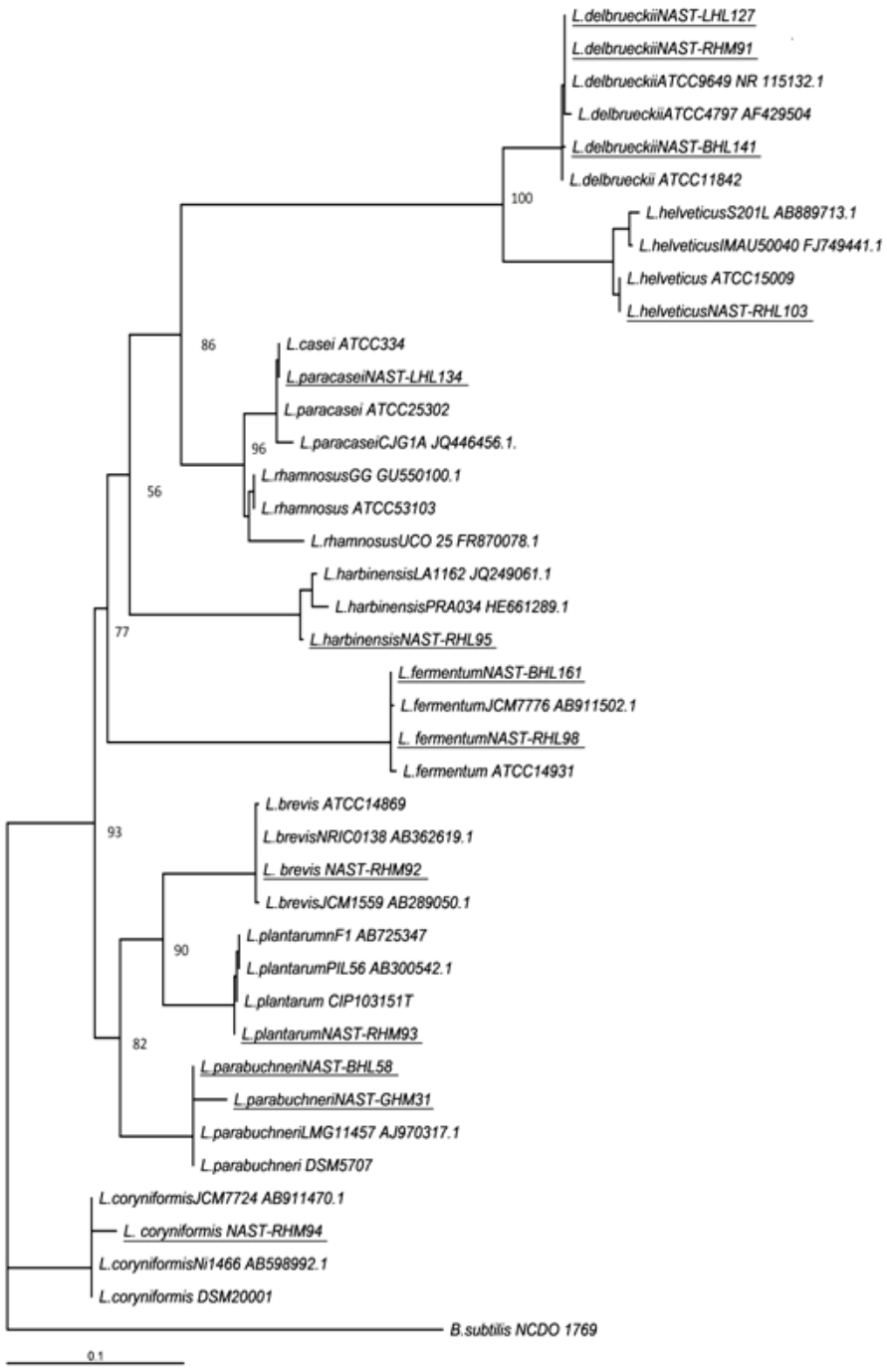

Figure 4.4b. Phylogenetic tree showing the relative positions of *Lactobacillus* spp. isolated from Nepalese curd (underlined) as inferred from the neighbor-joining method of phylogenetic analysis based on partial 16S rDNA sequences. Numerical values shown in the nodes represent Bootstrap values. References of the type strains used in the analysis are given with their respective gene bank accession numbers. *B. subtilis* is used as an out group.

effort to identify lactobacillus from curd at species level, molecular phylogeny analysis was conducted and phylogenetic tree was constructed based on partial 16S rDNA sequences from evolutionary distances by the neighbor-joining method.

Following phylogenetic analysis, lactobacilli isolates from Nepalese curd formed distinct cluster (reported with bootstrap values) with respective *Lactobacillus* species obtained from Genbank database (Figure 4.4b).

Table 4.7. Diversity of *Lactobacillus* species obtained from samples collected from Hilly (HL) and Himalayan (HM) region.

S.N.	Lactobacilli identified	Gorkha (MCA, HM)	Rasuwa (HM)	Rasuwa (HL)	Lalitpur (HL)	Bhaktapur (HL)	Total
1	*Lactobacillus paracasei*	23	4	-	3	-	30
2	*Lactobacillus rhamnosus*	12	-	-	-	9	21
3	*Lactobacillus delbrueckii*	-	7	10	6	23	46
4	*Lactobacillus fermentum*	4	-	1	1	6	12
5	*Lactobacillus parabuchneri*	3	-	-	-	2	5
6	*Lactobacillus helveticus*	-	-	2	-	-	2
7	*Lactobacillus brevis*	-	1	-	-	-	1
8	*Lactobacillus coryniformis*	-	1	-	-	-	1
9	*Lactobacillu harbinensis*	-	-	1	-	-	1
10	*Lactobacillus plantarum*	-	1	-	-	-	1
BACTERIAL DISTRIBUTION		4/42	5/14	4/14	3/10	4/40	10/120

All other isolates were also identified by specific PCR (Figure 4.6, 4.7 and 4.8) as *Lactobacillus delbrueckii* (46 isolates, cluster 1) (Figure 4.3i), *Lactobacillus paracasei* (30 isolates, cluster 2) (Figure 4.1d), *Lactobacillus rhamnosus* (21 isolates, cluster 3) (Figure 4.1b), *Lactobacillus fermentum* (12 isolates, cluster 4) (Figure 4.1a), *Lactobacillus parabuchneri*, (5 isolates, cluster 5) (Figure 4.1c), *Lactobacillus helveticus* (2 isolates, cluster 6) (Figure 4.3j), *Lactobacillus brevis* (1 isolate, cluster 7) (Figure 4.2e), *Lactobacillus harbinensis* (1 isolate, cluster 8) (Figure 4.2h), *Lactobacillus coryniformis* (1 isolate, cluster 9) (Figure 4.2g) and *Lactobacillus plantarum* (1 isolate, cluster 10) (Figure 4.2f). An identification detail with respect to sampling sites and number is given in Table 4.7 Figure 4.5.

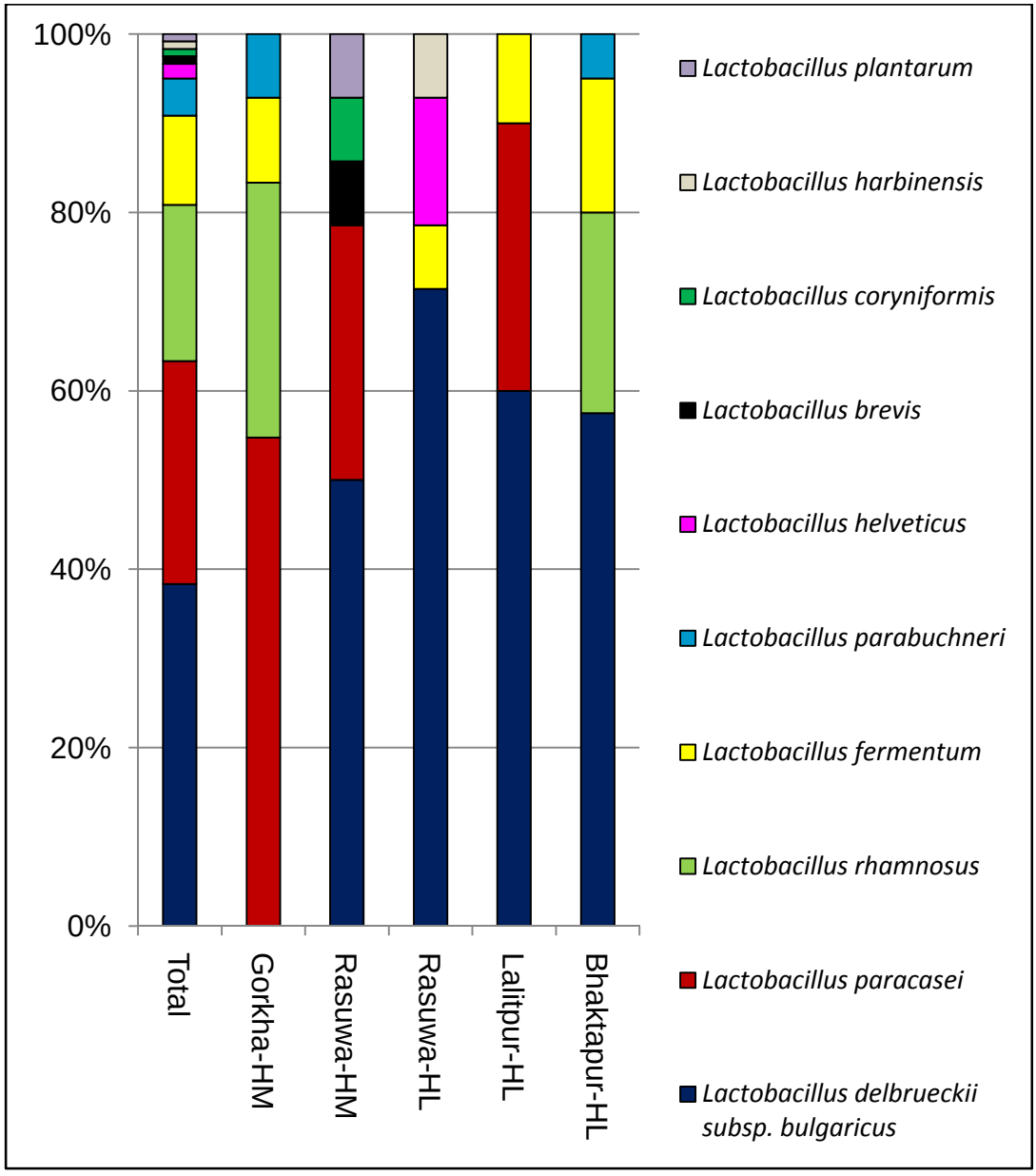

Figure 4.5. Relative distribution of the *Lactobacillus* species identified in curd samples according to the geographical origin. Ten *Lactobacillus* species were obtained in various percentage: *Lactobacillus delbrueckii* (38%), *Lactobacillus paracasei* (25%), *Lactobacillus rhamnosus* (18%), *Lactobacillus fermentum* (10%), *Lactobacillus parabuchneri*, (4%), *Lactobacillus helveticus* (less than 2%), *Lactobacillus brevis* (less than 1%), *Lactobacillus harbinensis* (less than 1%), *Lactobacillus coryniformis* (less than 1%) and *Lactobacillus plantarum* (less than 1%).

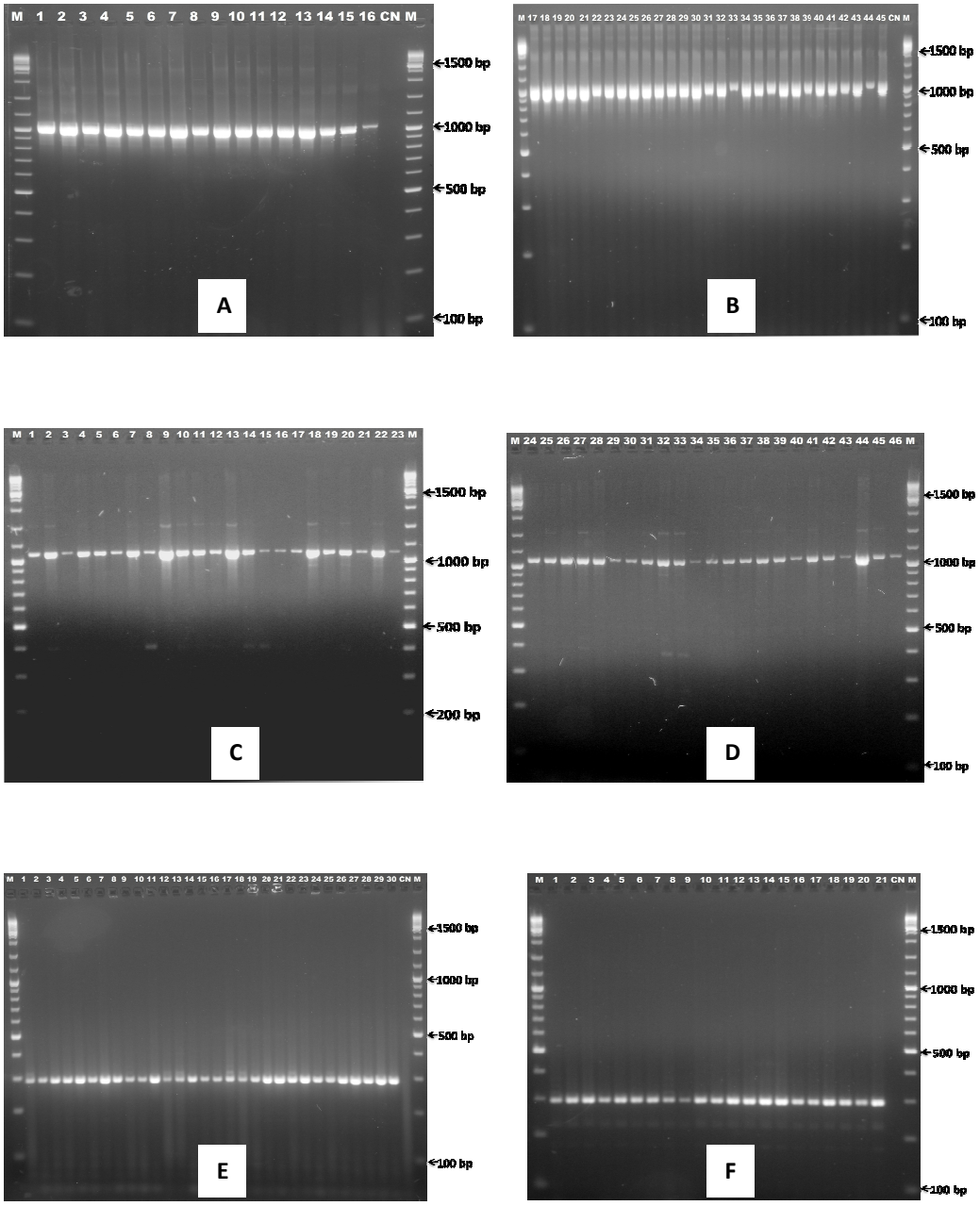

Figure 4.6. Gel picture of species specific PCR profile: A&B) *L. delbrueckii* (n=46), C&D) *L. delbrueckii* subsp. *bulgaricus* (n=46), E) *L. paracasei* (n=30), F) *L. rhamnosus* (n=21).

4.1.1.3.2. Genetic analysis of *Lactobacillus* strains using other PCR based maker tools

Five major groups of strains (*L. delbrueckii, L. paracasei, L. rhamnosus, L. fermentum* and *L. parabuchneri*) were typed by combined analysis of repetitive element PCR (rep-PCR), BOXAIR and RAPD typing which allowed a high discrimination at strain level. The majority of the observed fingerprinting profiles within each group were unique (Figure 4.13, 4.14, 4.15 and 4.16), revealing a high

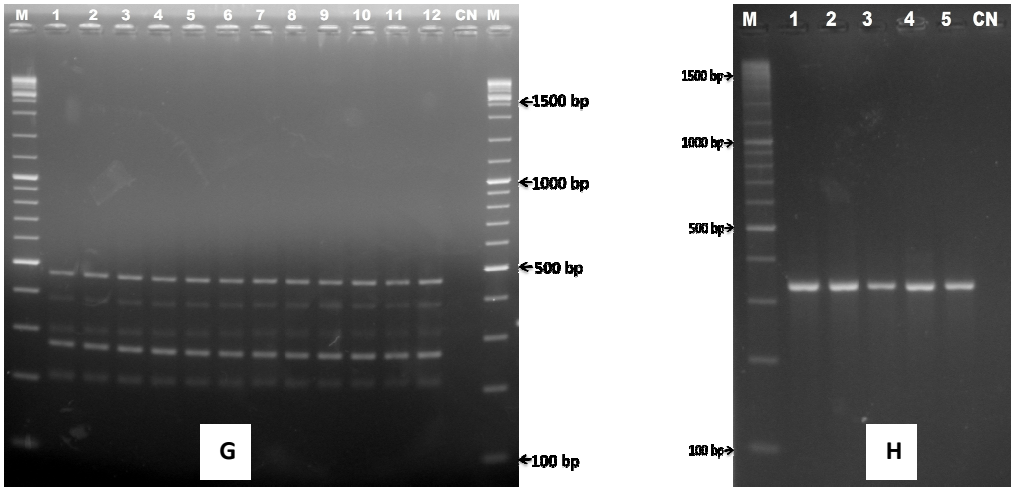

Figure 4.7. Gel picture of species specific PCR profile: G) *L. fermentum* (n=12), H) *L. parabuchneri* (n=5).

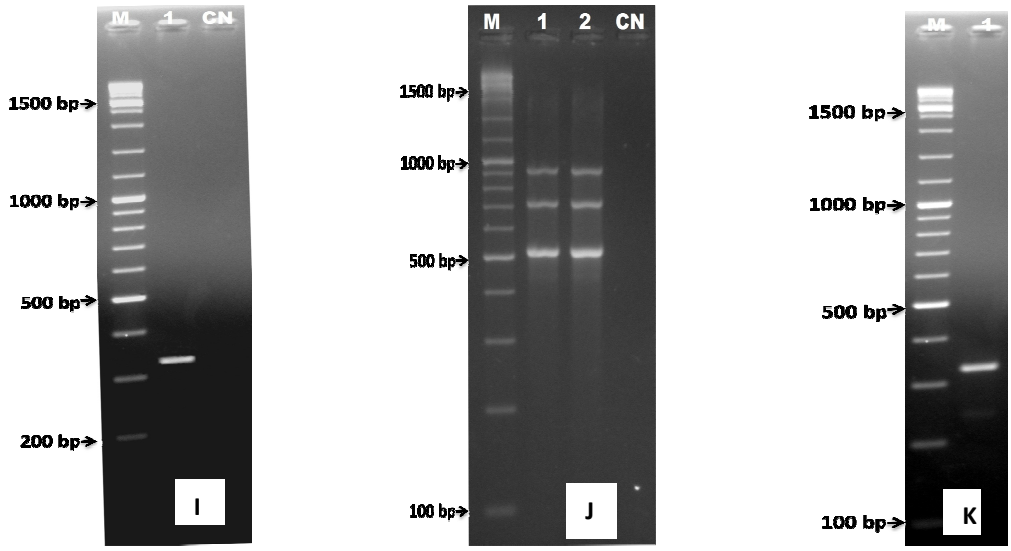

Figure 4.8. Gel picture of species specific PCR profile: I) *L. brevis* (n=1), J) *L. helveticus* (n=2), K) *L. plantarum* (n=1).

degree of genetic differences with respect geographical variation. Although isolated from different samples collected from various geographical regions, *Lactobacillus rhamnosus* isolates has identical/monomorphic fingerprints with the entire tested marker. The experimental results showed that *Lactobacillus delbrueckii* could be considered as major dominant species in Hilly regions whereas in Himalayan region *Lactobacillus paracasei* is dominant. *Lactobacillus rhmnosus* was not found in Rasuwa and Lalitpur districts.

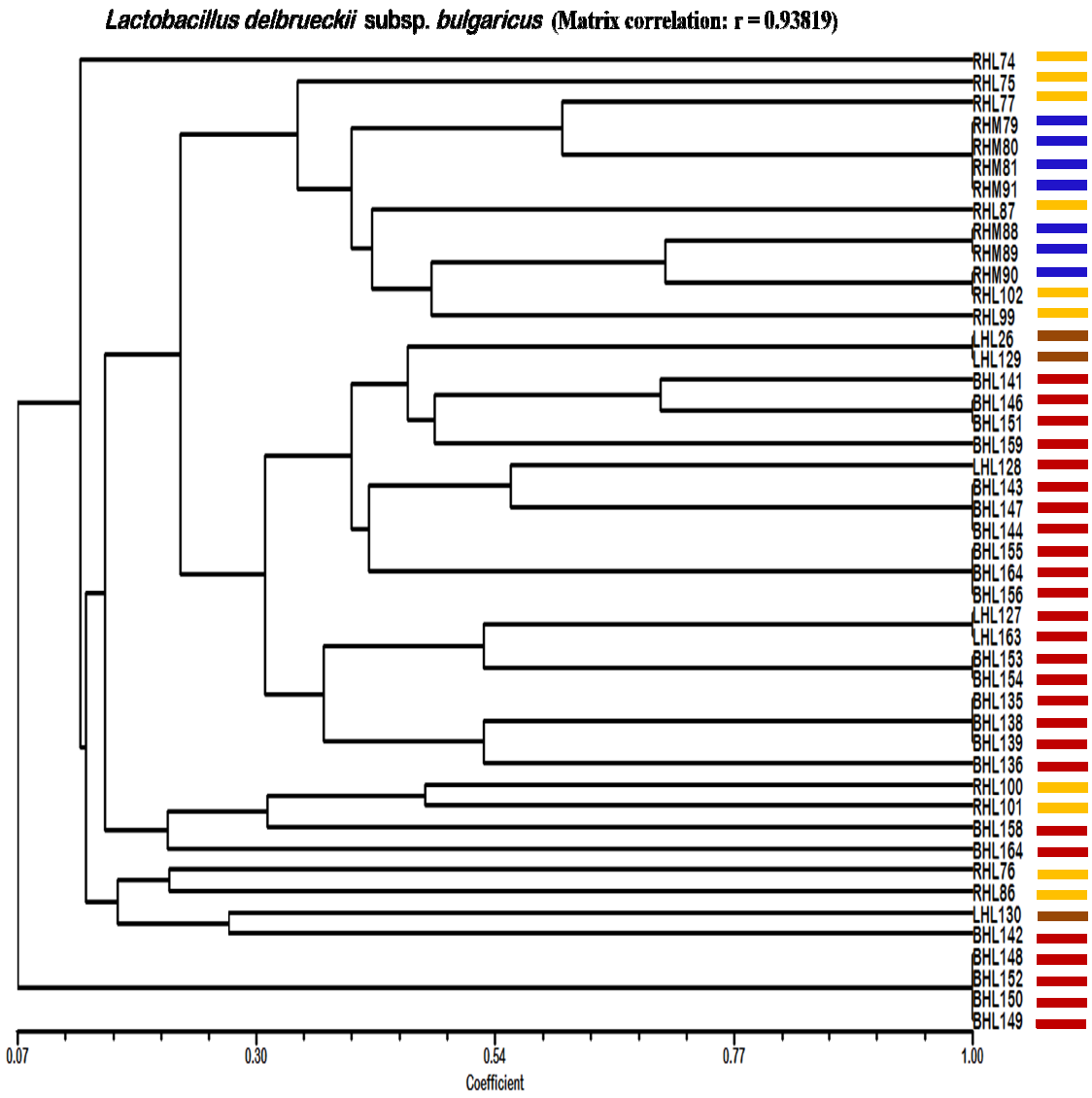

Figure 4.9. Phenogram generated for *Lactobacillus delbrueckii* subsp. *bulgaricus* strains using DNA profiles produced by primers OPI17, OPI02 and M13 using UPGMA algorithm of NTSYS PC software (version 2.11 – Applied Biostatics Inc., NY). LHL = Lalitpur, BHL = Bhaktapur, RHL = Rasuwa, RHM = Rasuwa.

The dendrogram related to each group of isolates, constructed after analysis of the digitized respective fingerprints with the UPGMA method, are shown in Figure 4.9, 4.10, 4.11 and 4.12. Similarity matrices were deduced by means of Simple Matching (SM), Jaccard's (J) and Dice (D) coefficient of similarity using SimQual (Similarity for qualitative data) module of NTSYS pc ver. 2.11. The correlation coefficient value (r) for of Jaccard, Dice and Simple Matching coefficients were not very different in

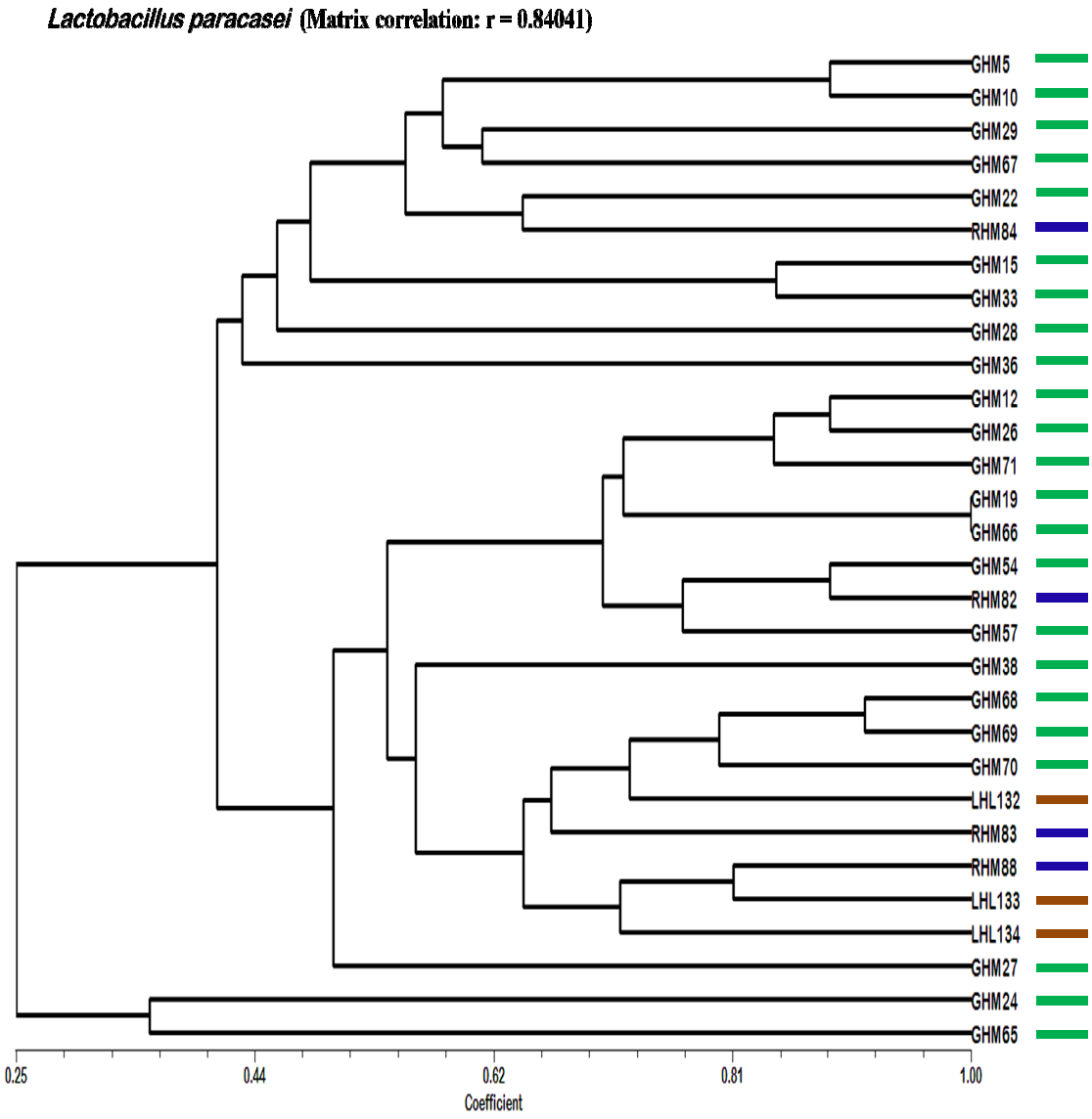

Figure 4.10. Phenogram generated for *Lactobacillus paracasei* strains using DNA profiles produced by primers1254, M13, BOXA1R, and (GTG)5 using UPGMA algorithm of NTSYS PC software (version 2.11 – Applied Biostatics Inc., NY).Strain origin: LHL = Lalitpur, RHM = Rasuwa, GHM = Gorkha.

all four *Lactobacillus* species tested for intra species genetic diversity study. The correlation coefficient values for Jaccard, Dice and Simple Matching coefficients found to be **0.93819**, 0.93027 and 0.92071 for *L. delbrueckii*, **0.84041**, 0.83754 and 0.83092 for *L. paracasei*, **0.94090**, 0.93814 and 0.93801 for *L. fermentum* and **0.92868**, 0.92163 and 0.91807 for *L. parabuchneri* respectively. Assessment of intra-species genetic diversity was carried out using Jaccard's coefficient matrix as it revealed very well fit of similarity matrix to phenogram. The symmetric (similarity)

value ranged from 0.001 to 0.700, 0.158 to 0.889, 0.044 to 0.730 and 0.071 to 0.391 for *L. delbrueckii*, *L. paracasei*, *L. fermentum* and *L. parabuchneri* respectively.

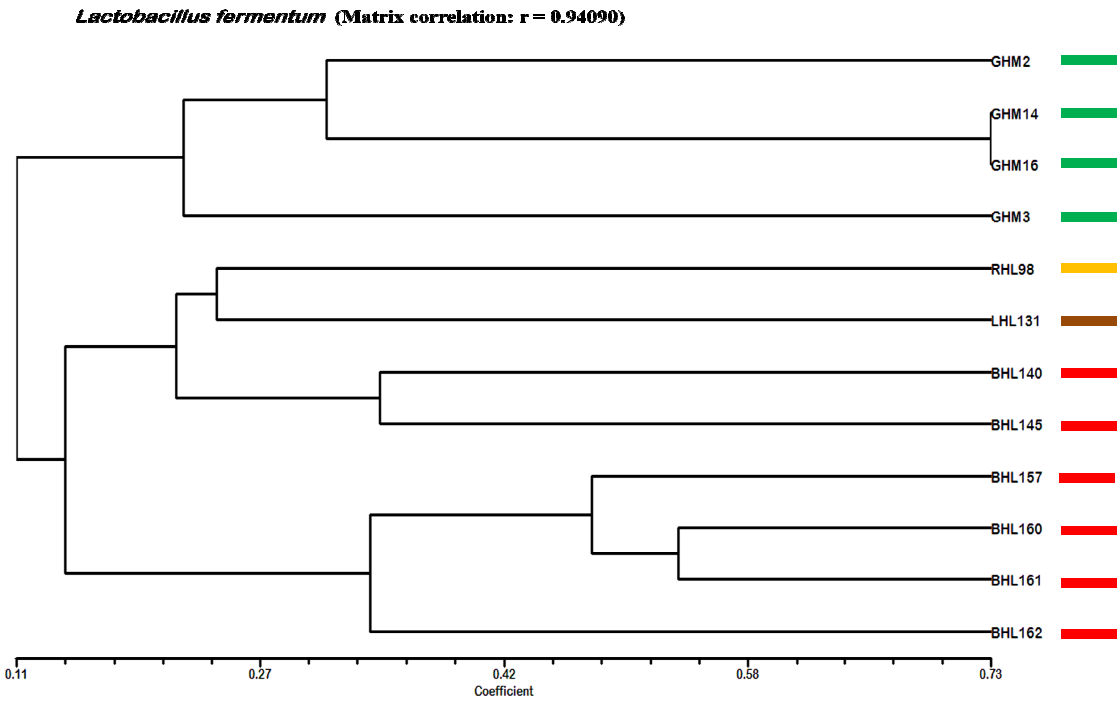

Figure 4.11. Phenogram generated for *Lactobacillus fermentum* strains using DNA profiles produced by primers OPI17, 1254, and M13 using UPGMA algorithm of NTSYS PC software (version 2.11 – Applied Biostatics Inc., NY).Strain origin: LHL = Lalitpur, GHM = Gorkha, RHL = Rasuwa, BHL = Bhaktapur.

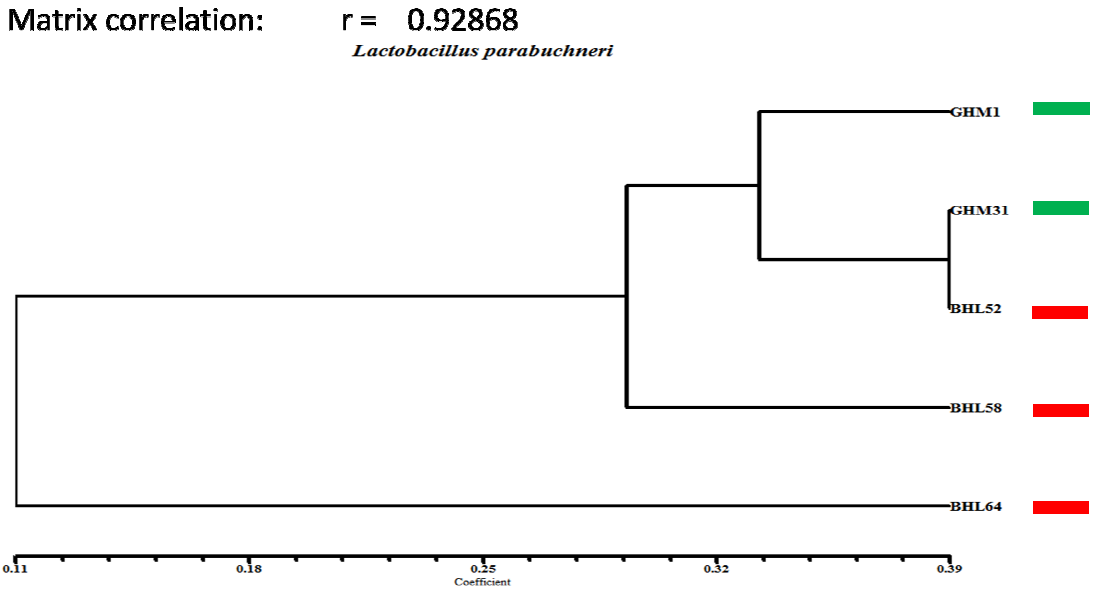

Figure 4.12. Phenogram generated for *Lactobacillus parabuchneri* strains using DNA profiles produced by primers *viz.* OPI17, 1254, and M13 (UPGMA algorithm of NTSYS PC software (version 2.11 – Applied Biostatics Inc., NY). Strain origin: GHM = Gorkha, KVBHL = Bhaktapur.

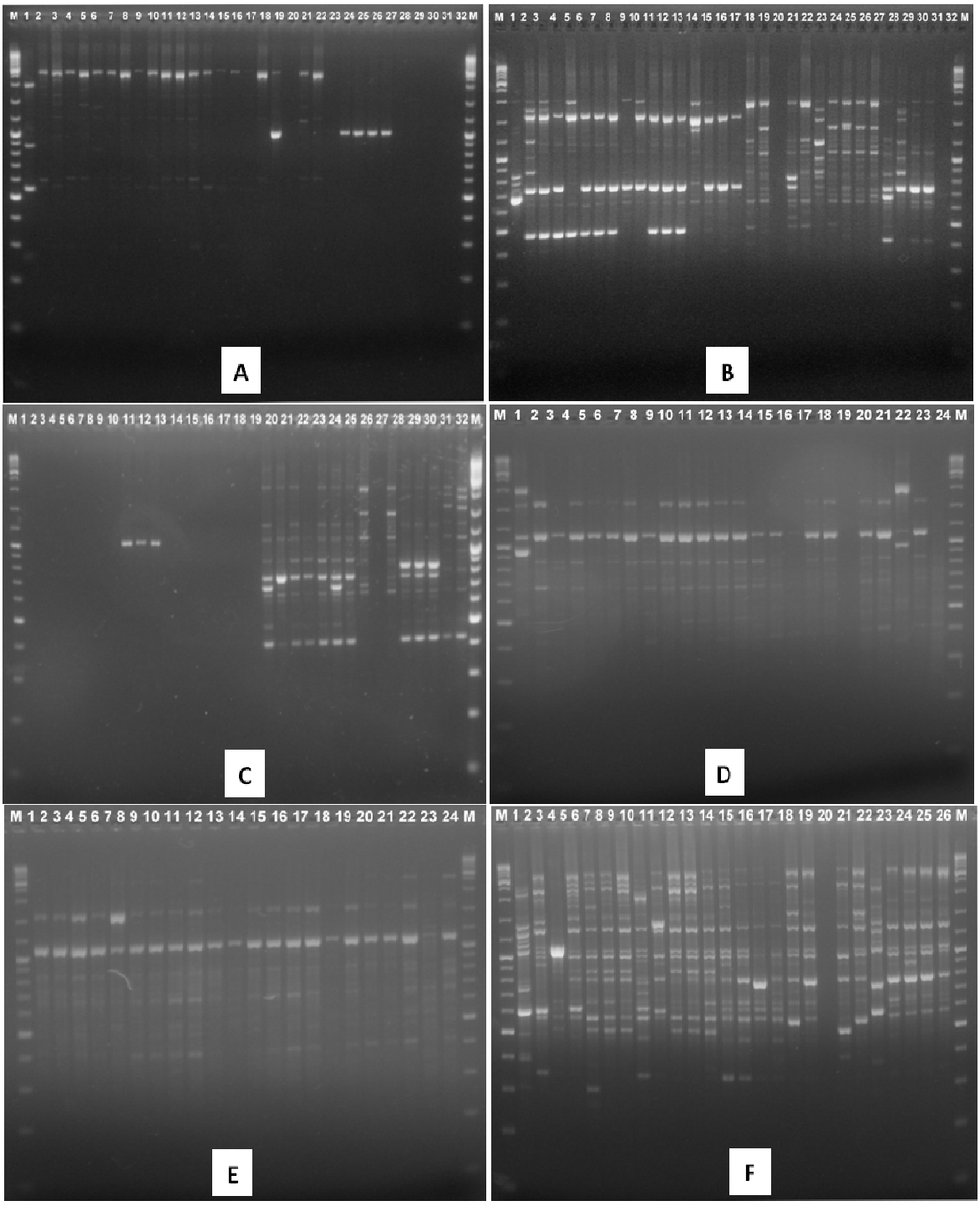

Figure 4.13. PCR profiles generated for *L. delbrueckii* subsp. *bulgaricus* isolates (n=46): A) Lane 1-32 with primer 1254 (1-32 isolates), B) Lane 1-32 with primer M13 (1-32 isolates), C) Lane 1-14 & Lane 19-32 with primer 1254 (33-46 isolates) & M13 (33-46 isolates), D) Lane 1-23 with primer OPI02 (1-23 isolates), E) Lane 1-23 with primer OPI02 (24 to 46) , F) Lane 1-26 with primer OPI017 (1-26 isolates); M represents 100 bp ladder molecular weight marker and CN represents control negative.

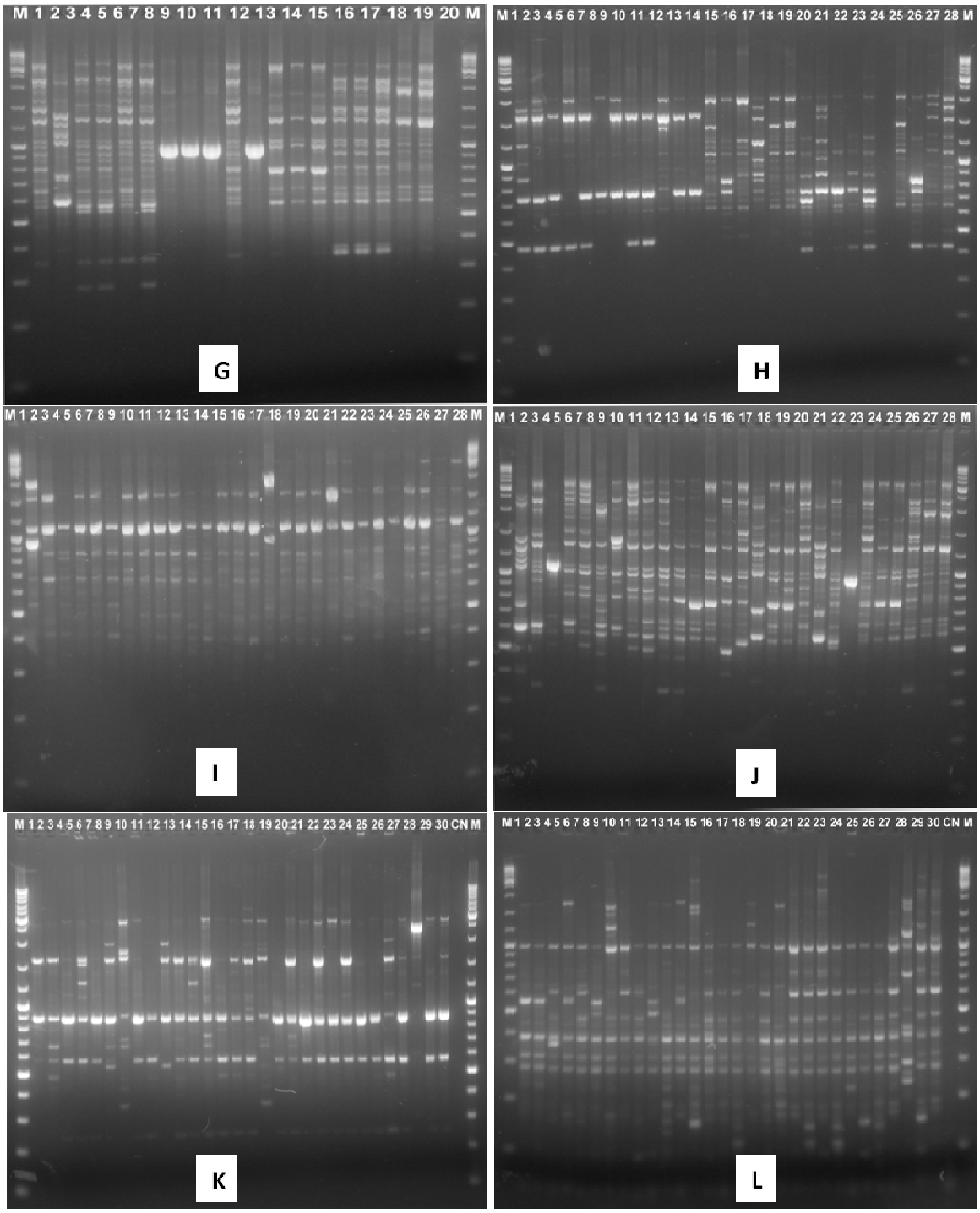

Figure 4.14. PCR profiles generated for *L. delbrueckii* subsp. *bulgaricus* isolates (n=46): G) Lane 1-26 with primer OPI017 (1-26 isolates), *L. delbrueckii* subsp. *bulgaricus* (reduced from 46 to 28 isolates based on previous profile for preparation of score matrix): H) Lane 1-28 with primer M13 (n=28), I) Lane 1-28 with primer OPI02 (n=28), J) Lane 1-28with primer OPI17 (n=28). PCR Profiles for *L. paracasei:* K) Lane 1-30 with primer 1254 (n=30), L) Lane 1-30 with primer BOXAIR and (GTG)5 (n=28); M represents 100 bp ladder molecular weight marker and CN represents control negative.

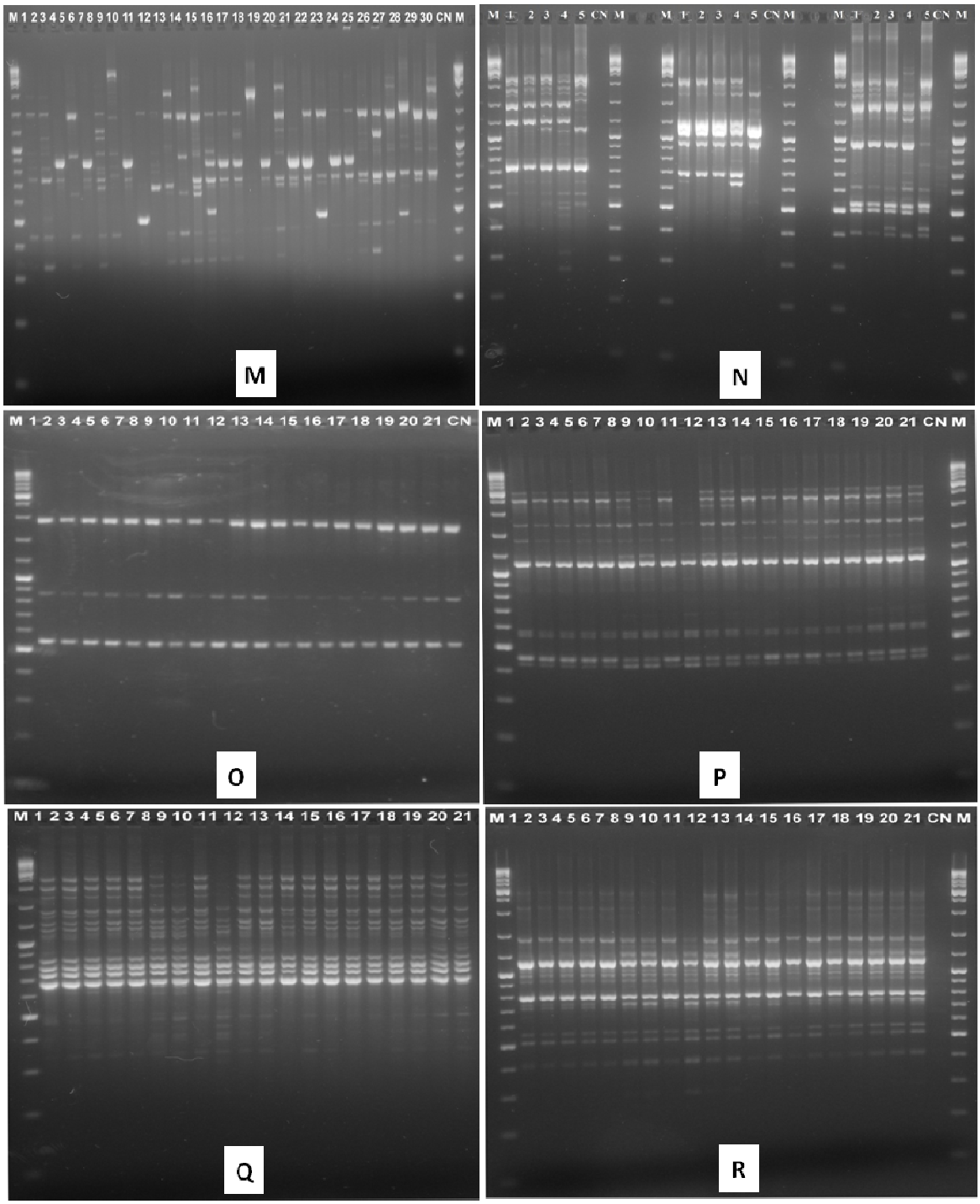

Figure 4.152. PCR profiles generated for *L. paracasei:* M) Lane 1-30 with primer M13 (n=30), N) PCR profile for *L. parabuchneri* with primer 1254, M13, OPI17 (n=5, from left to right in the same gel picture), PCR profile for *L. rhamnosus:* O) Lane 1-21 with primer 1254 (n=21), P) Lane 1-21with primer API02 (n=21), Q) Lane 1-21with primer M13 (n=21), R) Lane 1-21with primer OPI17 (n=21); M represents 100 bp ladder molecular weight marker and CN represents control negative.

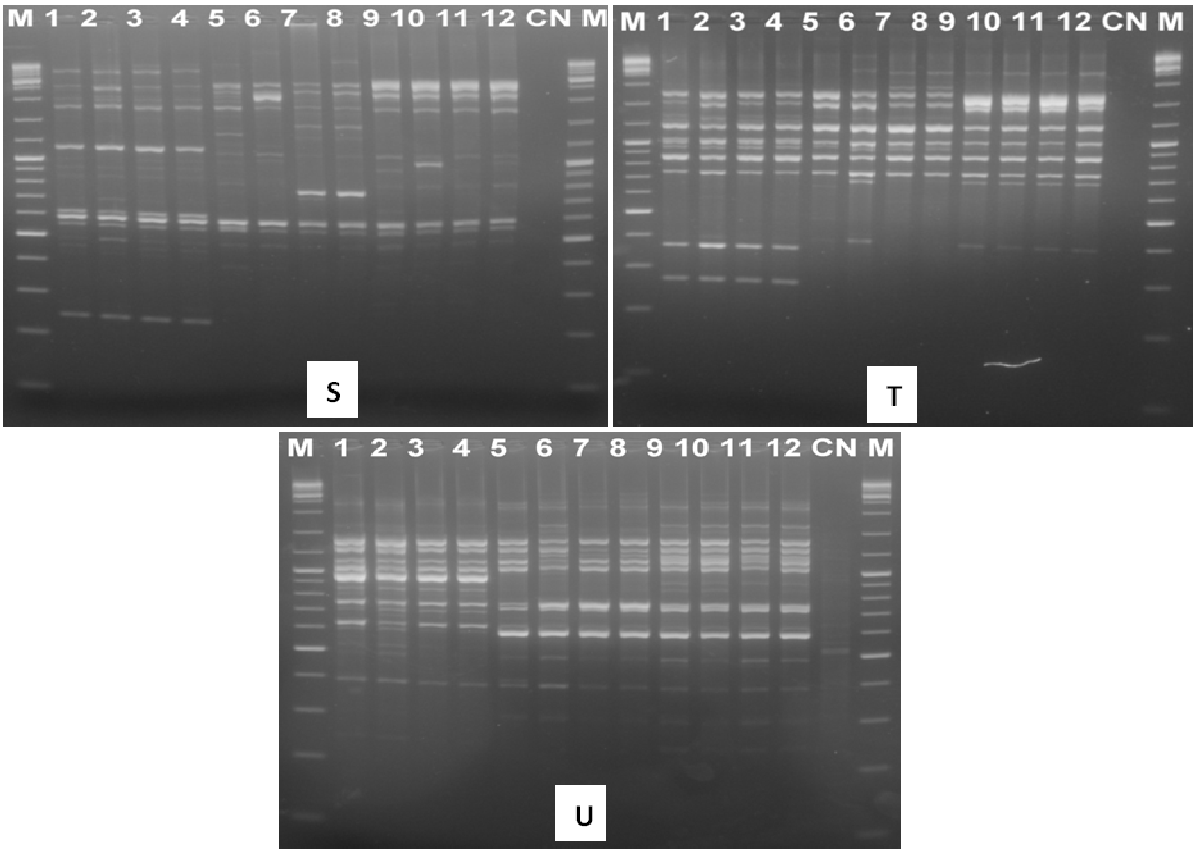

Figure 4.16. PCR profiles generated for: *L. fermentum*: S) Lane 1-12 with primer 1254 (n=12), T) Lane 1-12 with primer M13 (n=12), (U) Lane 1-12 with primer OPI17 (n=12); M represents 100 bp ladder molecular weight marker and CN represents control negative.

4.1.1.3.3. Rapid genotyping of strains of *Lactobacillus casei* group of species by Real time PCR based High Resolution Melt Curve analysis (HRMa)

For this experiment, *L. paracasei* isolates (five representative isolates from different geographical locations) and *L. rhamnosus* isolates (six representative isolates from different geographical locations) together with the reference strains (Koirala et al., 2015) of *L. paracasei* subsp. *tolerans* LMG 9191, *L. casei* (LMG 23516 and 6904^{T}/ATCC 393) and several other Lactobacilli were used. Our experiment showed that GroHMR primer allowed the discrimination among the *L. casei* group of species [Figure 4.17 (A)]. The melt curve analysis of three species were distinct which was not distinct using species specific PCRs. Also, *L. paracasei* isolates were distinguished into two subspecies; *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans* using melt curve analysis [Figure 4.17 (B)]. All representative isolates of *L. paracasei* obtained from traditionally prepared curd (*dahi*) belong to *L. paracasei* subsp. *paracasei*.

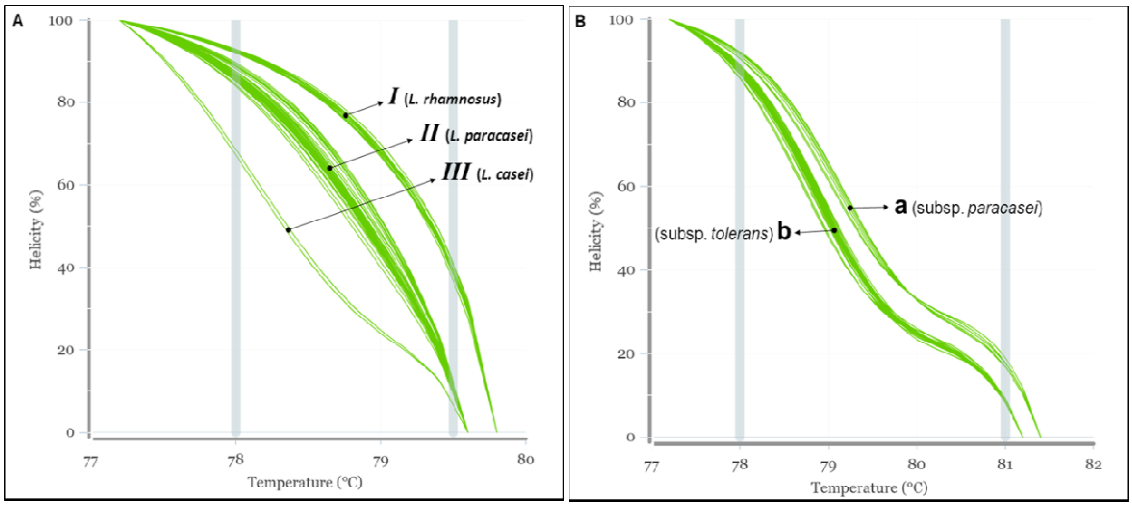

Figure 4.17. High Resolution Melting Curve Analysis (HRMa) of the 150 bp groEL DNA fragment amplified by qPCR from strains belonging to species of the *Lactobacillus casei* group. Panel A: HRMa performed on *Lactobacillus casei* group of species. The group I curves include *L. rhamnosus* strains; group II curves include *L. paracasei* strains; group III curves include *L. casei* strains. Panel B: HRMa performed on *L. paracasei* strains. The cluster of curves "a" includes *L paracasei* subsp. *paracasei* and the cluster of curves "b" include *L. paracasei* subsp. *tolerans*.

4.1.2. *In vitro* probiotic properties of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) obtained from different geo-climatic conditions of Nepal

4.1.2.1. Gastrointestinal transit resistance

Twenty four *Lactobacillus* isolates belonging to ten different species were tested for gastrointestinal transit resistance in acid and bile conditions successively (Table 4.8, Table 4.10). All the isolates were found to be resistant to pH 3 and 0.3% bile with the Log10 CFU/mL ranging from 4.9 to 7.8 (Table 4.8 and Figure 4.18 - 4.27) while only fifteen isolates showed resistant to pH 2 and 0.3% bile (Log10 CFU/mL for survivals ranging from 1.3 to 7.6) (Table 4.9 and Figure 4.18 - 4.27). During gastrointestinal transit resistance at pH 3 and 0.3% bile, *L. fermentum* NAST-GHM2 showed the highest resistance with average viable count of 7.85 (Log10 CFU/mL) at T_{180} while *L. paracasei* NAST-RHM84 showed lowest resistance with average viable count 4.95 (Log10 CFU/mL) at T_{180}. Here, thirteen lactobacilli showed resistance with the average viable count ≥7 (Log10 CFU/mL). Similarly, nine isolates with average viable count ≥6 (Log10 CFU/mL), one isolate with average count ≥5 (Log10 CFU/mL) and one isolate with average count ≥4 (Log10 CFU/mL) showed resistance at different level.

Table 4.8. Viable count (Log10 CFU/mL) of lactic isolates during gastrointestinal transit resistance at pH 3 and bile (0.3%) *L. casei* SHIROTA and *L. rhamnosus* GG were used as the standard organisms for this experiment.

Lactic cultures used for GIT experiment	Average viable count at pH 3 and 0.3% bile					
	Control (T_0)	Test (T_0)	Control (T_{90})	Test (T_{90})	Control (T_{180})	Test (T_{180})
	Log10 CFU/mL	Log10 CFU/mL	Log10 CFU/mL	Log10 CFU/mL	Log 10 CFU/mL	Log10 CFU/mL
L. coryniformis NAST-RHM94	7.54±0.04	7.46±0.08	7.60±0.07	7.64±0.04	7.69±0.01	7.38±0.02
L. plantarum NAST-RHM93	7.77±0.05	7.36±0.02	7.74±0.04	7.79±0.06	7.73±0.03	7.79±0.11
L. brevis NAST-RHM92	7.64±0.04	7.61±0.01	7.53±0.01	7.69±0.04	7.73±0.06	7.63±0.04
L. delbrueckii NAST-BHL138	7.26±0.04	7.16±0.01	6.84±0.08	7.22±0.07	6.74±0.04	6.68±0.11
L. delbrueckii NAST-BHL152	6.85±0.01	6.85±0.07	6.83±0.05	6.86±0.09	6.88±0.09	6.75±0.09
L. delbrueckii NAST-LHL128	7.23±0.10	7.20±0.04	7.17±0.06	7.30±0.01	7.36±0.01	6.43±0.09
L. delbrueckii NAST-RHL101	5.72±0.08	5.88±0.09	6.45±0.04	6.38±0.00	6.66±0.07	6.42±0.03
L. delbrueckii NAST-RHL74	7.33±0.02	7.27±0.08	7.96±0.08	7.93±0.04	7.92±0.06	7.80±0.02
L. delbrueckii NAST-RHL87	7.26±0.07	7.25±0.05	7.21±0.07	7.21±0.07	7.13±0.00	6.66±0.07
L. fermentum NAST-BHL162	7.83±0.02	7.79±0.02	7.83±0.05	7.80±0.09	7.78±0.01	6.85±0.04
L. fermentum NAST-GHM2	7.79±0.05	7.85±0.04	7.19±0.00	7.85±0.01	6.78±0.01	**7.85±0.07**
L. fermentum NAST-RHL98	7.51±0.08	7.57±0.04	7.29±0.20	7.73±0.02	7.17±0.06	7.34±0.02
L. harbinensis NAST-RHL95	7.45±0.04	7.46±0.02	7.53±0.01	7.51±0.08	7.51±0.08	7.51±0.08
L. helveticus NAST-RHL103	6.58±0.05	6.66±0.07	6.62±0.03	6.77±0.08	6.60±0.07	6.78±0.08
L. parabuchneri NAST-BHL58	7.64±0.03	7.63±0.03	7.63±0.03	7.67±0.06	7.68±0.05	7.70±0.03
L. parabuchneri NAST-GHM31	7.71±0.16	7.77±0.08	7.68±0.05	7.78±0.05	7.75±0.05	7.81±0.13
L. paracasei NAST-GHM24	6.78±0.02	6.84±0.41	6.68±0.25	6.86±0.06	6.81±0.06	6.40±0.16
L. paracasei NAST-GHM5	7.56±0.08	8.11±0.03	7.65±0.03	8.14±0.06	**8.20±0.04**	7.66±0.02
L. paracasei NAST-LHL132	7.57±0.04	7.61±0.08	7.59±0.01	7.64±0.03	7.77±0.08	6.62±0.03
L. paracasei NAST-LHL133	7.14±0.07	6.87±0.02	7.49±0.01	6.88±0.10	7.14±0.07	5.30±0.01
L. paracasei NAST-RHM82	7.50±0.05	7.50±0.05	7.36±0.01	7.41±0.04	7.32±0.02	7.32±0.02
L. paracasei NAST-RHM84	7.49±0.01	7.55±0.01	6.92±0.06	7.58±0.05	6.90±0.01	**4.95±0.06**
L. rhamnosus NAST-BHL6	7.68±0.05	7.69±0.04	7.79±0.05	7.67±0.06	7.82±0.10	7.67±0.02
L. rhamnosus NAST-GM25	7.72±0.04	7.68±0.05	7.73±0.06	7.72±0.10	7.75±0.05	7.75±0.09
L. casei SHIROTA	5.73±0.02	5.76±0.05	5.72±0.01	5.73±0.02	**5.73±0.04**	5.72±0.06
L. rhamnosus GG	5.95±0.04	6.23±0.10	6.39±0.07	6.23±0.01	6.38±0.20	6.34±0.02

In case of pH 2 and 0.3% bile, the strain *L. fermentum* NAST-GHM2 showed the highest resistance with average viable count 7.66 (Log10 CFU/mL) where as *L. plantarum* NAST-RHM93 showed least resistance with average viable count 1.69

Table 4.9. Viable count (Log10 CFU/mL) of lactic isolates during gastrointestinal transit resistance at pH 2 and bile (0.3%) *L. casei* SHIROTA and *L. rhamnosus* GG were used as the standard organisms for this experiment.

Lactic cultures used for GIT experiment	Average viable count at pH 2 and 0.3% bile					
	Control (T_0)	Test (T_0)	Control (T_{90})	Test (T_{90})	Control (T_{180})	Test (T_{180})
	Log10 CFU/mL	Log10 CFU/mL	Log10 CFU/mL	Log10 CFU/mL	Log 10 CFU/mL	Log10 CFU/mL
L. coryniformis NAST-RHM94	8.25±0.05	8.13±0.20	8.02±0.01	0.00±0.00	8.20±0.04	0.00±0.00
L. plantarum NAST-RHM93	7.66±0.07	7.74±0.08	7.70±0.03	1.69±0.08	7.19±0.00	1.69±0.08
L. brevis NAST-RHM92	7.76±0.03	7.77±0.04	7.80±0.06	1.00±0.10	7.60±0.02	2.17±0.06
L. delbrueckii NAST-BHL138	7.23±0.10	7.19±0.10	7.07±0.09	0.00±0.00	7.27±0.08	0.00±0.00
L. delbrueckii NAST-BHL152	7.14±0.06	7.29±0.15	7.25±0.05	0.00±0.00	**8.13±0.00**	0.00±0.00
L. delbrueckii NAST-LHL128	7.21±0.07	7.14±0.06	7.20±0.04	0.00±0.00	7.34±0.02	0.00±0.00
L. delbrueckii NAST-RHL101	6.40±0.06	6.77±0.10	6.30±0.01	0.00±0.00	6.51±0.08	0.00±0.00
L. delbrueckii NAST-RHL74	7.49±0.08	7.67±0.02	7.51±0.01	7.39±0.07	7.50±0.05	6.45±0.04
L. delbrueckii NAST-RHL87	6.69±0.10	6.65±0.03	6.68±0.05	0.00±0.00	6.71±0.06	0.00±0.00
L. fermentum NAST-BHL162	7.55±0.10	7.63±0.03	7.50±0.05	7.50±0.05	7.51±0.08	6.29±0.00
L. fermentum NAST-GHM2	7.47±0.07	7.63±0.03	7.55±0.12	7.70±0.07	7.60±0.07	**7.66±0.07**
L. fermentum NAST-RHL98	6.72±0.04	7.35±0.03	6.74±0.04	7.61±0.08	6.34±0.02	7.13±0.00
L. harbinensis NAST-RHL95	7.34±0.02	7.26±0.07	7.32±0.02	2.30±0.01	7.38±0.09	2.39±0.07
L. helveticus NAST-RHL103	6.55±0.20	6.77±0.08	6.66±0.07	3.11±0.03	6.49±0.08	3.13±0.00
L. parabuchneri NAST-BHL58	7.67±0.02	7.58±0.05	7.57±0.09	0.00±0.00	7.62±0.08	0.00±0.00
L. parabuchneri NAST-GHM31	7.67±0.02	7.63±0.03	7.60±0.07	0.00±0.00	7.66±0.02	0.00±0.00
L. paracasei NAST-GHM24	6.52±0.05	6.56±0.08	6.57±0.04	2.47±0.07	6.51±0.08	2.47±0.07
L. paracasei NAST-GHM5	7.72±0.30	7.72±0.08	7.71±0.01	2.74±0.00	7.82±0.02	2.47±0.07
L. paracasei NAST-LHL132	7.34±0.02	7.38±0.09	7.31±0.01	2.17±0.06	7.43±0.09	2.00±0.00
L. paracasei NAST-LHL133	7.23±0.10	7.13±0.00	7.20±0.04	2.30±0.01	7.25±0.05	2.00±0.00
L. paracasei NAST-RHM82	7.25±0.05	7.31±0.01	7.30±0.01	3.14±0.06	7.45±0.03	2.87±0.05
L. paracasei NAST-RHM84	7.52±0.05	7.60±0.07	7.62±0.03	2.30±0.01	7.66±0.07	2.00±0.00
L. rhamnosus NAST-BHL6	7.69±0.04	7.68±0.01	7.76±0.03	0.00±0.00	7.81±0.02	0.00±0.00
L. rhamnosus NAST-GM25	7.69±0.04	7.68±0.05	7.78±0.01	2.60±0.02	7.83±0.05	2.92±0.09
L. casei SHIROTA	5.85±0.01	5.84±0.05	5.90±0.03	2.36±0.01	**5.85±0.01**	**1.30±0.01**
L. rhamnosus GG	6.00±0.40	5.95±0.04	6.07±0.09	3.87±0.05	6.11±0.03	1.61±0.00

(Log10 CFU/mL) (Table 4.9). Here, two lactobacilli showed resistance with the average viable count ≥7 (Log10 CFU/mL). Similarly, two isolates with average count ≥6 (Log10 CFU/mL), one isolate with average viable count ≥3 (Log10 CFU/mL),

nine isolate with average count ≥2 (Log10 CFU/mL) and one isolate with average viable count ≥1 (Log10 CFU/mL).The average viable count of two standard probiotic organisms *viz*. *Lactobacillus casei* SHIROTA and *L. rhamnosus* GG remained ≤2 (Log10 CFU/mL). The results obtained are superior as compared to commercial probiotic organisms like *Lactobacillus rhamnosus* GG and *Lactobacillus casei* SHIROTA (Table 4.8, 4.9 and Figure 4.28, 4.29). Summary of the gastrointestinal resistance assay is presented in Table 4.10.

Table 4.10. Summary for Gastrointestinal transit resistance. *L. casei* SHIROTA and *L. rhamnosus* GG were used as the standard organisms for this experiment.

Lactic culture used for Gastrointestinal transit (GIT) experiment	Survival at the GIT (at T_{90} and T_{180} minutes in acidic and bile conditions respectively) with	
	pH 3 & 0.3% Bile	pH 2 & 0.3% Bile
L. coryniformis NAST-RHM94	+	+
L. plantarum NAST-RHM93	+	+
L. brevis NAST-RHM92	+	+
L. delbrueckii NAST-BHL138	+	-
L. delbrueckii NAST-BHL152	+	-
L. delbrueckii NAST-LHL128	+	-
L. delbrueckii NAST-RHL101	+	-
L. delbrueckii NAST-RHL74	+	+
L. delbrueckii NAST-RHL87	+	-
L. fermentum NAST-BHL162	+	+
L. fermentum NAST-GHM2	+	+
L. fermentum NAST-RHL98	+	+
L. harbinensis NAST-RHL95	+	+
L. helveticus NAST-RHL103	+	+
L. parabuchneri NAST-BHL58	+	-
L. parabuchneri NAST-GHM31	+	-
L. paracasei NAST-GHM24	+	+
L. paracasei NAST-GHM5	+	+
L. paracasei NAST-LHL132	+	+
L. paracasei NAST-LHL133	+	+
L. paracasei NAST-RHM82	+	+
L. paracasei NAST-RHM84	+	+
L. rhamnosus NAST-BHL6	+	-
L. rhamnosus NAST-GHM25	+	-
L. casei SHIROTA	+	+
L. rhamnosus GG	+	+

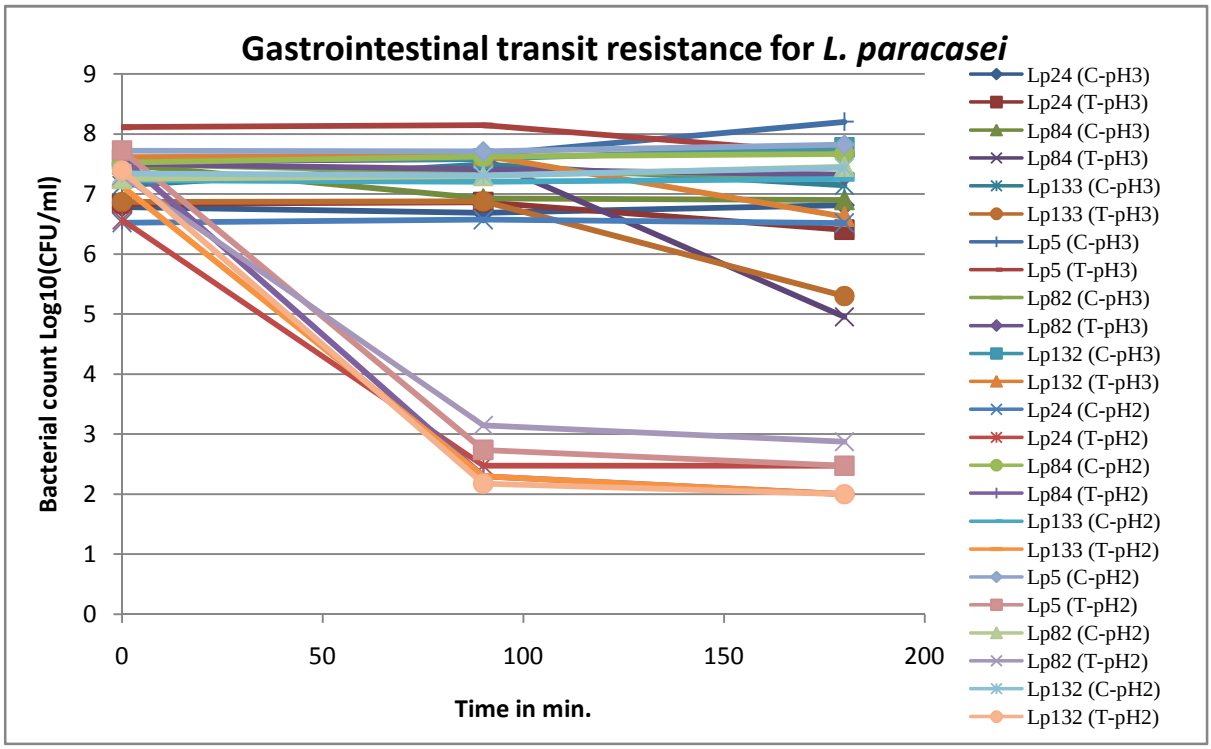

Figure 4.18. The graph plotted using viable count (Log10 CFU/mL) of six strains for *L. paracasei* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lp5 (*L. paracasei* NAST-GHM5), Lp24 (*L. paracasei* NAST-GHM24), Lp82 (*L. paracasei* NAST-RHM82), Lp84 (*L. paracasei* NAST-RHM84), Lp132 (*L. paracasei* NAST-LHL132), Lp133 (*L. paracasei* NAST-LHL133).

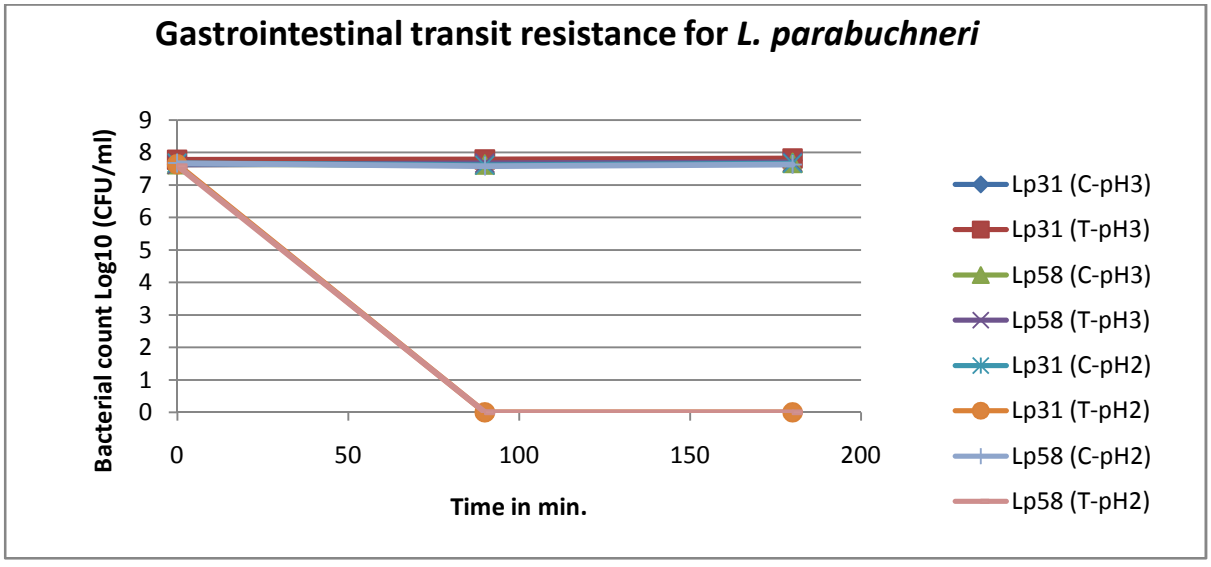

Figure 4.19. The graph plotted using viable count (Log10 CFU/mL) for two strains of *L. parabuchneri* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lp31 (*L. parabuchneri* NAST-GHM31), Lp58 (*L. parabuchneri* NAST-BHL58).

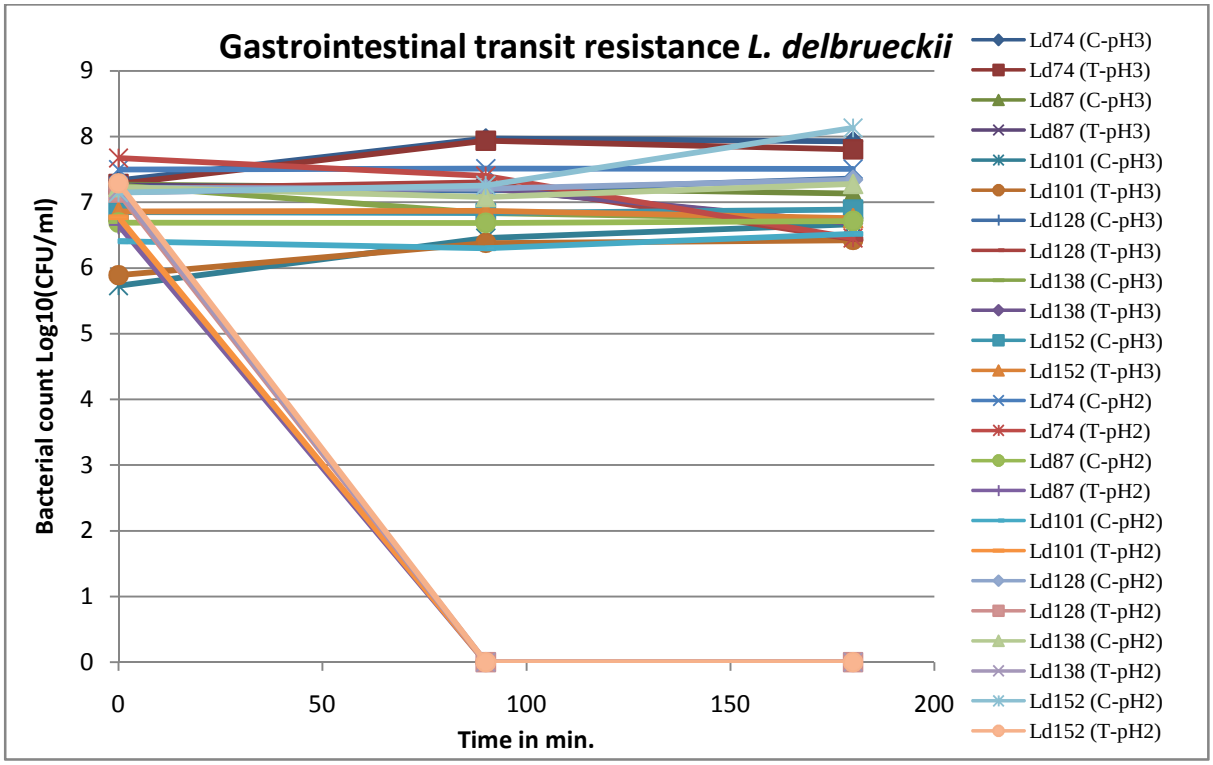

Figure 4.20. The graph plotted using viable count (Log10 CFU/mL) of six strains for *L. delbrueckii* in two GIT models (pH3+0.3% bile and pH2+0.3% bile) as indicated in legend C=control and T= test: Lp74 (*L. delbrueckii* NAST-RHL74), Lp87 (*L. delbrueckii* NAST-RHL87), Lp101 (*L. delbrueckii* NAST-RHL101), Lp128 (*L. delbrueckii* NAST-LHL128), Lp138 (*L. delbrueckii* NAST-BHL138), Lp152 (*L. delbrueckii* NAST-BHL152).

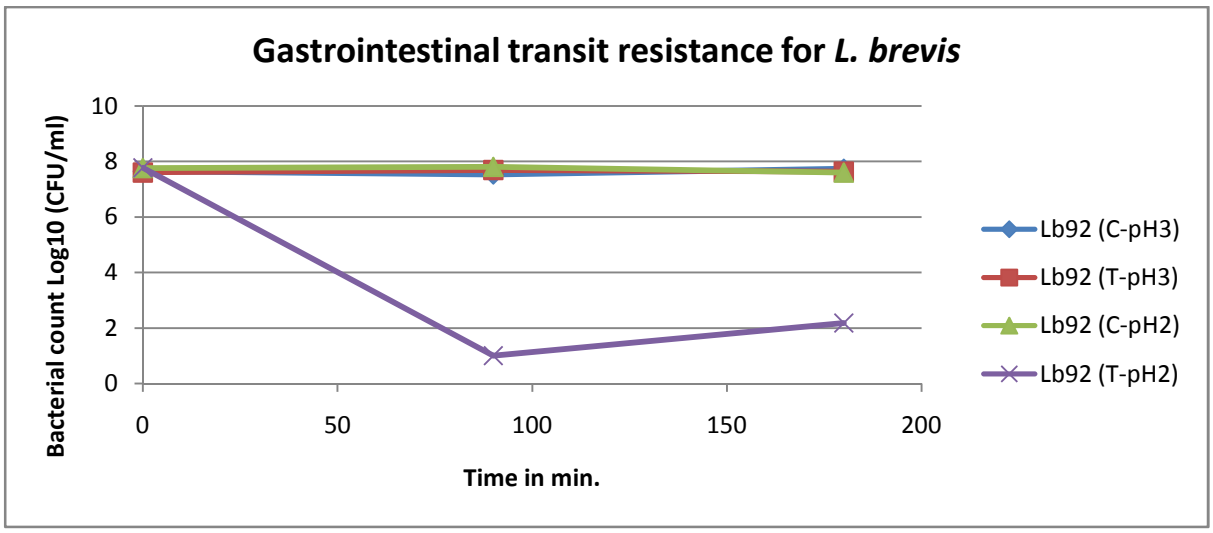

Figure 4.21. The graph plotted using viable count (Log10 CFU/mL) of one strain for *L. brevis* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lb92 (*L. brevis* NAST-RHM92).

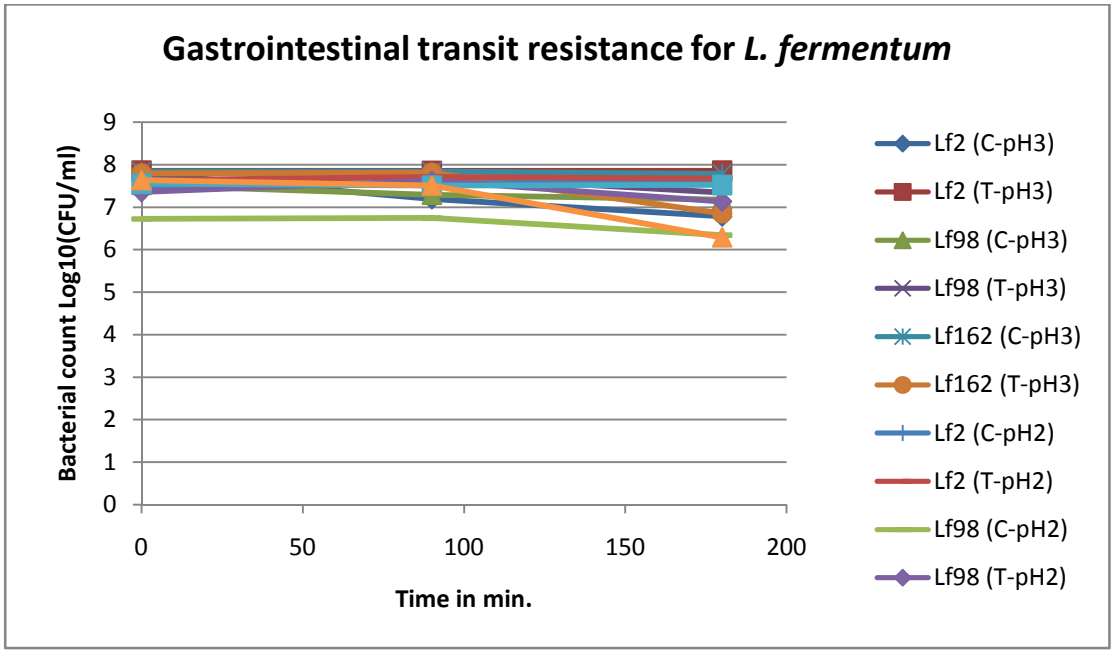

Figure 4.22. The graph plotted using viable count (Log10 CFU/mL) for *L. fermentum* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lf2 (*L. fermentum* NAST-GHM2), Lf98 (*L. fermentum* NAST-RHL98), Lf162 (*L. fermentum* NAST-BHL162).

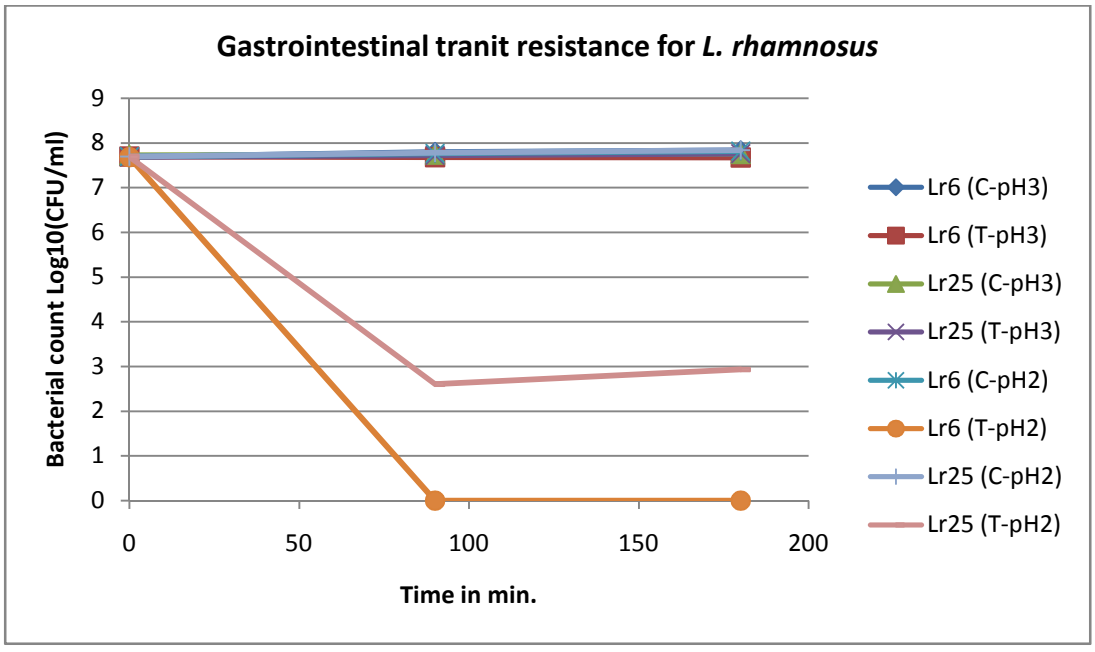

Figure 4.23. The graph plotted using viable count (Log10 CFU/mL) for two strains of *L. rhamnosus* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lr6 (*L. rhamnosus* NAST-BHL6), Lr25 (*L. rhamnosus* NAST-GHM25).

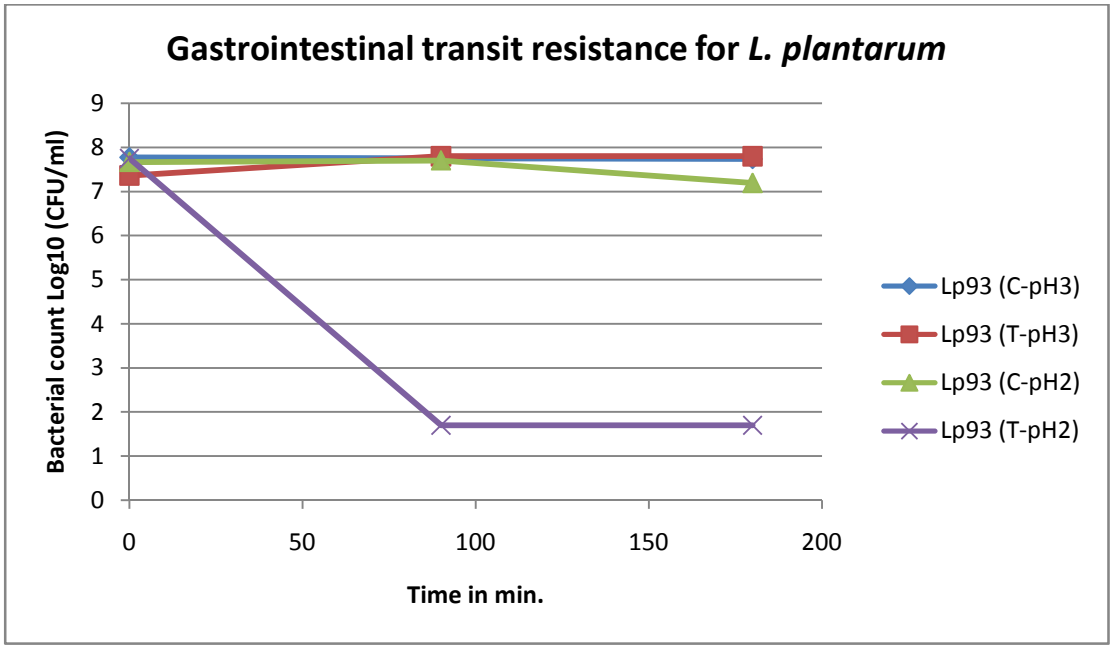

Figure 4.24. The graph plotted using viable count (Log10 CFU/mL) for one strain of *L. plantarum* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lp93 (*L. plantarum* NAST-RHM93**).**

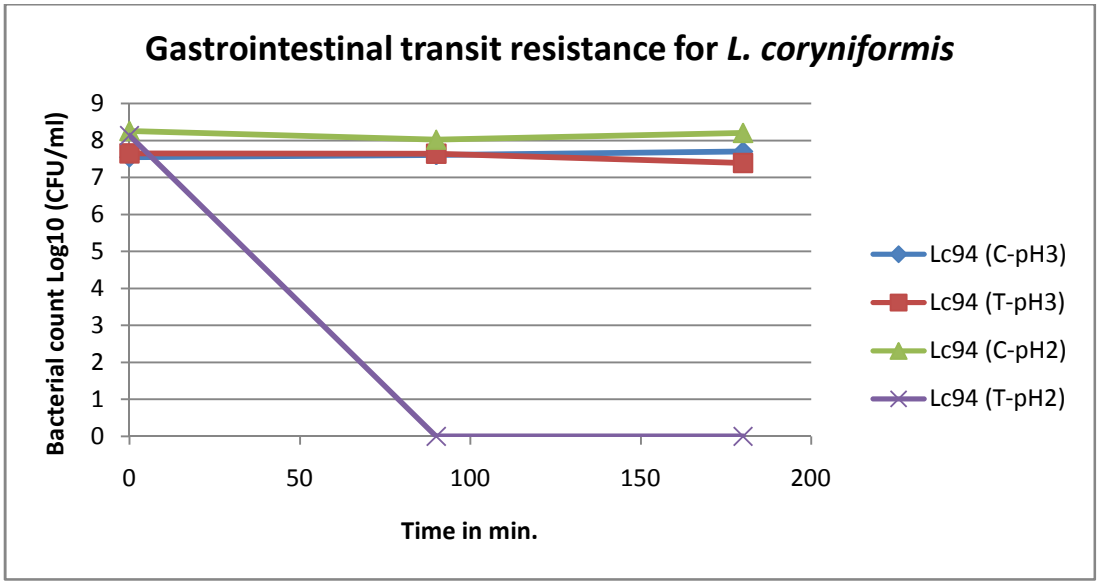

Figure 4.25. The graph plotted using viable count (Log10 CFU/mL) for one strain of *L. coryniformis* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lc94 (*L. coryniformis* NAST-RHM94).

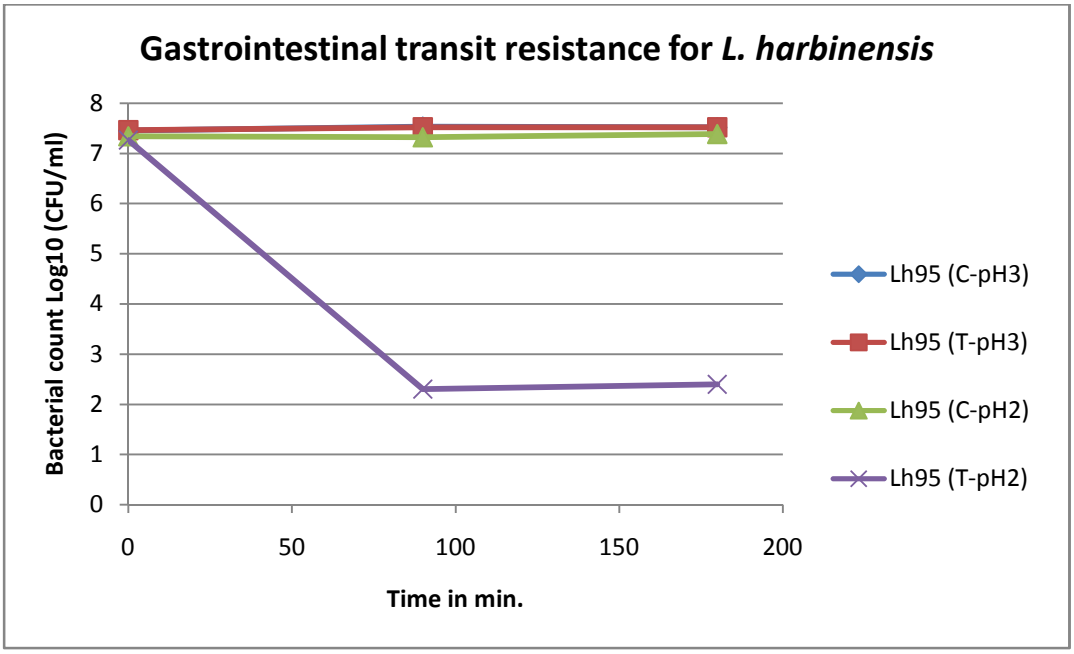

Figure 4.26. The graph plotted using viable count (Log10 CFU/mL) for one strain of *L. harbinensis* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lb95 (*L. harbinensis* NAST-RHL95).

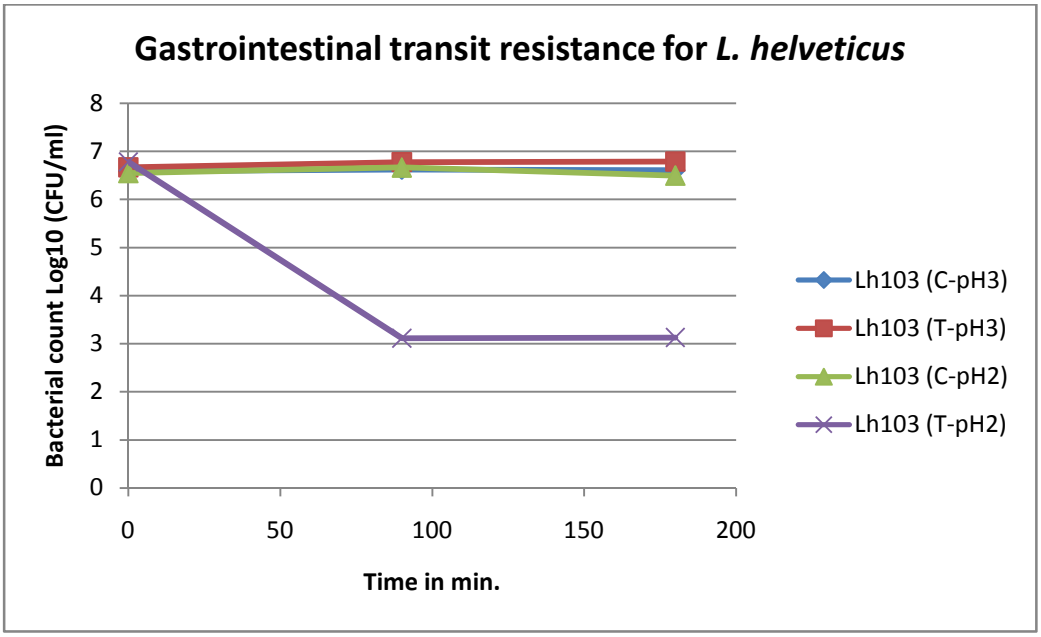

Figure 4.27. The graph plotted using viable count (Log10 CFU/mL) for one strain of *L. helveticus* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: Lh103 (*L. helveticus* NAST-RHL103).

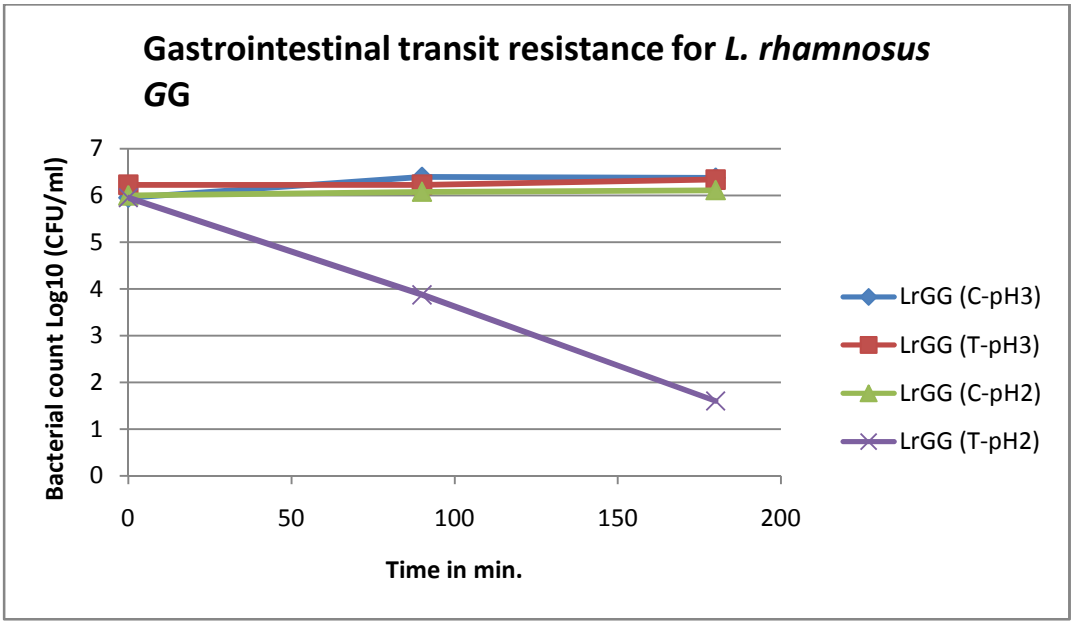

Figure 4.28. The graph plotted using viable count (Log10 CFU/mL) for one strain of *L. rhamnosus* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: LrGG (*L. rhamnosus* GG).

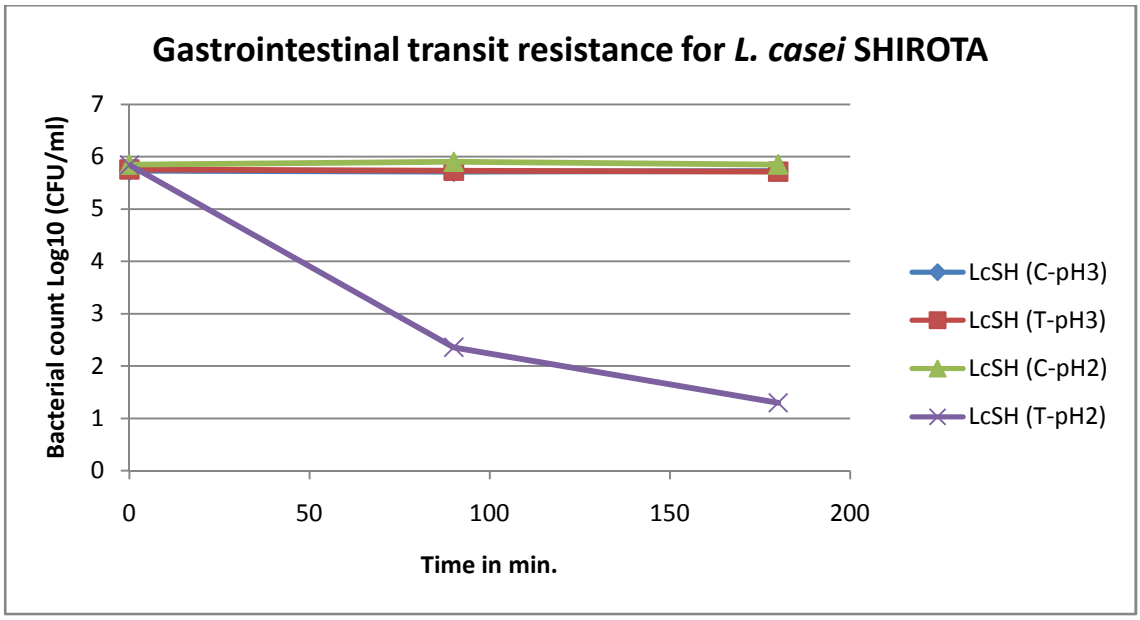

Figure 4.29. The graph plotted using viable count (Log10 CFU/mL) for *L. casei* in two GIT models (pH3+0.3% bile and pH2+0.3% bile), as indicated in legend C=control and T= test: LcSH (*L. casei* SHIROTA).

4.1.2.2. Adherence to Caco-2 monolayer cells

Caco-2 cell monolayer was used to know the adhesion property of the lactic isolates, out of 24 isolates tested, 17 isolates showed positive to adhesion while nine did not posses any adhesive property (Figure 4.30-4.35). Based on the number of bacteria adhered in each microscopic field an average of 20 microscopic field was obtained for each isolates. Log value of the average number of bacteria adhered indicated the weak (+), mild (++) or strong adhesion (+++) (Table 4.11).

Table 4.11. Results of adhesion assay of *Lactobacillus* species on Caco-2 cell monolayer.

Lactic culture Identification	Avg. no of nuclei	Avg. no. of bacterial cells	Log10 (avg. no. of bacterial cells)	Remarks
L. coryniformis NAST-RHM 94	16.0±1.80	0±0.00	NA	-
L. plantarum NAST-RHM93	12.3±2.10	22.2±1.50	1.34±0.06	++
L. brevis NAST-RHM92	13.2±1.40	936±13.80	2.94±0.06	+++
L. delbrueckii NAST-BHL138	19.1±1.30	12.8±1.10	1.10±0.07	++
L. delbrueckii NAST-BHL152	20.2±0.80	12.6±1.60	1.10±0.01	++
L. delbrueckii NAST-LHL128	11.6±1.10	18.7±2.10	1.27±0.02	++
L. delbrueckii NAST-RHL101	11.6±1.20	18.8±1.70	1.27±0.04	++
L. delbrueckii NAST-RHL74	12.3±1.50	142±5.80	2.15±0.02	+++
L. delbrueckii NAST-RHL87	17.7±2.80	12.5±0.70	1.09±0.07	++
L. fermentum NAST-BHL162	19.3±3.30	22.2±1.50	1.34±0.06	++
L. fermentum NAST-GHM2	19.4±1.70	186.6±11.80	2.27±0.01	+++
L. fermentum NAST-RHL98	24.2±0.90	230.8±8.50	2.36±0.03	+++
L. harbinensis NAST-RHL95	11.9±1.20	102.6±5.20	2.01±0.01	+++
L. helveticus NAST-RHL103	14.6±1.40	495.8±12.60	2.69±0.05	+++
L. parabuchneri NAST-BHL58	9.2±0.60	89.9±6.30	1.95±0.03	++
L. parabuchneri NAST-GHM31	12.1±1.20	537.6±3.40	2.7±0.03	+++
L. paracasei NAST-GHM24	10.0±1.00	0±0.00	NA	-
L. paracasei NAST-GHM5	10.0±1.00	0±0.00	NA	-
L. paracasei NAST-LHL132	9.0±1.00	21±2.00	NA	-
L. paracasei NAST-LHL133	11.0±1.50	0±0.00	NA	-
L. paracasei NAST-RHM82	20.3±3.00	4.9±0.50	0.69±0.10	+
L. paracasei NAST-RHM84	19.3±1.90	4.8±0.70	0.68±0.01	+
L. rhamnosus NAST-BHL6	14.0±20	0±0.00	NA	-
L. rhamnosus NAST-GHM25	13.0±1.60	0±0.00	NA	-

Note: NA = Not Applicable; - = Non-adhesive; + = Adhesive with Log10 value below 1, ++ = Adhesive with Log10 value between 1- 2, +++ = Adhesive with Log10 value between 2-3.

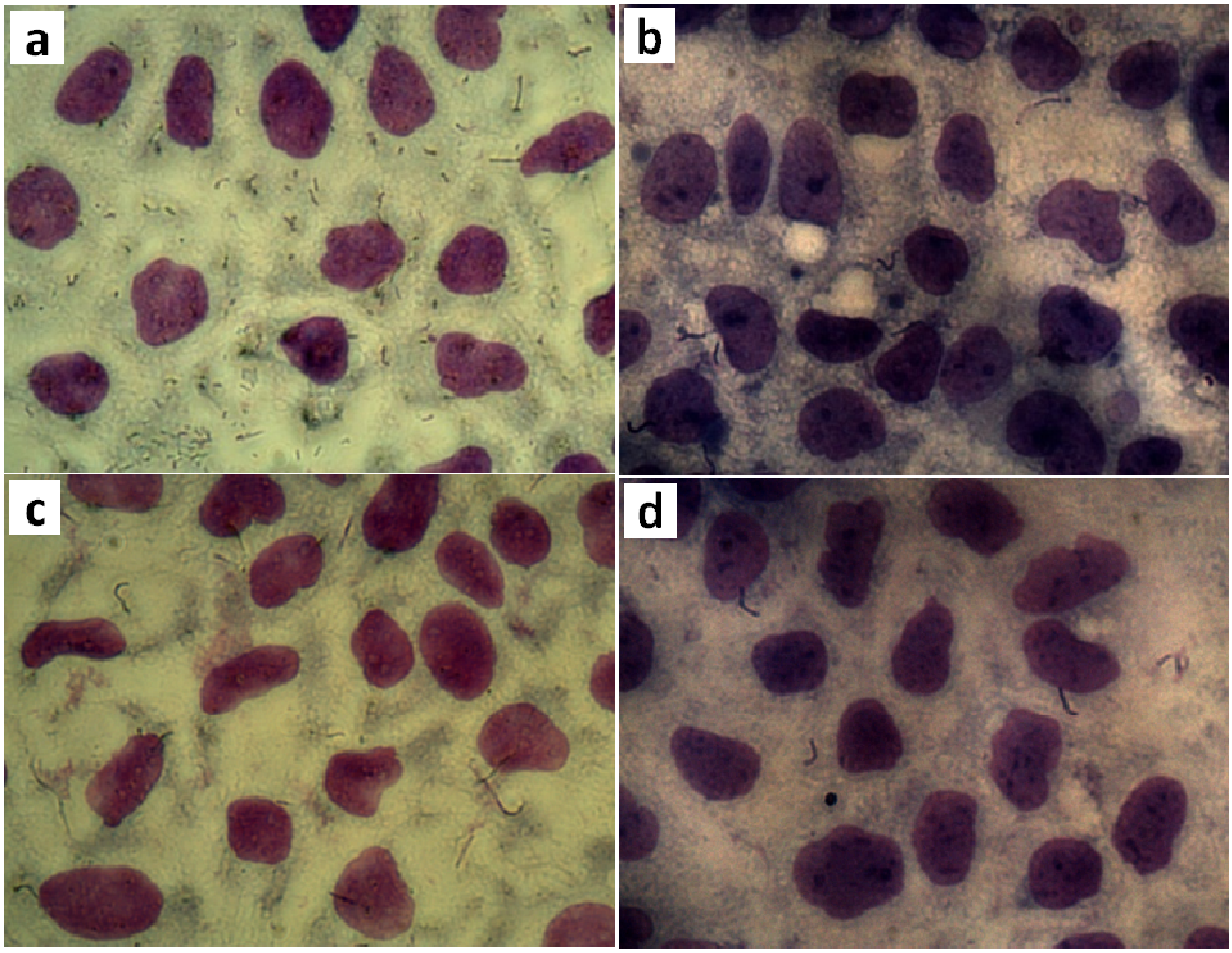

Figure 1.30. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (a) *L. delbrueckii* NAST-RHL74, (b) *L. delbrueckii* NAST-RHL87, (c) *L. delbrueckii* NAST-RHL101, (d) *L. delbrueckii* NAST-LHL128.

Seven isolates were completely non-adhesive (photograph not shown) whereas other seventeen isolates showed adhesion to Caco-2 cell monolayer. Two strains (*Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus paracasei* NAST-RHM84) were weak adhesive with Log10 average number of bacterial cells adhered equals to <1. Similarly eight strains were found to be mild adhesive with Log10

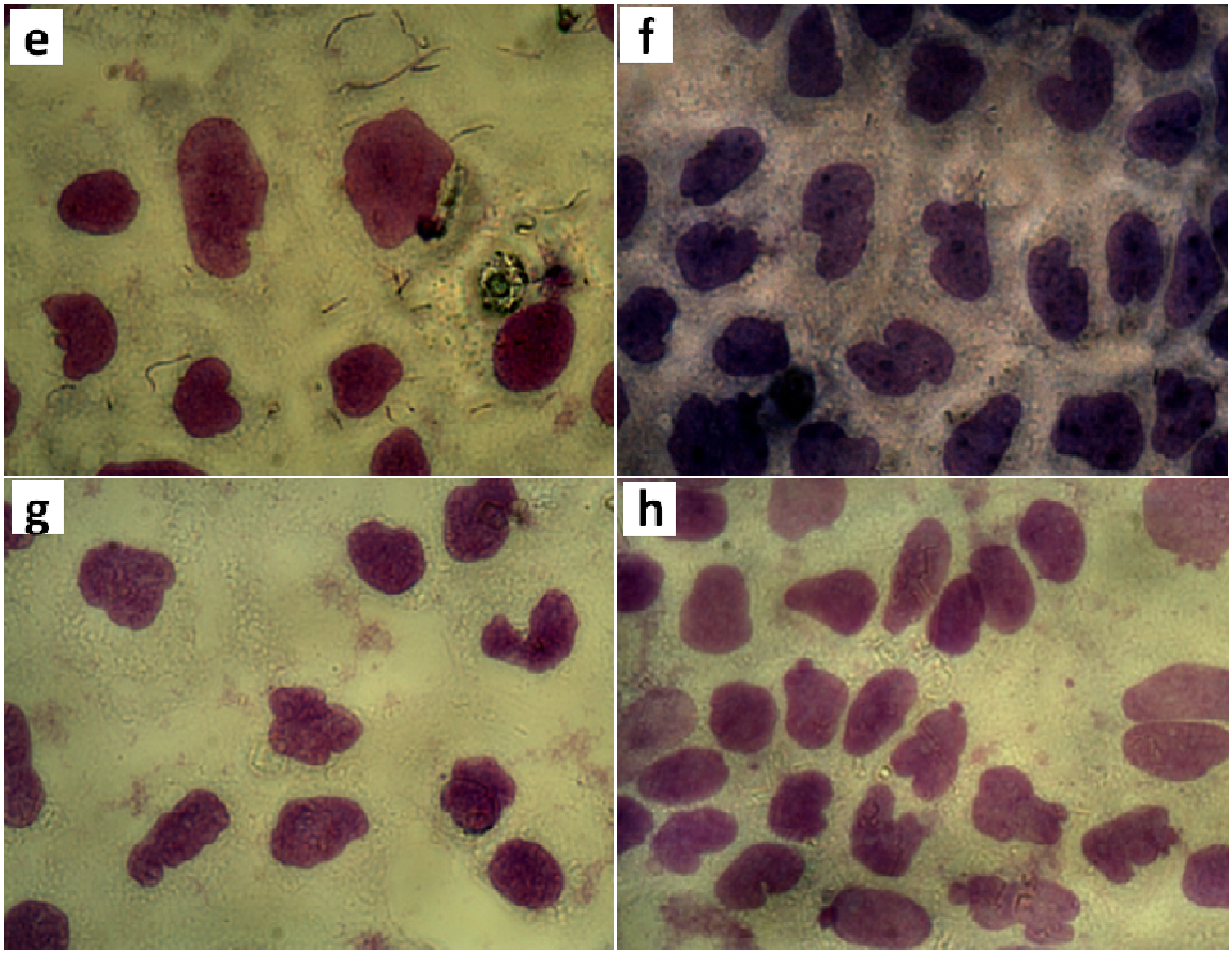

Figure 4.31. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (e) *L. delbrueckii* NAST-BHL138, (f) *L. delbrueckii* NAST-BHL152 (g), *L. paracasei* NAST-GHM5, (h) *L. paracasei* NAST-GHM24.

average number of bacterial cells adhered equals to 1-2. This includes five isolates of *L. delbrueckii*, one isolate of *L. plantarum*, one isolate of *L. parabuchneri* and one isolate of *L. fermentum*. In contrast, seven strains were found to be strong adhesive with Log10 average number of bacterial cells adhered equals to 2-3. This includes two isolates of *L. fermentum* and one isolate of *L. brevis, L. delbrueckii, L. parabuchneri, L. helveticus* and *L. harbinensis*. *Lactobacillus brevis* was found to be the strongest adhesive followed by *L. helveticus* with Log10 average number of bacterial cells adhered equals to 2.9 and 2.6 respectively.

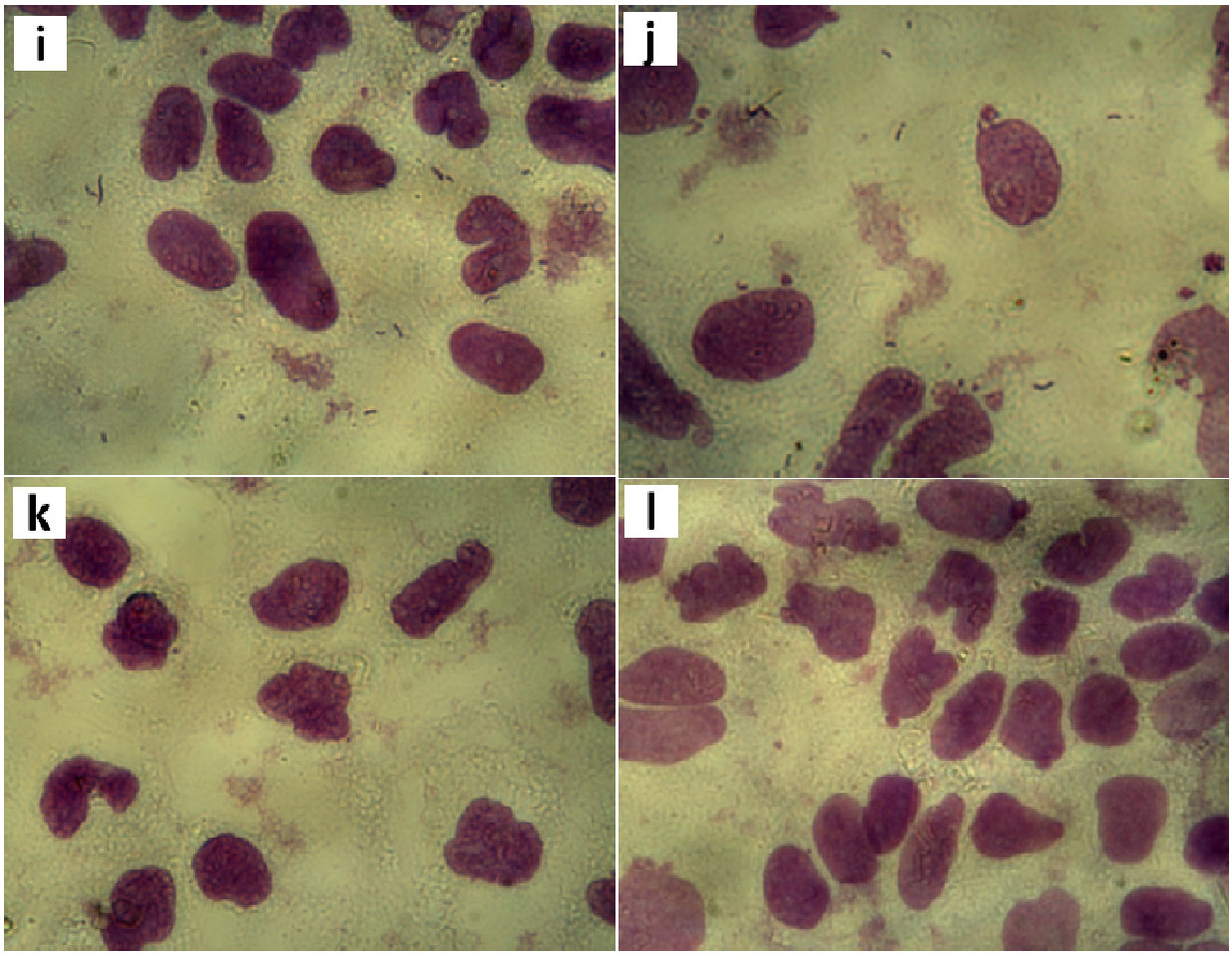

Figure 4.32. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (i) *L. paracasei* NAST-RHM82, (j) *L. paracasei* NAST-RHM84, (k) *L. paracasei* NAST-LHL132, (l) *L. paracasei* NAST-LHL133.

The strains adhered and colonized to the gastrointestinal tract may enhance the capacity to stimulate the immune system and heal the damaged intestinal tissue, so the adhesion of strains to the intestinal mucosa was considered to be one of the main selection criteria for a probiotic microorganism with health beneficial effects. Our results showed that the organisms could be potential probiotic based on the adhesion result (Dimitrov et al., 2014).

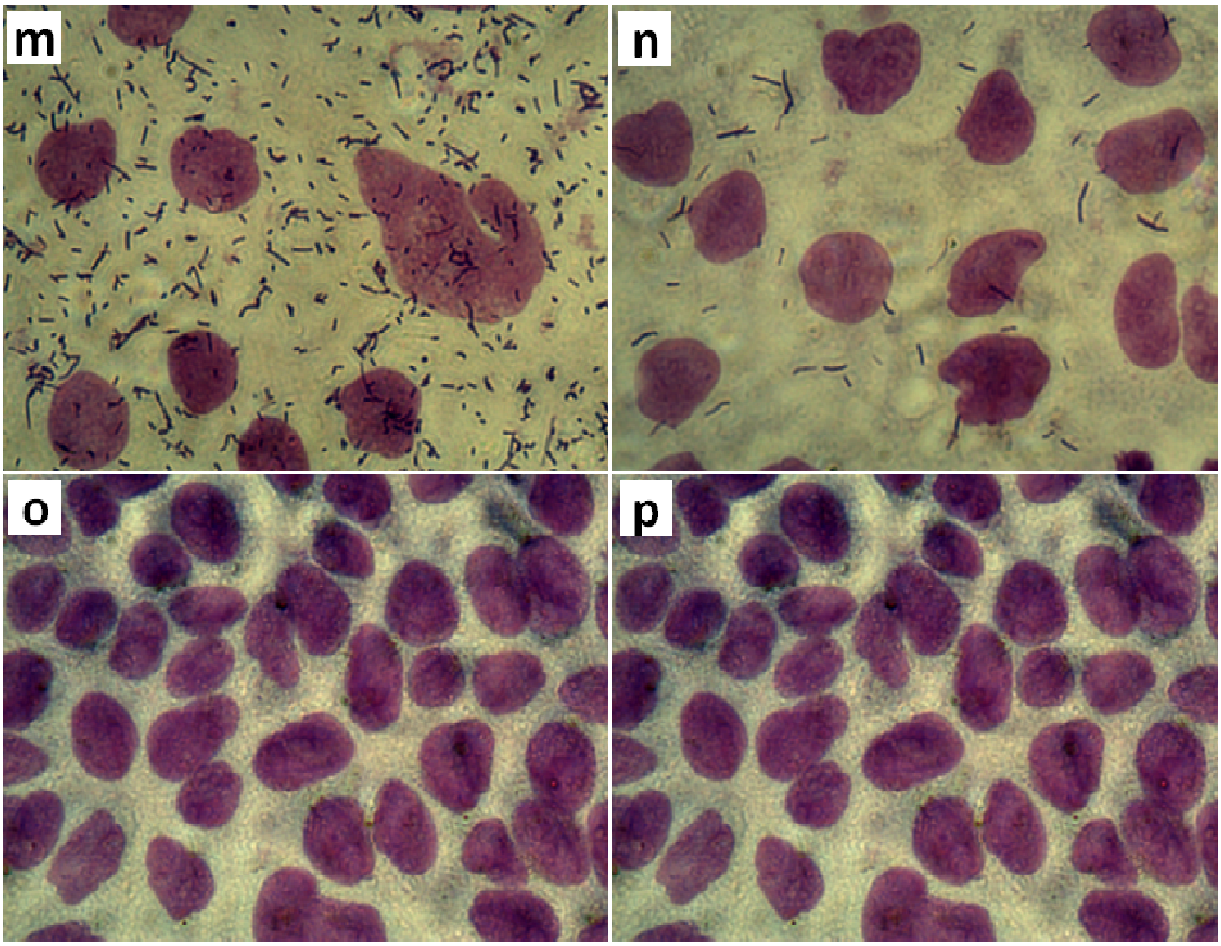

Figure 4.33. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (m) *L. parabuchneri* NAST-GHM31, (n) *L. parabuchneri* NAST-BHL58, (o) *L. rhamnosus* NAST-BHL6, (p) *L. rhamnosus* NAST-GHM25.

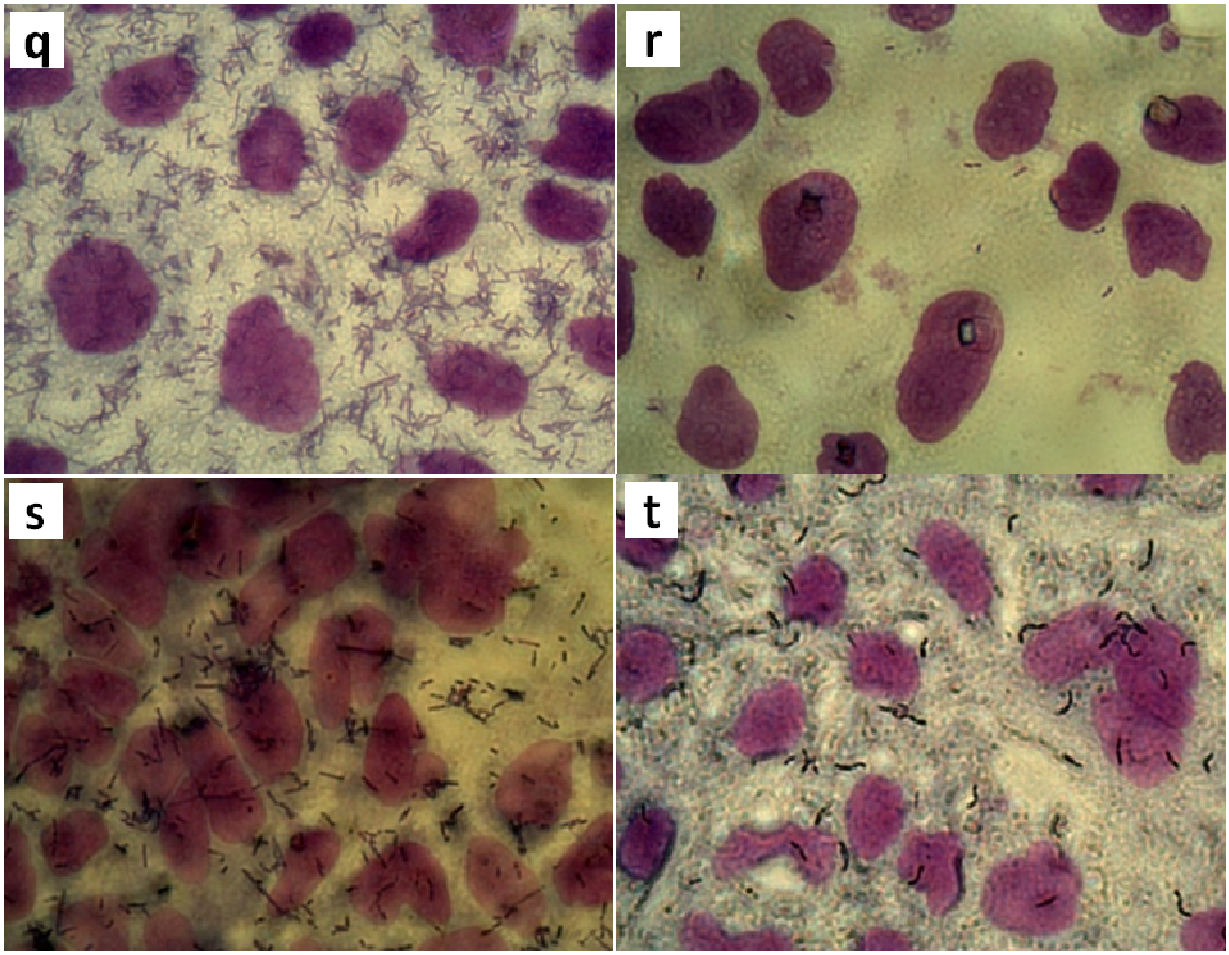

Figure 4.34. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (q) *L. brevis* NAST-RHM92, (r) *L. plantarum* NAST-RHM93, (s) *L. helveticus* NAST-RHL103, (t) *L. harbinensis* NAST-RHL95.

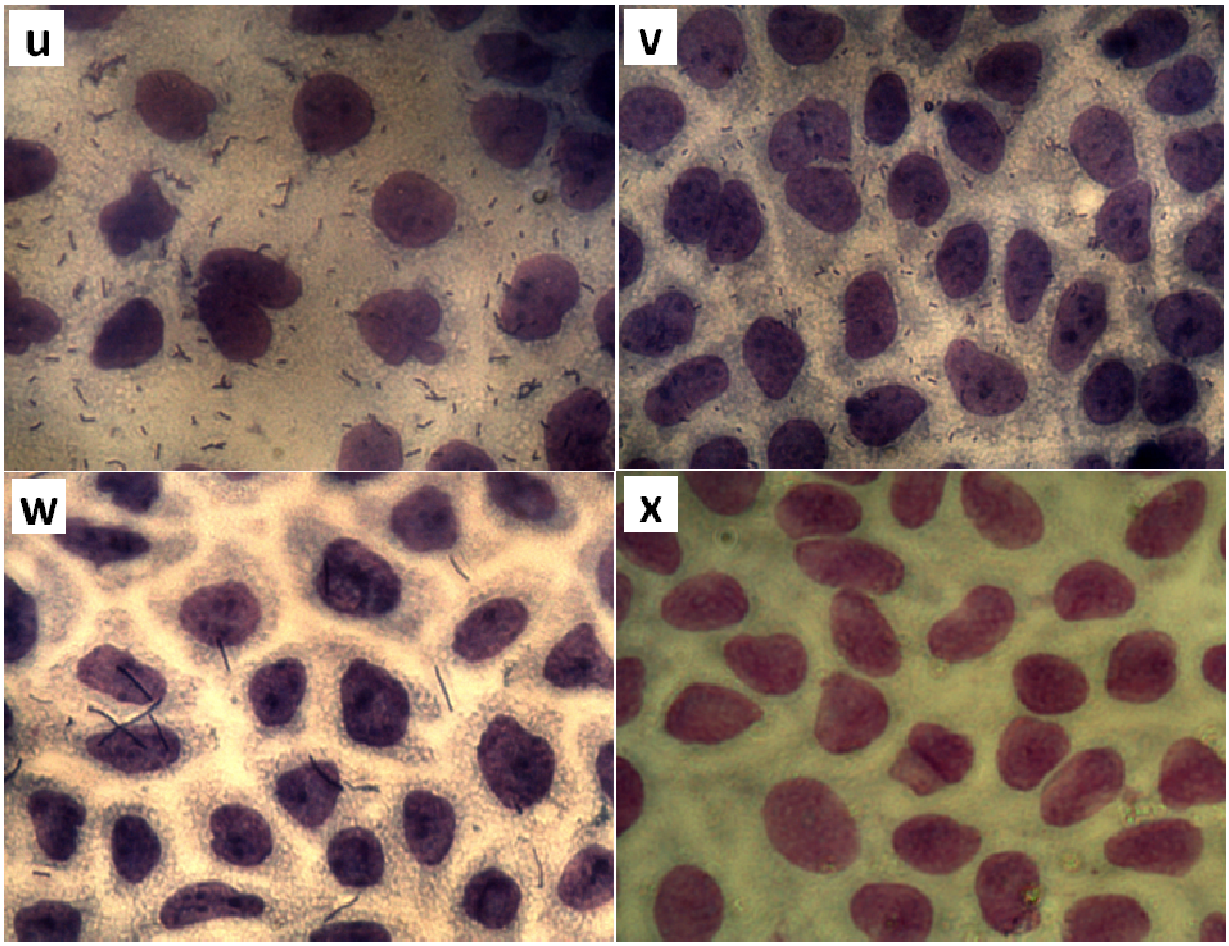

Figure 4.35. Adhesion to Caco-2 cells monolayer, as observed with Giemsa staining under light microscope (× 400 magnification). Pink coloured (big patch like) are nucleus of Caco-2 cells and small rod shaped structures present on the monolayer are adhered lactobacilli cells: (u) *L. fermentum* NAST-GHM2, (v) *L. fermentum* NAST-RHL98, (w) *L. fermentum* NAST-BHL162, (x) *L. coryniformis* NAST-RHM 94.

4.1.2.3. Antimicrobial activity

The result showed that only few isolates showed inhibition to enteric pathogens. The inhibition shown *by L. dellbrueckii* subsp. *bulgaricus* NAST-RHL101 against *S. pyogenes*, *P. aeruginosa* and *S. aureus* was notable. While other inhibition possesed by *L. plantarum* NAST-RHM93 (against *L. monocytogenes* and *S. enterica*) and *L. rhamnosus* NAST-GHM25 (against *P. aeruginosa*) were shown to be weak. The details of the result obtained for antimicrobial screening is shown below (Table 4.12).

Table 4.12. Preliminary screening for antimicrobial activity.

Lactobacillus species	Test pathogens					
	L. monocytogenes	*S. enterica*	*E. coli* VE	*S. pyogenes*	*P. aeruginosa*	*S. aureus*
L. coryniformis NAST-RHM 94	-	-	-	-	-	-
L. plantarum NAST-RHM93	+	+	-	-	-	-
L. brevis NAST-RHM92	-	-	-	-	-	-
L. delbrueckii NAST-BHL138	-	-	-	-	-	-
L. delbrueckii NAST-BHL152	-	-	-	-	-	-
L. delbrueckii NAST-LHL128	-	-	-	-	-	-
L. delbrueckii NAST-RHL101	-	-	-	+	+	+
L. delbrueckii NAST-RHL74	-	-	-	-	-	-
L. delbrueckii NAST-RHL87	-	-	-	-	-	-
L. fermentum NAST-BHL162	-	-	-	-	-	-
L. fermentum NAST-GHM2	-	-	-	-	-	-
L. harbinensis NAST-RHL95	-	-	-	-	-	-
L. helveticus NAST-RHL103	-	-	-	-	-	-
L. helveticus NAST-RHL103	-	-	-	-	-	-
L. parabuchneri NAST-BHL58	-	-	-	-	-	-
L. parabuchneri NAST-GHM31	-	-	-	-	-	-
L. paracasei NAST-GHM24	-	-	-	-	-	-
L. paracasei NAST-GHM5	-	-	-	-	-	-
L. paracasei NAST-LHL132	-	-	-	-	-	-
L. paracasei NAST-LHL133	-	-	-	-	-	-
L. paracasei NAST-RHM82	-	-	-	-	-	-
L. paracasei NAST-RHM84	-	-	-	-	-	-
L. rhamnosus NAST-BHL6	-	-	-	-	-	-
L. rhamnosus NAST-BHL25	+	-	-		+	-
L. plantarum PAC 1.0	+	-	-	-	-	-
L. plantarum PAC1.10	+	-	-	-	-	-
L. plantarum WHE92	+	-	-	-	-	-

Note: + and – in the result table represents positive and negative for inhibiting pathogens respectively.

4.1.2.4. Antibiotic resistance test

Twenty four lactobacilli isolates were investigated for antibiotics resistant test according to EFSA guidelines. The antibiotics tested were; a) inhibitors of cell wall synthesis, b) inhibitors of protein synthesis and c) inhibitors of nucleic acid synthesis. Of the 24, four bacterial isolates were found to be completely safe *viz. L. helveticus* NAST103, *L. paracasei* NAST82, *L. fermentum* NAST2 and *L. coryniformis* NAST 94 based on EFSA guideline. And sensitivity results for different antibiotics varied for rest of the lactic isolates (Table 4.13).

Table 4.13. Antibiotic susceptibility profiling for *Lactobacillus* strains.

Lactic culture used for antibiotic assay	Minimum Inhibitory Concentration (MIC) µgmL^{-1}) of antibiotics								
	Ampicillin	Vancomycin	Gentamycin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
Lactobacillus paracasei NAST-GHM5	<4 (<2)	nr	<64 (<32)	<64 (<64)	nr	<1 (<1)	<2 (<1)	<8 (<4)	<16 (<4)
Lactobacillus paracasei NAST-GHM24	<2 (<2)	nr	<32 (<32)	<128 (<64)	nr	<1 (<1)	<1 (<1)	<8 (<4)	<8 (<4)
Lactobacillus paracasei NAST-RHM82	<2 (<2)	nr	<32 (<32)	<64 (<64)	nr	<0.5 (<1)	<1 (<1)	<4 (<4)	<4 (<4)
Lactobacillus paracasei NAST-RHM84	<2 (<2)	nr	<32 (<32)	<64 (<64)	nr	<1 (<1)	<1 (<1)	<4 (<4)	<8 (<4)
Lactobacillus paracasei NAST-LHL132	<4 (<2)	nr	<4 (<32)	<256 (<64)	nr	<4 (<1)	<4 (<1)	<8 (<4)	<16 (<4)
Lactobacillus paracasei NAST-LHL133	<2 (<2)	nr	<8 (<32)	<64 (<64)	nr	<1 (<1)	<1 (<1)	<8 (<4)	<8 (<4)
Lactobacillus rhamnosus NAST-BHL6	<2 (<4)	nr	<8 (<16)	<128 (<64)	<32 (<32)	<1 (<1)	<1 (<1)	<8 (<8)	<16 (<4)
Lactobacillus rhamnosus NAST-BHL25	<4 (<4)	nr	<16 (<16)	<128 (<64)	<32 (<32)	<1 (<1)	<1 (<1)	<8 (<8)	<16 (<4)
Lactobacillus delbrueckii NAST-RHL74	<0.5 (<1)	>8 (<2)	<16 (<16)	>64 (<16)	<32 (<16)	<0.25 (<1)	<0.5 (<1)	<16 (<4)	<8 (<4)
Lactobacillus delbrueckii NAST-RHL87	<1 (<1)	<0.5 (<2)	<8 (<16)	<32 (<16)	<8 (<16)	<0.125 (<1)	<0.125 (<1)	<4 (<4)	<4 (<4)
Lactobacillus delbrueckii NAST-RHL101	<0.125 (<1)	<0.5 (<2)	<4 (<16)	<32 (<16)	<4 (<16)	<0.125 (<1)	<0.125 (<1)	<2 (<4)	<4 (<4)
Lactobacillus delbrueckii NAST-LHL128	<1 (<1)	<1 (<2)	<16 (<16)	<32 (<16)	<16 (<16)	<0.125 (<1)	<0.125 (<1)	<2 (<4)	<4 (<4)
Lactobacillus delbrueckii NAST-BHL138	<0.5 (<1)	>8 (<2)	<4 (<16)	<64 (<16)	<16 (<16)	<0.125 (<1)	<0.125 (<1)	<4 (<4)	<4 (<4)
Lactobacillus delbrueckii NAST-BHL152	<0.125 (<1)	<0.5 (<2)	<16 (<16)	<32 (<16)	<16 (<16)	<0.125 (<1)	<1 (<1)	<4 (<4)	<4 (<4)
Lactobacillus fermentum NAST-GHML2	<0.5 (<1)	nr	<4 (<16)	<8 (<32)	<32 (<64)	<0.125 (<1)	<0.125 (<1)	<8 (<8)	<4 (<4)
Lactobacillus fermentum NAST-RHL98	<1 (<1)	nr	<64 (<16)	>128 (<32)	<256 (<64)	<1 (<1)	<1 (<1)	<16 (<8)	<4 (<4)
Lactobacillus fermentum NAST-BHL162	<2 (<1)	nr	>128 (<16)	<128 (<32)	<64 (<64)	<0.125 (<1)	<0.125 (<1)	<8 (<8)	<4 (<4)
Lactobacillus parabuchneri NAST-GHM31	<4 (<2)	nr	<32 (<16)	<32 (<16)	<128 (<64)	<2 (<1)	<2 (<1)	<32 (<8)	<8 (<4)
Lactobacillus parabuchneri NAST-BHL58	<4 (<2)	nr	<32 (<16)	<32 (<16)	<128 (<64)	<2 (<1)	<2 (<1)	<32 (<8)	<8 (<4)
Lactobacillus helviticus NAST-RHL103	<1 (<1)	<0.5 (<2)	<4 (<16)	<16 (<16)	<4 (<16)	<0.5 (<1)	<0.25 (<1)	<2 (<4)	<4 (<4)
Lactobacillus brevis NAST-RHM92	<8 (<2)	nr	>128 (<16)	>128 (<16)	<256 (<64)	>4 (<1)	>4 (<1)	<32 (<8)	<16 (<4)
Lactobacillus corniformis NAST-RHM94	<4 (<4)	nr	<4 (<16)	<64 (<64)	<64 (<64)	<0.25 (<1)	<0.25 (<1)	<8 (<8)	<4 (<4)
Lactobacillu harbinensis NAST-RHL95	<8 (<4)	nr	<64 (<16)	<64 (<64)	<256 (<64)	<1 (<1)	<0.5 (<1)	<8 (<8)	<8 (<4)
Lactobacillus plantarum NAST-RHM93	<2 (<2)	nr	<8 (<16)	<32 (<64)	nr	<0.25 (<1)	<0.5 (<1)	<128 (<32)	<8 (<8)

Note: Highlighted readings in every cells of table indicate the EFSA standard guideline of the minimum inhibitory concentration of the antibiotic concentrations, nr represents not required.

Five isolates of *L. paracasei* were found to be susceptible to Kanamycin (EFSA limit <64 µgmL-^{1}) except *L. paracasei* NAST24 while five isolates of *L. paracasei* were found to be resistant to Chloramphenicol (EFSA limit <4 µgmL-^{1}) except *L. paracasei* NAST82. In addition, *L. paracasei* NAST5 and *L. paracasei* NAST132 showed resistance to Ampicillin (EFSA limit <2 µgmL-^{1}). All other *L. paracasei* were found to be sensitive towards rest of the antibiotics. Both the isolates of *L. rhamnosus* tested were resistant to Kanamycin (EFSA limit <64 µgmL-^{1}) and Chloramphenicol (EFSA limit <4 µgmL-^{1}) while both the *L. rhamnosus* strains were sensitive to all other antibiotics based on EFSA guidelines.

Among six isolates of *L. dellbrueckii*, five isoaltes were found to be sensitive to Ampicillin (EFSA limit <1 µgmL-^{1}), four isolates were sensitive to Vancomycin (EFSA limit <2 µgmL-^{1}) and all the isolates were sensitive to Gentamycin, five isolates were resistant to Kanamycin (EFSA limit <16 µgmL-^{1}), five isolates were sensitive to Streptomycin (EFSA limit <16 µgmL-^{1}), all the isolates were sensitive to Erythromycin (EFSA limit <1 µgmL-^{1}), five isolates were sensitive to Tetracycline (EFSA limit <4 µgmL-^{1}) and Chloramphenicol (EFSA limit <4 µgmL-^{1}).

One isolate of *L. fermentum* NAST2 was sensitive to all the antibiotics while two other isolates were resistant to Gentamycin, Kanamycin, Streptomycin and Tetracycline. Both the isolates of *L. parabuchneri* were resistant to Gentamycin (EFSA limit <16 µgmL-^{1}), Kanamycin (EFSA limit <16 µgmL-^{1}), Streptomycin (EFSA limit <64 µgmL-^{1}), Erythromyin (EFSA limit <1 µgmL-^{1}), Clindamycin (EFSA limit <1 µgmL-^{1}), Tetracycline (EFSA limit <8 µgmL-^{1}) and sensitive to Ampicillin (EFSA limit <2 µgmL-^{1}) & Chloramphenicol (EFSA limit <2 µgmL-^{1}). *L. brevis* NAST92 was resistant to all nine antibiotics tested. *L. harbinensis* NAST95 was sensitive to Erythromycin (EFSA limit <1 µgmL-^{1}) and Clindamycin (EFSA limit <1 µgmL-^{1}). *L. plantarum* NAST93 was sensitive to all eight antibiotics tested except Tetracycline (EFSA limit <32 µgmL-^{1}) which was found to be resistant.

4.1.3. Preparation of Probiotic Curd (*Dahi*) and Sweet Curd (*Dahi*) using novel lactic cultures (*Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103) having probiotic properties.

4.1.3.1. Partial purification of antimicrobial substance from indigenous lactic cultures

4.1.3.1.1. Preliminary screening for bacteriostatic activity from CFSs derived from MRS culture broth

Out of six strains (cultured in MRS broth) tested, three strains *viz. L. rhamnosus* NAST-GHM25, *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 showed antibacterial activity against all four food borne pathogens considered for this study (Table 4.14 and Figure 4.36). Lactic acid in various combinations *viz.* 1% LA in distilled water, 1% LA in phosphate buffer (PB) pH 7 and 10 µL LA in 100 mL distilled water did not show any inhibition with the indication that the inhibition is not due to acidity of the cell CFSs.

Table 4.14. Preliminary screening of cell free supernatants (CFSs) derived from MRS broth culture (Bacteriostatic assay).

Negative control used and cell free supernatant of lactic isolate	Zone of Inhibition against the indicator strains (mm)			
	B. cereus	*E. coli*	*L. monocytogenes*	*S. aureus*
1 % LA in DW (pH 2.48)	-	-	-	-
1% LA in PB-pH7 (pH 4.18)	-	-	-	-
10 µl LA in 100 ml DW (pH 3.2)	-	-	-	-
MRS Media (pH 6.8)	-	-	-	-
L. fermentum NAST-GHM2	-	-	-	-
L. rhamnosus NAST-GHM25	16±0.26	17±0.18	16.5±0.25	15±0.32
L. delbreuckii NAST-RHL74	-	-	-	-
L. paracasei NAST-RHM82	15±0.13	15±0.31	16±0.27	13±0.56
L. delbreuckii NAST-RHL101	-	-	-	-
L. helveticus NAST-RHL103	19±0.15	18±0.41	20.5±0.29	16±0.16

Note: mm = millimeter PB = phosphate buffer and DW = distilled water

L. helveticus NAST-RHL103 showed the highest zone of inhibition (ZOI) against all the pathogens as compared to other two lactic cultures. Among the inhibitions, inhibition against *L. monocytogenes* was highest (20.5 mm) followed by the same strain against *B. cereus* (19 mm), *E. coli* (18 mm) and *S. aureus* (16 mm). Subsequent to *L. helveticus* NAST-RHL103, *L. rhamnosus* NAST-GHM25 showed highest inhibition against *E. coli* (17 mm) followed by *L. monocytogenes* (16.5 mm), *B. cereus* (16 mm) and *S. aureus* (15 mm). Finally, *L. paracasei* NAST-RHM82 showed highest dilution against *L. monocytogenes* (16 mm) followed by the same strain against *B. cereus* (15 mm), *E. coli* (15 mm) and *S. aureus* (13 mm).

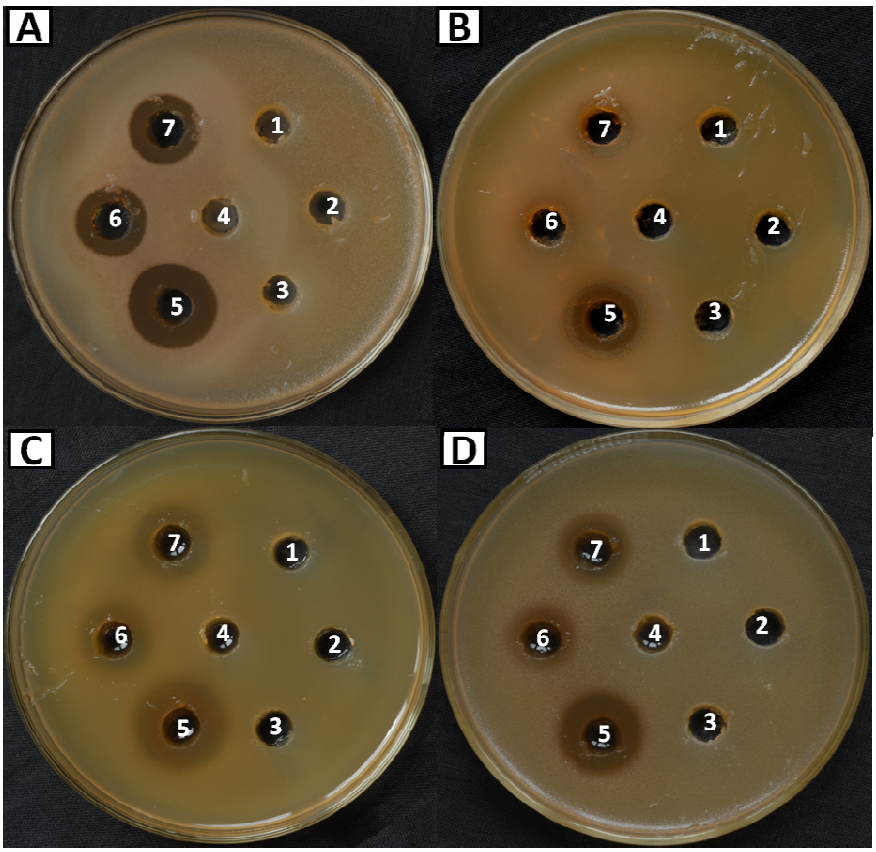

Figure 4.36. Preliminary screening by plate assay (bacteriostatic assay) of cell free supernatants (CFSs) derived from MRS broth culture; Lawn of pathogens in different plates: A) *B. cereus*, B) *E. coli*, C) *L. monocytogenes*, D) *S. aureus*. In the well of all plate (A, B, C & D), well number indicates: 1) 1% Lactic acid (LA) in distilled water (pH 2.48), 2) 1% LA in Phosphate Buffer (PB) pH 7 (pH 4.18), 3) 10 µL LA in distilled water (pH 3.2) and 4) MRS broth (pH 6.8) as negative controls and well no. 5) CFSs of *L. helveticus* NAST-RHL103,6) CFSs of *L. paracasei* NAST-RHM82 and 7) CFSs of *L. rhamnosus* NAST-GHM25.

4.1.3.1.1. Preliminary screening for bactericidal activity from CFSs derived from MRS culture broth in plate and broth

All the three cultures *viz. L. helveticus* NAST-RHL103, *L. paracasei* NAST-RHM82 and *L. rhamnosus* NAST-GHM25 showed bactericidal action against *B. cereus* (Table 4.15 and Figure 4.37). *L. helveticus* NAST-RHL103 showed highest inhibition (24 mm) followed by *L. paracasei* NAST-RHM82 (18 mm) and *L. rhamnosus* NAST-GHM25 (17 mm). These zones of inhibition shown by all three lactic cultures against *B. cereus* are relatively higher than the zone of inhibition in bacteristatic experiment (as mentioned earlier).

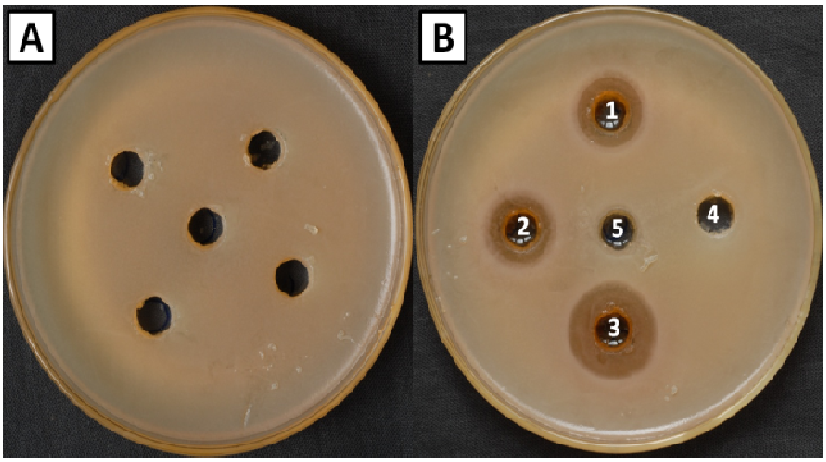

Figure 4.37. A) Lawn of *B. cereus* and B) Bactericidal Plate assay of cell free supernatants (CFSs) derived from culture broth, well number 1) CFSs of *L. rhamnosus* NAST-GHM25, 2) *L. paracasei* NAST-RHM82 and 3) *L. helveticus* NAST-RHL103.

Figure 4.38. Bactericidal assay in BHI broth: *B. cereus* cells treated with cell free supernatants (CFSs) of: A) *L. rhamnosus* NAST-GHM25, B) *L. paracasei* NAST-RHM82, C) *L. helveticus* NAST-RHL and D) positive control (growth of pathogen observed by turbidity).

Likewise, the bactericidal assay performed in BHI broth showed that there was no growth of the pathogens in the broth culture tube after 12 hours of incubation in room temperature (Figure 4.38). The indicator organism showed its growth in the negative control (without CFSs; turbidity observed by naked eye) in the broth while indicator strains did not show growth in the test (with antimicrobial agent; no turbidity observed by naked eye).

Table 4.15. Plate assay of cell free supernatants (CFSs) derived from MRS culture broth (Bactericidal assay) against *B. cereus*.

CFSs derived from lactic isolates	Zone of Inhibition (mm) (against *B. cereus*)
L. rhamnosus NAST-GHM 25	17±0.41
L. paracasei NAST-RHM82	18±0.36
L. helveticus NAST-RHL103	24±0.52

4.1.3.1.2. Antibacterial properties of cell free supernatants (CFSs) derived from skimmed milk culture broth and concentration using various solvents

CFSs of two isolates *viz. L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 cultured in skimmed milk broth were used independently. The ten times concentrated CFSs showed zone of inhibition (ZOI) against four different pathogens in the plate assay (Table 4.16 and Figure 4.39). The zone of inhibition of ten times concentrated CFSs of *L. helveticus* NAST-RHL103 was found to be notably higher than *L. paracasei* NAST-RHM82 against all the indicator pathogenic strains. CFSs of *L. paracasei* NAST-RHM82 showed highest inhibition against *E. coli* (16.5 mm) followed by the same strains against *L. monocytogenes* (16 mm), *S. aureus* (15 mm) and *B. cereus* (14 mm). CFSs of *L. helveticus* NAST-RHL103 showed highest inhibition against *L. monocytogenes* (25.5 mm) followed by the same strains against *E. coli* (25 mm), *S. aureus* (25 mm) and *B. cereus* (24 mm).

Methanol concentrate of CFSs (derived from skimmed milk culture broth) of *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 showed relatively higher ZOI than CFSs derived from skimmed milk concentrate against all the indicator strains (Table 4.16 and Figure 4.39). The methanol concentrate of *L. helveticus*

NAST-RHL103 showed higher inhibition than *L. paracasei* NAST-RHM82 against all the indicator strains. The methanol concentrate of *L. helveticus* NAST-RHL103 showed highest inhibition against *S. aureus* (31.5 mm) followed by the same strain against *L. monocytogenes* (29.5 mm), *E. coli* (29 mm) and *B. cereus* (27.5 mm). Similarly, methanol concentrate of *L. paracasei* NAST-RHM82 showed highest inhibition against *L. monocytogenes* (21 mm) followed by the same strain against *E. coli* (18 mm), *B. cereus* (17 mm) and *S. aureus* (16 mm).

Table 4.16. Plate assay for antimicrobial activity of skimmed milk, methanol & methanol-acetone concentrate of *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 against food borne pathogens.

CFSs of lactic culture and its derivative concentrates in different solvents	Zone of Inhibition against the indicator strains in mm			
	B. cereus	*E. coli*	*L. monocytogenes*	*S. aureus*
Skimmed Milk concentrate				
L. paracasei NAST-RHM82	14±0.32	16.5±0.54	16±0.60	15±0.43
L. helveticus NAST-RHL103	24±0.42	25±0.37	25.5±0.18	25±0.25
Methanol concentrate				
L. paracasei NAST-RHM82	17±0.23	18±0.18	21±0.35	16±0.52
L. helveticus NAST-RHL103	27.5±0.25	29±0.23	29.5±0.49	31.5±0.29
Methanol-acetone concentrate				
L. paracasei NAST-RHM82	19±0.44	19.5±0.32	22±0.17	19±0.50
L. helveticus NAST-RHL103	29±0.72	30±0.25	32±0.32	34±0.52

The zone of inhibition against all the indicator pathogen strains by methanol-acetone concentrate was found higher as compared to the skimmed milk concentrate and methanol concentrate of lactic isolates used. The zone of inhibition exhibited by using methanol-acetone concentrate was considerably higher for *L. helveticus* NAST-RHL103 for with *L. paracasei* NAST-RHM82 against all the indicator strains used. Methanol-acetone concentrates of *L. paracasei* NAST-RHM82 showed highest inhibition against *L. monocytogenes* (22 mm) followed by the same strain against *E. coli* (19.5 mm), *B. cereus* (19 mm) and *S. aureus* (19 mm). Similarly, Methanol-acetone concentrates of *L. helveticus* NAST-RHL103 showed highest inhibition

against *S. aureus* (34 mm) followed by the same strain against *L. monocytogenes* (32 mm), *E. coli* (30 mm) and *B. cereus* (29 mm) (Table 4.16).

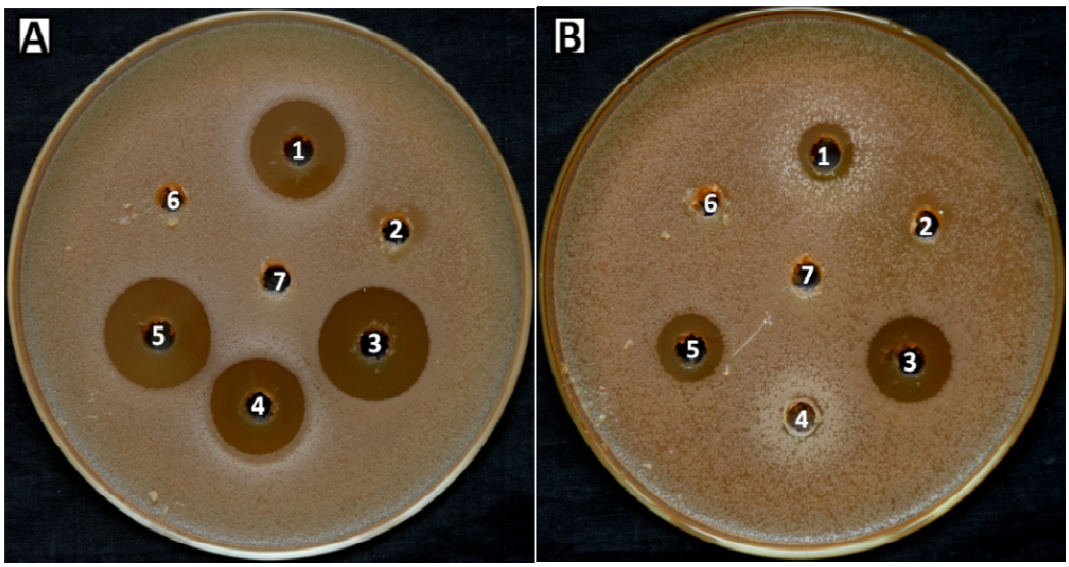

Figure 4.39. Plate assay for antimicrobial activity of skimmed milk, methanol & methanol-acetone concentrate of: (A) *L. helveticus* NAST-RHL103 and (B) *L. paracasei* NAST-RHM82 against food borne pathogens. In both the plates *B. cereus* lawn was inhibited by respective concentrates, the wells in both cases represents; (1) Skim milk concentrate (10X), (2) Acetone, (3) Methanol-acetone concentrate, (4) Methanol acetone sediment, (5) Methanol concentrate, (6) Methanol, (7) 1% lactic acid in D/W (pH 2.48).

Table 4.17. Protein estimation of CFSs and solvent extracts.

Lactic culture used to obtain Cell free supernatants (CFSs) from skimmed milk broth for antimicrobial assay	Bradford protein assay for protein content in various concentrates at different step of purification (μgmL^{-1})		
	Skim milk concentrate	Methanol concentrate	Methanol-acetone concentrate
L. paracasei NAST-RHM82	818±4.5	397±3.9	331±4.5
L. helveticus NAST-RHL103	853±6.5	631±4.2	489±3.8

The protein content in CFSs concentrate derived from skimmed milk culture broth, methanol concentrate and the methanol-acetone concentrate was found to be in decreasing order respectively (Table 4.17). The protein content of *L. helvetius* NAST-RHL103 was found to be higher than *L. paracasei* NAST-RHM82 the respective extracts. The protein level of the skimmed milk concentrate, methanol concentrate and methanol-acetone concentrate derived from *L. paracasei* NAST-RHM82 was

found to be 818, 397 and 331µg/mL respectively. Similarly the protein level of the skimmed milk concentrate, methanol concentrate and methanol-acetone concentrate derived from *L. helveticus* NAST-RHL103 was found to be 853, 631, 489 µg/mL respectively.

4.1.3.1.3. Determination of Arbitrary Unit mL^{-1} ($AUmL^{-1}$)

In terms of antibacterial activity in arbitrary unit per mL ($AUmL^{-1}$), only the CFSs and its concentrates (methanol and methanol-acetone extracts) of *L. helveticus* NAST-RHL103 showed the remarkable zone of inhibition against all of the four indicator strains (active cell number of each indicator strains used was 2×10^6 CFU/mL) (Table 4.18) while CFSs and its concentrates (methanol and methanol-acetone extracts) of *L. paracasei* NAST-RHM82 did not exhibit potential antibacterial activity against all four indicator strains under study.

Table 4.18. Arbitrary unit per mL of various concentrate derived from *L. helveticus* NAST-RHL103 against various food borne pathogens. A 100 µL of each concentrate was used against 2×10^6 CFU/mL every test pathogens.

Test Pathogen	Activity Profile [AU mL^{-1}]		
	Skimmed Milk concentrate	Methanol concentrate	Methanol-Acetone concentrate
B. cereus	252±2.30	425±3.24	440±4.00
E. coli	275±2.50	420±2.18	470±2.45
L. monocytogenes	287±1.75	325±2.80	350±2.50
S. aureus	327±2.16	445±3.55	470±3.40

Skimmed milk concentrate showed its highest activity against *S. aureus* (327 AU mL^{-1}) followed by the same against *L. monocytogenes* (287 AU mL^{-1}), *E. coli* (275 AU mL^{-1}) and *B. cereus* (252 AU mL^{-1}). Similarly methanol concentrate showed its highest activity against *S. aureus* (445 $AUmL^{-1}$) followed by the same against *B. cereus* (425 $AUmL^{-1}$), *E. coli* (420 $AUmL^{-1}$) and *L. monocytogenes* (325 $AUmL^{-1}$). Likewise, Methanol-acetone concentrate showed its highest activity against *E. coli* (470 $AUmL^{-1}$) and *S. aureus* (470 $AUmL^{-1}$) followed by the same against *B. cereus* (440 A mL^{-1}) and *L. monocytogenes* (350 AU mL^{-1}).

4.1.3.1.4. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration in $AUmL^{-1}$ estimated for *L. helveticus* NAST-RHL103 was found different for different concentrates against all indicator strains (Table 4.19).

Table 4.19. Minimum inhibitory concentration (MIC) of various concentrate derived from *L. helveticus* NAST-RHL103 against various food borne pathogens. A 100 µL of each concentrate was used against 2×10^3 CFU/mL every test pathogen.

Test Pathogen	MIC in $AUmL^{-1}$ in phases of partial purification		
	Skimmed Milk concentrate	Methanol extract	Methanol-Acetone Extract
B. cereus	205±3.00	217±2.18	220±2.85
E. coli	210±2.20	215±2.80	215±2.72
L. monocytogenes	225±2.5	240±1.80	250±2.60
S. aureus	285±2.50	285±2.35	290±2.60

The MIC value in $AUmL^{-1}$ of skimmed milk concentrate against *S. aureus* (285) was found to be highest followed by the same against *L. monocytogenes* (225), *E. coli* (210) and *B. cereus* (205). Similarly, the MIC value in $AUmL^{-1}$ of methanol concentrate was found to be highest against *S. aureus* (285) followed by the same against *L. monocytogenes* (240), *B. cereus* (217) and *E. coli* (215). Likewise, the MIC value in $AUmL^{-1}$ of the methanol-acetone concentrate was found to be highest against *S. aureus* (290) followed by the same against *L. monocytogenes* (250), *B. cereus* (220) and *E. coli* (215).

4.1.3.1.5. Scanning Electron microscopy for pathogens treated with methanol-acetone extract

The four pathogenic strains (2×10^3 CFU/mL) treated overnight with methanol-acetone extract (1:1 dilution) derived from *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 did not grow i.e. the turbidity in the tube was not seen indicating that the pathogens were killed by the methanol-acetone extract from respective lactic cultures used. In addition, the morphological appearance via scanning electron microscopy showed that the cells were damaged or injured as compared with the

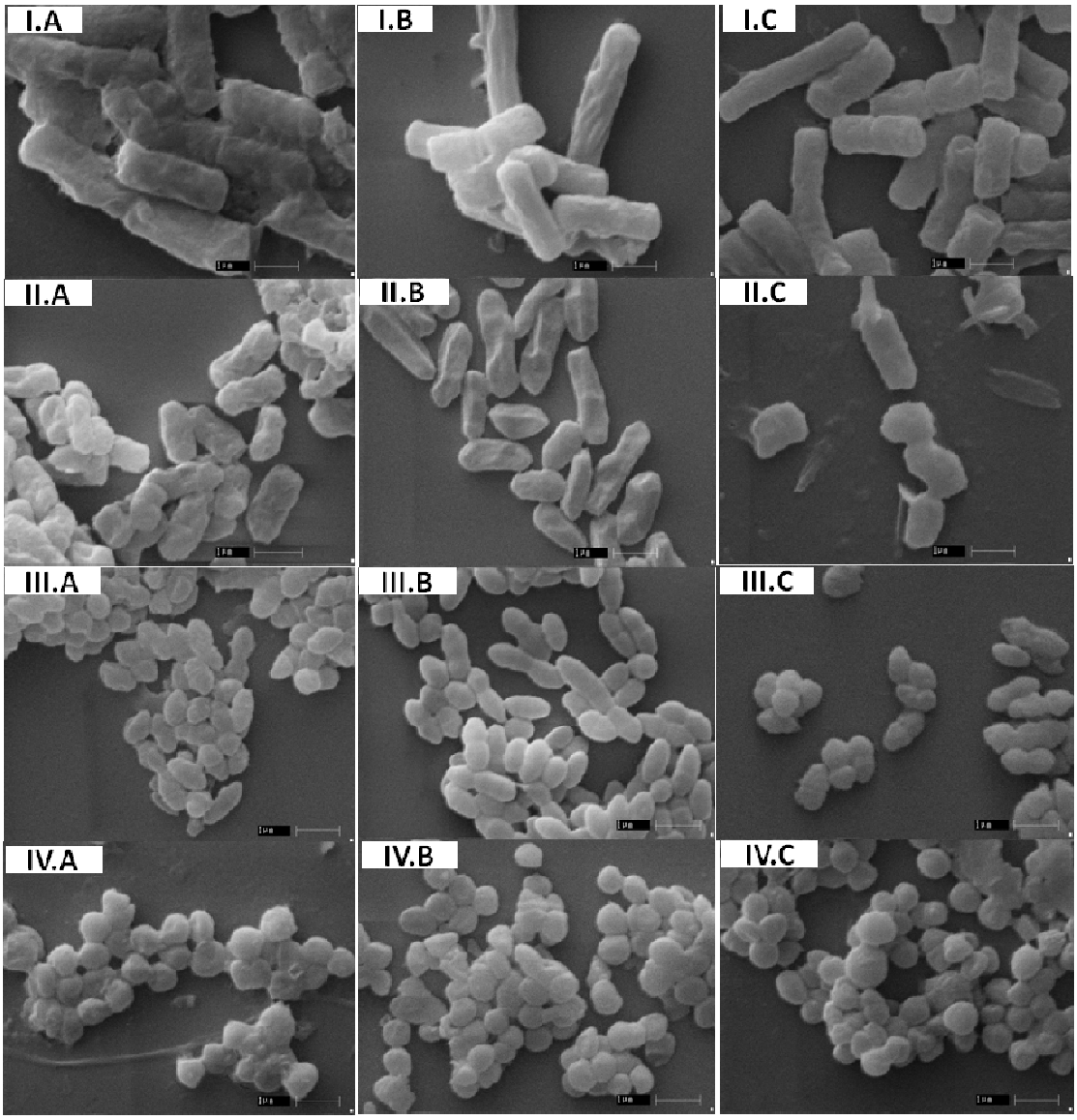

Figure 4.40. Scanning electron microscopy (40×10^3 magnification): I) *B. cereus*, II) *E. coli*, III) *L. monocytogenes*, IV) *S. aureus* and in the row: A) pathogens treated with methanol-acetone concentrate of *L. paracasei* NAST-RHM82, B) untreated pathogens, C) pathogens treated with methanol-acetone concentrate of *L. helveticus* NAST-RHL103.

untreated cells (Figure 4.40). The intensity of the damage or injury was more in case of pathogens treated with methanol-acetone extract of *L. helveticus* NAST-RHL103 than *L. paracasei* NAST-RHM82. Besides, cell injury the cells were found to have unusual morphological appearance like the rough edge of the cells, clumping of the cells, or holes in the cells. This results support the previous findings regarding antimicrobial activity mentioned in earlier sections.

4.1.3.2. Product development using *Lactobacillus helveticus* NAST-RHL103 and *Lactobacillus paracasei* NAST-RHM82

4.1.3.2.1. Preparation and characterization of Probiotic Curd (*Dahi*)

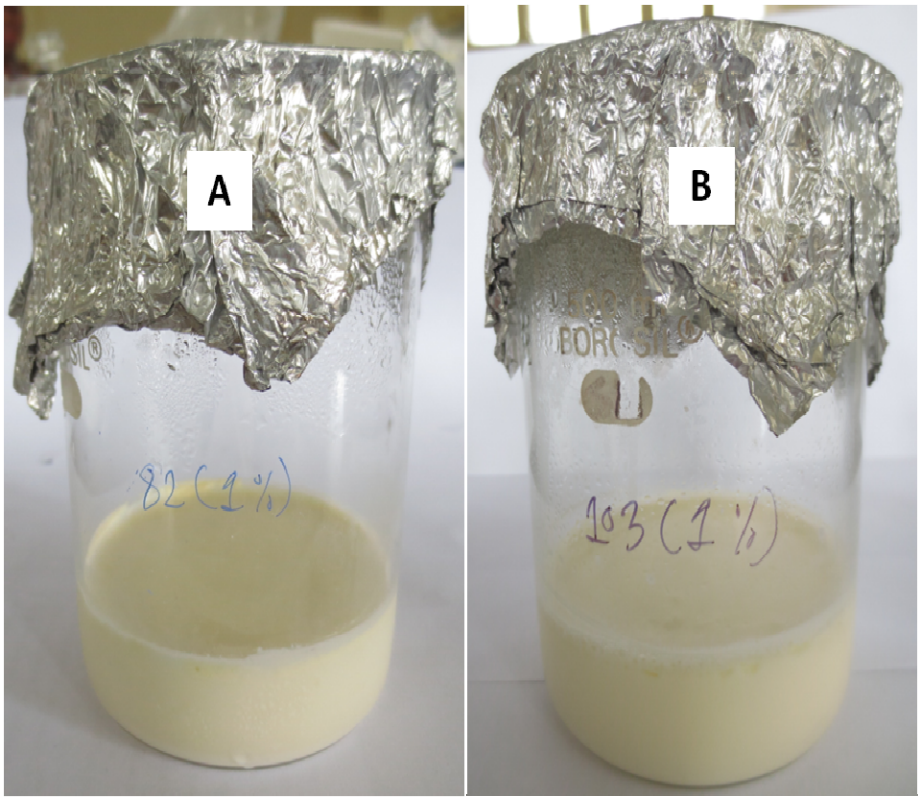

Figure 4.41. Probiotic Curd (*Dahi*) prepared by using (A) *L. paracasei* NAST-RHM82 and (B) *L. helveticus* NAST-RHL103.

Probiotic Curd was prepared using two lactic cultures *Lactobacillus helveticus* NAST-RHL103 and *Lactobacillus paracasei* NAST-RHM82 separately (Figure 4.41). The analysis of the products including microbiological and chemical properties is given in following section.

4.1.3.2.1.a. Microbiological and chemical properties

At first, the preparation method was optimized for inoculum size, setting (incubation) time, pH, titratable acidity, viable count and others. The optimized condition (only optimized data shown here) were found to be varied for the two lactic cultures (Table 4.20) particularly the setting time for curdling differed a lot in between these two cultures. One percent of inocolum at 37 ºC for 30 hours formed a good quality curd using *L. paracasei* NAST-RHM82 with titratable acidity = 0.792 %, pH = 4.72 and

viable count Log10 CFU/mL = 13.14 while one percent of inoculum at 37 ºC for 8 hours formed a good quality of curd using *L. helveticus* NAST-RHL103 with titratable acidity = 0.720%, pH = 4.52 and viable count Log10 CFU/mL = 16.02.

Table 4.20. Chemical and microbiological properties of Probiotic curd (*Dahi*) prepared using *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103.

Lactic culture used	Inoculum size (%)	Temp (^{0}C)	Time (hr)	pH	Titratable acidity (%)	Viable count Log10 CFU/mL
L. paracasei NAST-RHM82	1	37	30	4.72±0.02	0.792±0.003	13.14±0.18
L. helveticus NAST-RHL103	1	37	8	4.52±0.03	0.720±0.005	16.02±0.24

The curd produced with optimized conditions (Table 4.20) was also analyzed for likely changes during storage in refrigerator. For this, the parameters such as pH, titratable acidity and viable count were considered. Some minor changes in pH and tritratable acidity were noted but the viable count remained nearly same. A little decrement in pH was found while titratable acidity was increased slightly. The pH and titratable acidity remained within the acceptable limits (Table 4.21). The curd produced using *L. paracasei* NAST-RHM82 showed some decrement in pH from 4.72 to 4.60 while titratable acidity increased slightly from 0.729 to 0.92 % in 12 hours of storage time in the refrigerator. Similarly, the curd produced using *L. helveticus* NAST-RHL103 showed some decrement in pH from 4.52 to 4.30 while titratable acidity increased slightly from 0.720 to 1.08 % in 12 hours of storage time in the refrigerator. The viable count of the respective lactic cultures nearly remained same during the storage time. The changes in both the cases were expected and up to the acceptable limits. The curd prepared with optimized conditions was used for fat extraction and fatty acid profiling using gas chromatography. The percentage of fat was estimated for both cultures and was found to be 0.804±0.015 % and 0.691±0.012% for *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103 respectively. The fatty acid composition and their chromatogram present in the probiotic curd prepared from two lactic cultures and a commercial curd named, 'Commercial curd' is presented in Table 4.22 and Figure 4.42, 4.43 and 4.44. The peaks (peak no. 1-25) in

Table 4.21. Changes in chemical and microbiological properties during storage in refrigerator of Probiotic Curd (*Dahi*) prepared by using *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103.

Lactic culture used for curd preparation	changes during storage in refrigerator after product formation								Viable count Log10 CFU/mL
	pH				Titratable acidity				
	0 h	3 h	6 h	12 h	0 h	3 h	6 h	12 h	
Lb. paracasei NAST-RHM82	4.72 ±0.01	4.65 ±0.02	4.60 ±0.01	4.58 ±0.01	0.792 ±0.010	0.828 ±0.012	0.900 ±0.010	0.92 ±0.013	13.14±0.18 (0 h)
Lb. helveticus NAST-RHL103	4.52 ±0.02	4.40 ±0.03	4.33 ±0.01	4.30 ±0.02	0.720 ±0.012	0.792 ±0.011	0.936 ±0.010	1.08 ±0.014	16.02±0.24 (0 h)

in the chromatogram (Figure 4.42) obtained from gas chromatography represents the fatty acids found in the curd prepared using *L. paracasei* NAST-RHM82. The details of the peaks and their corresponding retention time, carbon atom and respective fatty acids are listed in (Table 4.22). Fourteen different fatty acids were found in this *dahi* sample. Similarly, the peaks (peak no. 1-27) in the chromatogram (Figure 4.43) obtained from gas chromatography represents the fatty acids found in the curd prepared using *L. helveticus* NAST-RHL103. The details of the peaks and their corresponding retention time, carbon atom and respective fatty acids are listed in Table 4.22. Fourteen different fatty acids were found in this curd sample. Unlike the experimental curd samples, the control showed one additional fatty acid *viz.* butyric acid. The peaks (peak no. 1-25) in the chromatogram (Figure 4.44) represent the fatty acids found in commercial curd (Mysore, India) (Table 4.22).

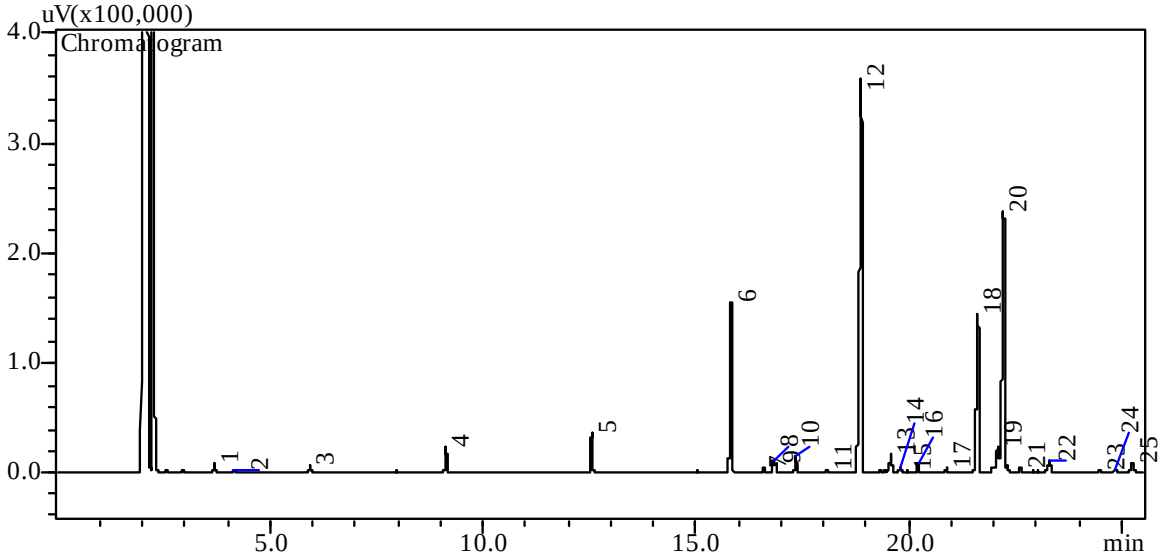

Figure 4.42. Chromatogram: Fatty acid profiling of Probiotic curd prepared using *L. paracasei* NAST-RHM82 by gas chromatography (GC).

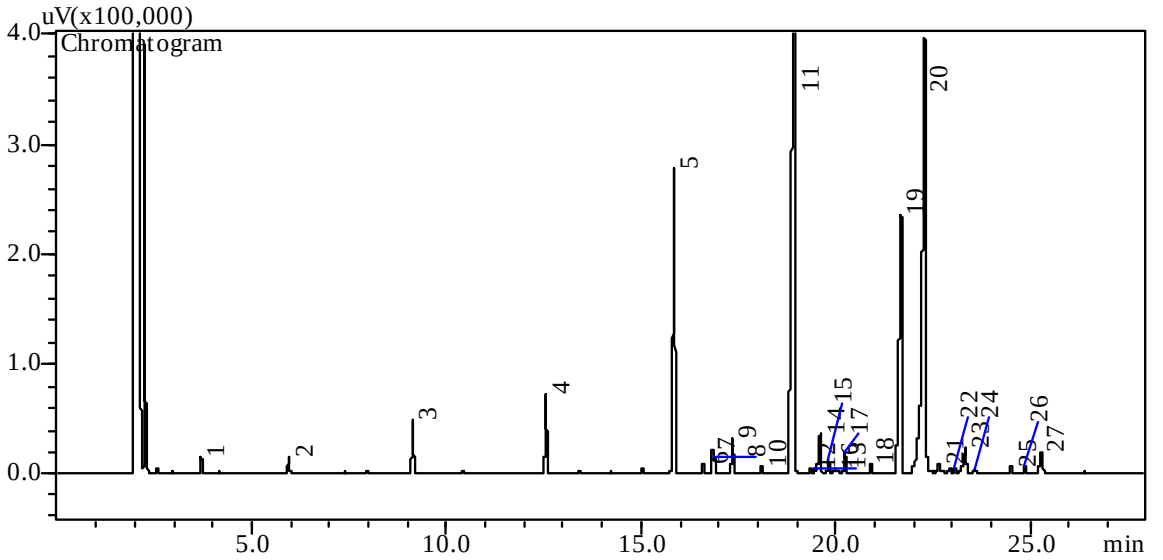

Figure 4.43. Chromatogram: Fatty acid profiling of Probiotic curd prepared using *L. helveticus* NAST-RHL103 by gas chromatography (GC).

Table 4.22. Qualitative analysis of fatty acid by Gas Chromatography indigenous curd prepared from two lactic cultures.

Chromatogram Peak	FA profiling form curd prepared from *L. paracasei* NAST-RHM82			FA profiling form curd prepared from *L. helveticus* NAST-RHL103			FA profiling form commercially available Commercial curd		
	Ret. time	Carbon atom	Fatty acid	Ret. time	Carbon atom	Fatty acid	Ret. time	Carbon atom	Fatty acid
1	3.7	6.0	Caproic	3.7	6.0	Caproic	2.6	4	Butyric
2	4.2		Caprylic	6.0	8.0	Caprylic	3.7	6.0	Caproic
3	5.9	8	Capric	9.1	10	Capric	6.0	8.0	Caprylic
4	9.1	10	Lauric	12.6	12	Lauric	9.2	10.0	Capric
5	12.6	12	Myristic	15.9	14	Myristic	12.6	12.0	Lauric
6	15.8	14		16.6			15.9	14.0	Myristic
7	16.6			16.8	14-1	Myristoeic	16.7		
8	16.8	14-1	Myristoeic	16.9			16.9	14-1	Myristoeic
9	16.9			17.4	15	Pentadecenoic	16.9		
10	17.3	15	Pentadecenoic	18.1			17.4	15.0	Pentadecenoic
11	18.1			18.9	16	Palmitic	18.2		
12	18.9	16	Palmitic	19.4			18.9	16.0	Palmitic
13	19.6	16-1	Palmitoleic	19.5			19.6	16-1	Palmitoleic
14	19.8			19.6	16-1	Palmitoleic	19.9		
15	20.0			19.8			20.3	17.0	Heptadecenoic
16	20.2	17	Heptadecenoic	20.0			21.0		
17	20.9			20.2	17	Heptadecenoic	21.7	18.0	Stearic
18	21.6	18	Stearic	20.9			22.1	18-1t	18-1 trans
19	22.1	18-1t		21.7	18	Stearic	22.2	18-1t	18-1 trans
20	22.2	18-1c	Oleic	22.3	18-1	Oleic	22.3	18-1	Oleic
21	22.6			22.7			22.4		
22	23.3	18-2	Linoleic	23.1			22.7		
23	24.5			23.3	18-2	Linoleic	23.4	18-2	Linoleic
24	24.8			23.6			24.6		
25	25.3	22	Behenic	24.5			25.4		
26				24.9					
27				25.3	22	Behenic			

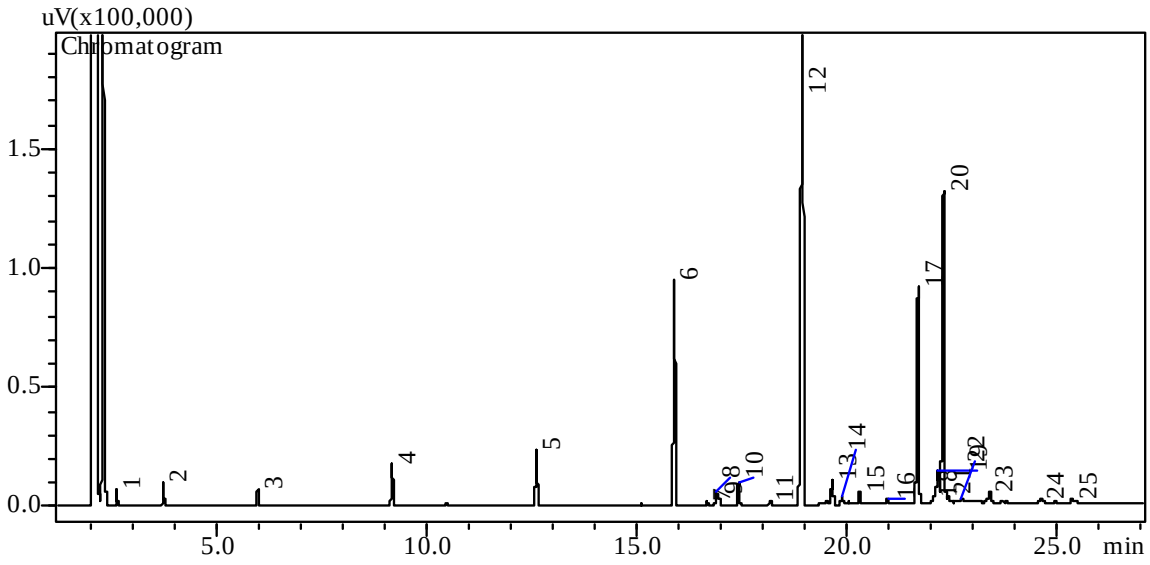

Figure 4.44. Chromatogram: Fatty acid profiling of commercially available Commercial curd by gas chromatography (GC).

4.1.3.2.1.b. Organoleptic properties (Sensory profiling)

The results of sensory analysis, indicated that Curd samples A (prepared by using *L. paracasei* NAST-RHM82) and B (prepared by using *L. helveticus* NAST-RHL103) had slightly lower intensity of whiteness value compared to sample C (locally available commercial curd, Mysore, India) (Figure 4.45), which was further reflected in instrumental colour measurement of Lightness value (L) showing higher lightness value for sample C (88.70). The instrumental colour measurement showed 87.71 and 87.80 lightness value for sample A and B respectively. There were no significant differences between sample-A and sample-B for set and for mouth feel properties such as creaminess and smoothness. However sample C was of loose set. Samples B and C had less creamy aroma and milky note compared to sample A. Sample A had less score for fermented aroma (5) and sourness (4). Results showed that all the three curd samples are acceptable, however, sample-A had higher acceptance indicating a score of 12 for overall quality compared to other samples.

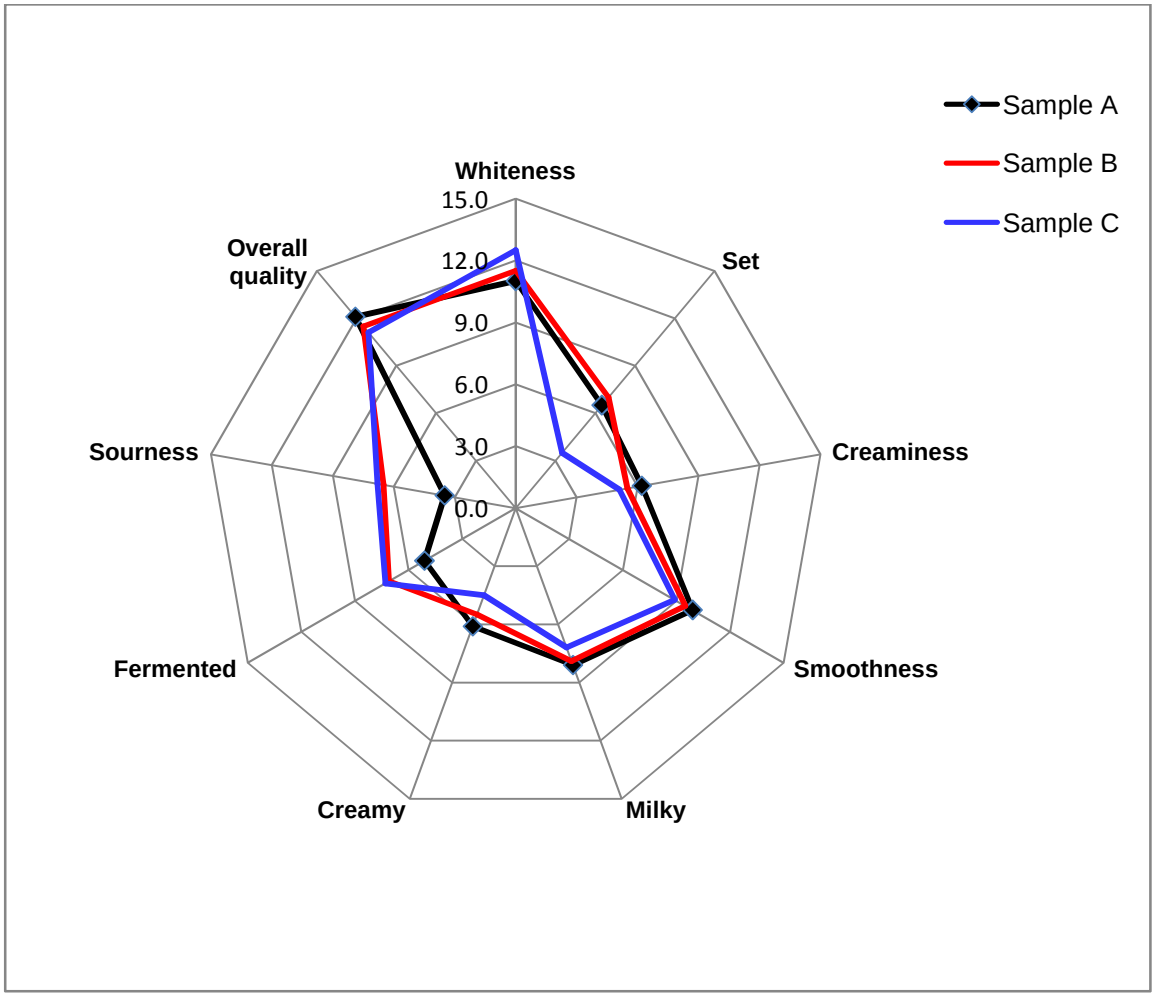

Figure 4.45. Sensory Profile of Probiotic Curd Sample: A) *L. paracasei* NAST-RHM82, B) *L. helveticus* NAST-RHL103 and C) Commercial curd (Mysore, India).

4.1.3.2.1.c. Preprocessing contaminant: Effect of Probiotic Curd towards selected food borne pathogens

To understand the antagonistic effect of probiotic curd (*dahi*), preprocessing contaminant effect study was performed. The starter lactic culture and the indicator pathogenic strains (four indicator strains inoculated separately) were inoculated simultaneously and incubated for curding time. The initial inoculums count of each pathogen was 2×10^4 CFU/mL, which was slightly decreased in both the cases (two lactic culture product separately with each indicator strains) (Table 4.23). In the product with *L. paracasei* NAST-RHM82, the preprocessing contaminant *E. coli* (Log10 CFU/mL = 4.96) was found to be highest followed by *B. cereus* (Log10 CFU/mL = 4.11), *S. aureus* (Log10 CFU/mL = 4.14) and *L. monocytogenes* (Log10 CFU/mL = 4.12) whereas the lactic culture count in terms of Log10 CFU/mL was found between 12.38 and 13.06. On the contrary, the preprocessing contaminants

number was decreased more in the product with *L. helveticus* NAST-RHL103. In this product, *E. coli* (Log10 CFU/mL = 4.19) were found to be highest followed by *S. aureus* (Log10 CFU/mL = 3.87), *B. cereus* (Log10 CFU/mL = 3.38) and *L. monocytogenes* (Log10 CFU/mL = 2.26) whereas the lactic culture count in terms of Log10 CFU/mL was found to between 13.41 and 14.05.

Table 4.23. Preprocessing contaminant effect of the Probiotic Curd towards selected food borne pathogens.

Indicator Pathogenic strains	Respective Count (Log10 CFU/mL) in curd prepared using			
	L. paracasei NAST-RHM82		*L. helveticus* NAST-RHL103	
	Pathogen	LAB	Pathogen	LAB
B. cereus	4.11±0.13	13.06±0.85	3.38±0.15	13.41±0.45
E. coli	4.96±0.11	12.38±0.76	4.19±0.10	13.67±0.68
L. monocytogenes	4.12±0.13	12.54±0.55	2.26±0.12	14.05±0.54
S. aureus	4.14±0.12	12.91±0.60	3.87±0.12	13.51±0.36

4.1.3.2.1.d. Post-processing contaminant: Effect of Probiotic Curd towards selected food borne pathogens

Equal chances are there to enter contaminant after preparation of the product till use. Sometimes contaminant may likely to enter even when the product is stored inside the refrigerator. To understand antibacterial action of the product against the pathogens (contaminated after product formation) was investigated as post processing contaminant effect. The post-processing contamination effect was studied using 2×10^4 CFU/mL of pathogens at 12 and 24 hours of time interval after storage in refrigerator. All the pathogens were killed after 24 hours for both curd prepared by different the cultures (Table 4.24).

Post processing contaminants (pathogens) in the product prepared using *L. paracasei* NAST-RHM82 were killed after 12 hours of storage in refrigerator. In contrary, the post processing contaminants remained in few numbers in the product prepared using *L. helveticus* NAST-RHL103 after 12 hours, but all the contaminants were killed only after 24 hours of storage (Table 4.24).

Table 4.24. Post processing contaminant effect of the Probiotic Curd against selected food borne pathogens.

Indicator Pathogenic strains	Respective Count (CFU/mL) in Curd prepared using			
	L. paracasei NAST-RHM82		*L. helveticus* NAST-RHL103	
	12 h/4 ºC	24 h/4 ºC	12 h/4 ºC	24 h/4 ºC
B. cereus	0	0	3±0.1.50	0
E. coli	0	0	4±1.00	0
L. monocytogenes	0	0	11±2.35	0
S. aureus	0	0	1±0.50	0

Indicator strains (individual pathogens) decreased considerably in the experiment performed to analyze the effect of probiotic curd on the food borne pathogens (with an increased cell number; 2×10^6 CFU/mL than the previous experiment performed with only 2×10^6 CFU/mL) by adding ten times concentrated cell free supernatant of skimmed milk culture broth of *L. helveticus* NAST-RHL103 into the curd prepared from the same strain i.e. *L. helveticus* NAST-RHL103 (Table 4.25). In this product, 1:1 dilution of the skimmed milk concentrate did not kill the pathogens much even after 24 hours while the direct (1X) skimmed milk concentrate reduced *B. cereus* to zero number after 24 hours of storage in refrigerator and the other pathogens such as *E coli, L. monocytogenes* and *S. aureus* remained as 8, 24 and 108 CFU/mL

Table 4.25. Post processing contaminant with skimmed milk concentrate (10X): Effect of the Probiotic Curd (using *L. helveticus* NAST-RHL103) towards selected food borne pathogens.

Indicator Pathogenic strains	Respective Count (CFU/mL) in Curd			
	Skimmed Milk Concentrate (1:1 X)		Skimmed Milk Concentrate (1X)	
	12 h/4 ºC	24 h/4 ºC	12 h/4 ºC	24 h/4 ºC
B. cereus	$11\times10^1\pm40$	20±3	6±1	0±0
E. coli	$24\times10^3\pm34$	$51\times10^2\pm35$	$49\times10^1\pm11$	8±1
L. monocytogenes	$292\times10^2\pm46$	$204\times10^2\pm25$	$130\times10^1\pm9$	24±2
S. aureus	$108\times10^2\pm30$	$136\times10^1\pm19$	$236\times10^1\pm14$	108±13

respectively after 24 hours in the refrigerator. This experiment was found to be notable against the *B. cereus*.

4.1.3.3.2.2. Sweet Curd (*Dahi*) preparation using *L. helveticus* NAST-RHL103

The thick milk (evaporated milk) with different sugar concentration (10%, 15% and 20%) inoculated with one percent inoculums of *L. helveticus* NAST-RHL103 was set after 16 hours at 37 ºC while the milk with 25% sugar did not formed the product. The setting time was double (16 hours in this case) as compared to the Probiotic Curd preparation, however the sensory evaluation indicated that sweet curd prepared with 20% sugar concentration was the best. The sugar utilization was found to be 5.4% of the respective sugar concentration in 15% and 20% milk broth (Table 4.26 and Figure 4.47). The colour of the fermented sweet curd resulted slightly faint cream colour as compared to control which was dark cream colour (Figure 4.46).

4.1.3.2.2.a. Microbiological, chemical and organoleptic properties

The microbiological and chemical parameters were also found to be varied but within the acceptable limit (Table 4.26). Chemical properties such as pH, titratable acidity and sugar utilization were measured. The product with 20% sugar content had the

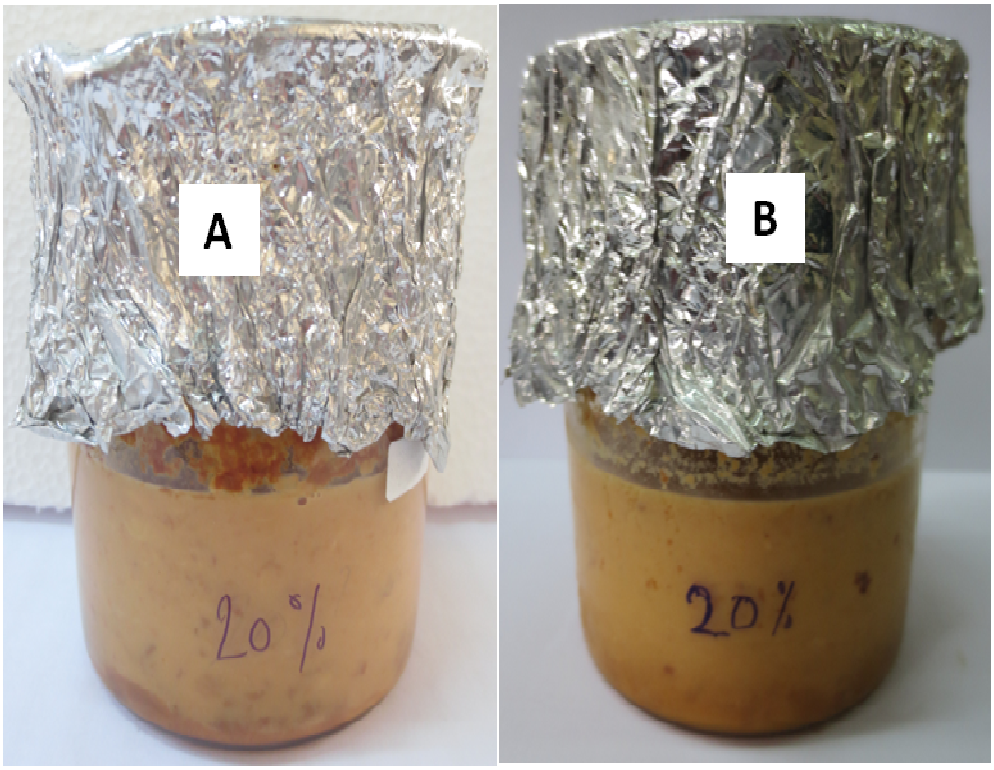

Figure 4.46. Sweet Curd prepared by *using L. helveticus* NAST-RHL103 at 20% Glucose level: A) Concentrated milk before fermentation and B) Sweet curd after fermentation.

highest pH value (pH 4.62) while the product with 10% and 15 % had pH 4.03 and pH 4.42 respectively. The titratable acidity was highest for the product with 15% (0.65%) sugar while 10% and 15% had titratable acidity 0.58% and 0.44 % respectively. The total sugar utilization was 5.4% of the initial sugar content for the product with 15% and 20% sugar while only 1.8% of sugar was utilized in the product with 10% sugar content (Figure 4.47). The viable count slightly varied among the three different sugar content products.

Table 4.26. Chemical and Microbiological properties of Sweet Curd prepared by *L. helveticus* NAST-RHL103.

% of sugar used in Sweet curd	Inoculum (Log10 CFU/mL)	Temp (ºC)	Time (hr)	pH	Titratable acidity (%)	Sugar utilized (%)	Viable lactic count Log10 CFU/mL
10	7.2	37	16	4.03±0.01	0.58±0.09	1.8±0.55	10.04±0.11
15	7.2	37	16	4.42±0.01	0.65±0.06	5.4±0.52	9.63±0.23
20	7.2	37	16	4.62±0.02	0.44±0.04	5.4±0.60	8.95±0.16

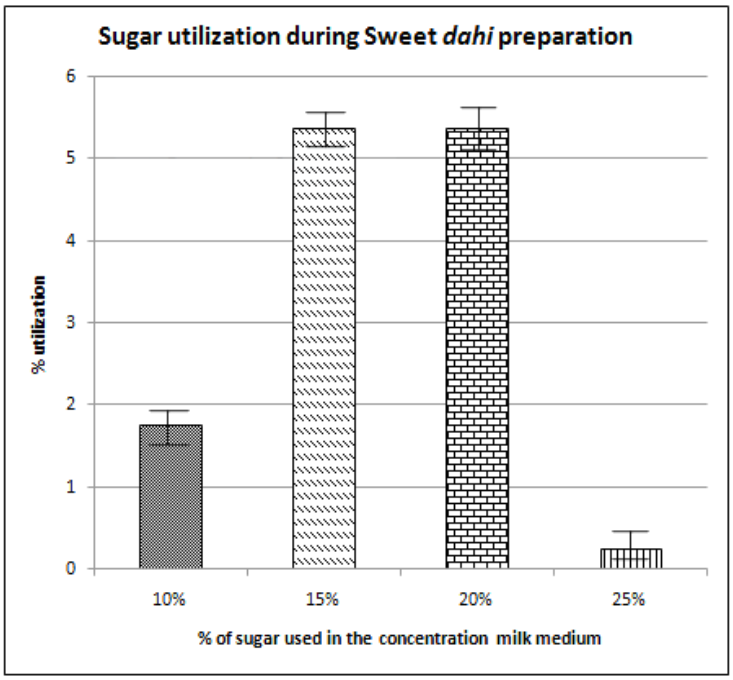

Figure 4.47.2 Graph for sugar utilization during Sweet curd fermentation at different level sugar by *L. helveticus* NAST-RHL103.

The highest viable count was found in the product with 10% sugar (Log10 CFU/mL = 10.04), followed by 15% sugar content product (Log10 CFU/mL = 9.63) while least count was found in the product with 20% sugar (Log10 CFU/mL = 8.95). Among three, product with 20% sugar was found to be accepted during sensory analysis.

4.2. Discussion

4.2.1. Isolation, biochemical and molecular characterization *Lactobacillus* spp. from traditionally prepared curd (*dahi*) at geo-climatic conditions of Nepal

The identification and characterization of autochthonous *Lactobacillus* strains are important for understanding their contribution to the sensorial characteristics of the final product and for providing new strains to be used as industrial starters (Fortina et al., 2001). The genus *Lactobacillus*, in fact, has a dominant role over other bacterial genera in food fermentation technologies (e.g., *L. delbrueckii, L. helveticus, L. plantarum*) and probiotic applications (e.g., *L. paracasei, L. rhamnosus*). For these reasons, 120 rod-shaped isolates were selected for further studies (Table 4.6).

The isolation and screening of lactic acid bacteria (LAB) from natural sources have been one of the powerful means to obtain strains for the food and medical applications. LAB are generally recognized as safe (GRAS) microorganism which have been used for centuries in food fermentation and preservation (Nes & Johnsborg, 2004; Deegan et al., 2006; Lee & Kim, 2010; Fernandez et al., 2014). For identification and analysis of LAB activities, many of the modern molecular tools are based on 16S ribosomal sequences, complete or partial genomes, or specific fluorescent probes that monitor the physiological activity of microbial cells. These high throughput approaches are increasingly applied to strains of LAB (Clevenland et al., 2001; Nami et al., 2015) and bifidobacteria that provide health benefits and are marketed as probiotic bacteria. The primary purpose of these approaches is to provide proper strain identification as required for legal and good manufacturing practices. In addition, these identification tools can be used to trace and track LAB including probiotics in the production phase and in food products as well as after consumption in the intestinal tract. Moreover, many of these identification tools can be instrumental in the selection of new strains or species of LAB or bifidobacteria as starters, flavor developments, or to be developed into probiotics. A series of functional approaches

are being developed and validated that can further be used in controlling quality (Taverniti & Guglielmetti, 2010).

4.2.1.1. Isolation and Biochemical (Phenotypic) Characterization of Lactic Acid Bacteria (*Lactobacillus* species)

The traditionally prepared curd samples were collected from different geographical regions for the present investigation. The viable mesophilic counts of LAB on MRS medium varied in the range of 6.3 to 10.4 Log10 $CFUg^{-1}$ (Table 4.5). The curd samples produced in the Lalitpur and Bhaktapur districts showed higher LAB count than those produced in Gorkha district. However, in case of Rasuwa districts, the LAB count was slightly higher in Hilly region as compared to Himalayan region. In total, two hundred and five bacterial isolates were obtained from 64 samples; among them, 193 were considered as presumptive LAB by their Gram positive reaction, of which negative catalase reaction, and lack of motility. The majority of isolates was rod shaped and, therefore, probably belongs to the genus *Lactobacillus* and the remaining were cocci (73 isolates). Previous study of Dewan & Tamang (2007) also found dominance of lactobacilli in traditional dairy products of the Himalayan region of India including few *dahi* samples collected from Nepal. They reported 73.5% of lactobacilli out of total 128 isolates obtained from 58 samples of Himalayan ethnic fermented milks. The LAB count in the milk products ranged from 10^7 to 10^8 $CFUg^{-1}$. They identified *Lactobacillus bifermentans, Lactobacillus paracasei* subsp. *pseudoplantarum, Lactobacillus kefir, Lacto- bacillus hilgardii, Lactobacillus alimentarius, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus plantarum, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* in the dairy products. However, their study was limited to phenotypic characterization including API sugar test. Our investigation for identification and characterization of the LAB present in traditionally prepared curd is based phenotypic and genotypic attributes.

4.2.1.2. Identification and genetic characterization of the lactobacilli using various molecular markers

At first, the 120 *Lactobacillus* isolates were considered for molecular analysis and identification at species level. To this aim, the isolates were clustered in ten groups according to electrophoretic profiles obtained by PCR amplification of the 16S-23S

rRNA spacer region (ITS) (Figure 4.4a). The taxonomic identification was then reached by 16S rRNA gene sequencing of representative isolates for each group and confirmed by species-specific amplifications of all strains belonging to the same group. For all the groups, the ITS profile was characteristics of one *Lactobacillus* species. The profile of Group 2 and 3 look similar in banding pattern but little differences was observed and which later identified as *L. paracasei* (Group 2) (Figure 4.6e) and *L. rhamnosus* (Group 3) (Figure 4.6f). These two species are related species and belong to *L. casei* group of species. *Lactobacillus casei* group (Lcs) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei* and *L. rhamnosus*) (Blaiotta et al., 2008). High resolution melting analysis (HRMAa) technique (Koirala et al., 2015) was applied to a 150 bp groEL gene fragment. The analysis was performed on *L. paracasei* and *L. rhamnosus* isolates together with reference strains as mentioned in materials and methods section. The HRMa clustered Lcs strains in three groups that exactly corresponded to the species of the *L. casei* group. Also, two distinct clusters were observed (Figure 4.17 A) for *L. paracasei* species which later revealed that two clusters (within *L. paracasei* cluster, Figure 4.17 B) represents two subspecies; *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*. All the *L. paracasei* isolates from *dahi* samples were identified as *L. paracasei* subsp. *paracasei*. Finally, an intraspecies-specific PCR analysis (Torriani et al., 1999) was carried for all *L. delbrueckii* isolates (Group 1, Figure 4.4a) to a precise subspecies (4.6 C & D). Phylogenetic trees based on the 16S rDNA partial sequence displayed high consistency regarding relationship between the organisms obtained in our study and obtained from genbank database. All the nodes leading to Nepalese curd isolates strain cluster are supported by high bootstrap values (Figure 4.4b). Also, curd isolates were designated to the correct species with close homology (Ennahar, 2003).

On the basis of molecular analysis (i.e. RSA profiling and species specific PCR), the 120 isolates were classified as belonging to ten different species (Table 4.7 Figure 4.5): 1) *L. delbrueckii* subsp. *bulgaricus* (46 isolates, ITS Group 1), 2) *L. paracasei* (30 isolates, Group 2), 3) *L. rhamnosus* (21 isolates, Group 3), 4) *L. fermentum* (12 isolates, Group 4), 5) *L. parabuchneri* (5 isolates, Group 5), 6) *L. helveticus* (2 isolates, Group 6), 7) *L. coryniformis* (1 isolate, Group 7), 8) *L. harbinensis* (1 isolate,

Group 8), 9) *L. brevis* (1 isolate, Group 9) and 10) *L. plantarum* (1 isolate, Group 10).

Based on our data, homofermentative *L. delbrueckii* subsp. *bulgaricus* can be considered as one of the dominant lactobacilli in *dahi* products. It accounted for 38% of the total isolates and was the predominant *Lactobacillus* population in the majority of the samples. The isolation of predominant *L. delbrueckii* from the traditional dairy products was previously reported (Dellaglio et al., 2005; Watanabe et al., 2008). Particularly, *L. delbrueckii* subsp. *bulgaricus* was shown as the most abundant LAB species also in market *dahi* products from Pakistan (Soomro & Masud, 2012). However, in this study Gorkha district samples were found to lack *L. delbrueckii* strains which indicates that these strains are either not found in that region or are very less in number or the geo-climatic conditions of Gorkha region (Table 4.7, Figure 4.5) do not support the growth of this bacteria in the dairy product or more study involving large number of samples substantiate this result to reconfirm the availability of this bacteria in this geo-climatic condition. Besides the species *L. casei* or *paracasei* was already reported as dominant group of lactobacilli in *dahi* (Dewan & Tamang, 2007; Soomro & Masud, 2012). Particularly, we observed that the facultative heterofermentative *L. paracasei* (accounting for 25 % of the total isolates) characterizes samples of *dahi* produced in Gorkha district. In general, we found a clearly different distribution of lactobacilli in the diverse dairy samples, which can be explained by the well-known importance of the environment on the relative distribution of different bacterial groups in the microbial ecosystems. Thus, animal origin of the milk, altitude, different technical conditions of product preparation, and any potential factor determining temperature shifts could have affected the bacterial competition during fermentation process. The two other most abundant lactobacilli resulted *L. rhamnosus* (18% of isolates) and *L. fermentum* (10%) (Table 4.7). These two species were reported to be isolated from *dahi* samples of India. However, their identification was based exclusively on sugar fermentation patterns (Bhardwaj et al., 2012). In addition, a few other *Lactobacillus* species were also found at very low frequency in some samples (Table 4.7, Figure 4.5). Several other *Lactobacillus* species have been previously reported in traditionally prepared curd or *dahi* and other milk products from South Asian countries (Bao et al., 2010; Bao et al., 2012; Satish et al., 2013, Milind & Jyoti, 2014) while some *Lactobacillus* species not found in our

study such as *L. bifermentans* and *L. alimentarius* have also been reported (Dewan & Tamang 2007). Our study indicates that the Himalayan ethnic fermented milk products are a rich source of different lactobacilli confirming previous results of Satish et al. (2013).

The technological properties and the probiotic potential of microorganisms are markedly strain specific, for this reason, we undertook the molecular finger printing of the main *Lactobacillus* species characterizing the curd (*dahi*) samples using RAPD and REP-PCR, which are techniques possessing intraspecies discriminatory power (Ljungh & Walstrom, 2009). Notably, these analyses revealed that *L. rhamnosus* isolates, although originating from samples collected from different geographical regions, had identical/monomorphic fingerprints with all the tested primers (Figure 4.15, O, P, Q, R). These isolates might have been transported from low altitudes to high altitudes during the back slopping method of starter culture addition during traditional method of *dahi* preparation (Table 2-5) or could have very less impact of geo-climatic conditions in their genetic makeup. On the contrary, *L. delbrueckii* subsp. *bulgaricus, L. paracasei, L. fermentum* and *L. parabuchneri* (Figure 14-17) isolates showed a high degree of genetic diversity at the strain level. More specifically, the cluster analysis resulting from the combined patterns of the different primer sets revealed that most of the profiles within each species were unique (Figure 10-13). The difference between the correlation coefficient values of Jaccard (J), Dice (D) and Simple Matching coefficient (SM) were not very different in respective study of the four lactobacilli assessed for intra-species genetic diversity. According to Rohlf (2009), if the correlation coefficient value, r is such that $0.8 \leq r < 0.9$, the similarity coefficient is interpreted as well fit. Because of the highest correlation value (Jaccard's correlation coefficient values were 0.93819, 0.84041, 0.94090, and 0.92868 for *L. delbrueckii* subsp. *bulgaricus, L. paracasei, L. fermentum* and *L. parabuchneri* respectively), Jaccard's coefficient of similarity was evaluated as the best for deducing the genetic relationship among the *Lactobacillus* species accessing UPGMA module. Considering that technological and probiotic features of microorganisms are strain specific, this result is of interest in light of possibility to select new potential probiotic strains or starter cultures to be used at industrial level.

4.2.2. *In vitro* probiotic properties of *Lactobacillus* spp. isolated from traditionally prepared Curd (*Dahi*) of Nepal

Various *in vitro* studies have been used to evaluate various characteristics of potential probiotic bacteria. Among these, tolerance to low pH of the stomach and the bile content of the upper parts of the intestines and the ability of bacteria to colonize the intestinal tract are important characteristics that have been studied widely (Naidu et al., 1999). Adherent strains are preferred, since their establishment in the intestine seems to be necessary characteristics for the probiotic effects to be exerted (Lee & salminen, 1995). Besides, strains with antimicrobial properties and lack of antibiotic resistant genes are considered as potential probiotic candidates (Felis & Dellaglio, 2005).

Also, lactic acid starter bacteria are widely used in combination with probiotic (*Bifidobacterium, Lactobacillus*) bacteria to manufacture fermented dairy products. Commercial strains of *L. acidophilus, L. casei*, *L. rhamnosus* and *Bifodobacterium* spp. are added to fermented milks, like youghurt (manufactured with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* cultures) and probiotic cheese (manufactured with *Streptococcus* and *Lactococcus* cultures) (Vinderola et al., 2000a & 2000b). Due to their poor ability to survive through the passage of the stomach and the gastrointestinal tract, lactic acid starter bacteria were usually not thought to be probiotics (IDF, 1999). However, more criteria (Lee et al., 1999; Naidu et al., 1999) have been assessed which prompted to include various LAB as probiotic bacteria.

In the present study, the *in vitro* probiotic attributes of twenty four lactic culture isolates belonging to ten different *Lactobacillus* species obtained from traditionally prepared curd or *dahi* at different geo-climatic conditions in Nepal were; *L. delbrueckii* subsp. *bulgaricus* (6 isolates), *L. paracasei* (6 isolates), *L. fermentum* (3 isolates), *L. rhamnosus* (2 isolates), *L. parabuchneri* (2 isolates), *L. helveticus* (one isolate), *L. brevis* (one isolate), *L. harbinensis* (one isolate), *L. plantarum* (one isolate), *L. coryniformis* (one isolate) were investigated (Table 3.2). (Koirala et al., 2014). The isolates were examined for resistance to pH 3 and 0.3% bile, pH 2 and 0.3% bile, adhesion to Caco-2 cells, antibiotic susceptibility and antimicrobial activities against enteric pathogenic bacteria.

4.2.2.1. Gastrointestinal transit resistance

Before reaching the distal part of the intestinal tract and exert probiotic effect, these bacteria must survive transition through the stomach and the upper part of the intestinal tract (Kirtzalidou et al., 2011). The pH value in human stomach ranges from 2.0 to 4.5 (Jacobsen et al., 1999). Hence it is necessary to screen these bacteria at pH 3.0 and later pH 2.0. The effect of acid and bile (pH 3 and 0.3% OxGall) on the viability of various lactic cultures is shown in Table 4.8, 4.10. The results showed that all the lactic isolates under study exhibited tolerance to pH 3 and 0.3% bile (viable count after treatment; Log10 CFU/mL > 5) (Figure 4.18-4.27). The viable count, after gastrointestinal transit treatment (i.e. 90 minutes of gastric juice treatment followed by 3 hours in 0.3 % bile), was equal or even more or sometimes less as compared to the control (pH 7 buffer) in all the cases; similar kind of results are reported by various researchers previously (Kirtzalidou et al., 2011; Soomro & Masud, 2012). 85% of the tested lactobacilli isolated from infant gut microbiota remained unaffected at pH 3 after 3 hours of incubation in Kirtzalidou et al. (2011) study while Soomro & Masud (2012) reported that viability of majority of strains decreased at pH 3 after 3 hours of incubation.

Unlike in pH 3, only 15 strains showed resistant to pH 2 and 0.3% bile out of 24 isolates (Table 4.9). The viable count of three lactic isolates were comparatively lower (Log10 CFU/mL < 2) (Table 4.10 and Figure 4.24, 4.28, 4.29) while the viable count of eight lactic isolates were considerable to some extent (Log10 CFU/mL = 2 to 4) (Table 4.10 Figure 4.18, 4.21, 4.26, 4.27). One isolate belonging to *L. delbrueckii* (Log10 CFU/mL = 6.4) (Table 4.10 and Figure 4.20) and three isolates of *L. fermentum* (Log10 CFU/mL = 6-7) (Table 4.10 and Figure 4.22) were found to be highly resistant in pH 2 and 0.3% bile. Similar results (Log10 CFU/mL upto 7.0) for *L. delbrueckii* subsp. *bulgaricus* and *L. fermentum* were reported by Harun-ur-Rashid et al., (2007). Our observations about the low viability of the lactobacilli strains at pH 2 and 0.3% bile are also in agreement with previous reports (Jin et al., 1998; Xanthopoulos et al., 2000; Kotsou et al., 2008; Soomro & Masud, 2012). Jaya et al., (1998) reported that survival value (Log10 CFU/mL) of two commercial probiotic strains, *L. acidophilus* LA-1 and *L. rhamnosus* GG, were 7.6 which declined after incubation at pH 3 for 3 hours. Likewise, Conway et al. (1987) investigated the fate of *L. acidophilus* NCFM in low pH phosphate buffer saline. A four-log cycle drop in

viable counts after 3 hours at pH 3 in buffered saline was screened for *L. acidophilus* NCFM (from Log10 CFU/mL equals to 7.5 to Log10 CFU/mL equals to 3.3). From our study, *Lactobacillus casei* SHIROTA and *L. rhamnous* GG which were used as reference culture in our study, viable count Log10 CFU/mL value remained same at pH 3 (Table 4.8, 4.9 and Figure 4.28, 4.29) but was dropped drastically at pH 2 at different time intervals. Considering all these, majority of lactic isolates from Nepalese *dahi* are superior to these commercial probiotics in gastrointestinal transit resistance.

4.2.2.2. Adherence to Caco-2 monolayer

The main criteria for selecting probiotic strains, adherence to intestinal epithelia is thought to be dominant. Indeed, adhesion to epithelial cells or mucus appears to mediate colonization of the gastrointestinal tract by lactobacilli and may be prerequisite for competitive exclusion of enteropathogenic bacteria and immune modulation of the host successful probiotic bacteria usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa (Dimitrov et al., 2014).

The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important and desirable property for probiotics. The capacity of any probiotic strain to adhere to the intestinal mucosa is a key factor in its ability to survive and function as a desired strain in the intestine (Dunne et al., 2001). Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. Several workers have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Pelletier et al., 1997; Perez et al., 1998; Del Re et al., 2000). In most cases, adherence property is influential factor for a potential probiotic candidate (Kotsou et al., 2008). In the present investigation, considerable differences were observed in adhesive properties of *Lactobacillus* isolates (Table 4.11 and Figure 4.30-4.35), independent of the species. This variation among *Lactobacillus* spp. has been observed before (Chauviere et al., 1992; Tuomola & Salminen, 1998). Tuomola & Salminen (1998) tested for 12 *Lactobacillus* strains including some commercial probiotics and found that *L. casei* (Fyos) was the most adhesive strain and *L. casei* var. *rhamnosus* was the least adhesive strain. Twenty randomly selected microscopic filed (×400 magnification) were used to analyze the results for adhesion, the number of bacteria adhered to the

caco-2 cells monolayer were counted and an average was taken into consideration. Based on the results obtained, isolates were classified as, non-adhesive or (-) (no cells adhered), weak adhesive or (+) (Log10 average cell number adhered <1), mild-adhesive or (++) (Log10 average cell number adhered = 1-2) and strong adhesive or (+++) (Log10 average cell number adhered = 2-3). Accordingly, seven isolates were non-adhesive (Table 4.11 and Figure 4.31g, 4.31h, 4.32k, 4.32l, 4.33o, 4.33p, 4.34x), two isolates were weak adhesive (Table 5 and Figure 15i, 15j), eight isolates were mild-adhesive (Table 4.11 and Figure 4.30b, 4.30c, 4.30d, 4.31e, 4.31f, 4.31g, 4.33n) and seven isolates were strong adhesive (Table 4.11 and Figure 4.30a, 4.33m, 4.34q, 4.34s, 4.35u, 4.35v, 4.35w). Pair wise comparisons of the negative control (*Streptococcus sanguinis* IS3; photograph not shown here) with the other revealed considerably higher adhesion to Caco-2 monolayers. Out of 24 strains, seventeen isolates were found to be adhesive. Of them again, seven isolates such as *L. brevis, L. delbrueckii* (one isolate out of six), *L. fermentum* (all three isolates), *L. parabuchneri* (one isolate out of two) and *L. helveticus* (one isolate) were found to be strong adhesive (Table 4.11), similar findings is reported by Delgado et al., 2007; Jakava-Viljanen & Palva, 2007; Bao et al., 2010; Dimitrov et al., 2014.

4.2.2.3. Antimicrobial activity

Lactobacilli have been demonstrated to possess antimicrobial activity towards a series of pathogenic bacteria, which can be attributed to competitive adhesion to the epithelium or to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins or ruterin (Bernet-Camard et al., 1997; Dunne et al., 2001; Fernandez et al., 2014).The antimicrobial properties of *Lactobacillus* isolates tested were very variable (Table 4.12). Many of the isolates showed weak or no inhibition against pathogenic strains. Our observation is in agreement with Soomro & Masud (2012). While some strains such as *L. plantarum* NAST-RHM93 (against *L. monocytogenes* and *S. enterica*), *L. delbrueckii* NAST-RHL101 (against *S. pyogenes, P. aeruginosa* and *S. aureus*) and *L. rhamnosus* NAS-BHL25 (against *L. monocytogenes* and *P. aeruginosa*) showed inhibition. The inhibition of *L. delbrueckii* NAST-RHL101 was replicable with visible clear zone but other inhibitions were weak. Jacobsen et al., 1999 also reported that the antimicrobial activities of the *Lactobacillus* species were very variable. Three strains of *L. plantarum* PAC1.0, PAC1.10 & WHE92 were used as positive control which showed strong inhibition

against *L. monocytogenes* (Table 4.12). In contrast to this results obtained, when ten times concentrated cell free supernatants (CFSs) (36 hours old culture) were used against various food borne pathogens for antimicrobial assay using agar well diffusion method (100 μL CFSs), a clear zone of inhibition in considerable values were obtained (data shown in section 4.2.3.2.). This result indicates that the disc diffusion method may not be appropriate or the quantity of CFSs used was very less or the incubation time of lactic cultures should be extended to allow bacteriocin to be released in the culture broth.

4.2.2.4. Antibiotic susceptibility profiling

According to EFSA (2008), the emergence and the spread of resistance to antimicrobials in bacteria pose a great threat to human and animal health and present a major financial cost. Among various actions to decrease the development of resistance, screening lactic cultures for various breakpoint values of antibiotics were given and are to be tested in order to confirm the absence of transmissible antibiotic resistance genes. The absence of transmissible antibiotic resistances is considered a key safety prerequisite for the selection of probiotic microorganisms (FAO/WHO, 2002).

We studied the antibiotic resistance for all the twenty four lactic isolates with reference to the European food safety authority (EFSA) breakpoints (EFSA, technical guidelines, 2008). The results obtained showed different susceptible and resistant ranges for various isolates (Table 4.13), the values highlighted below the obtained values represent the EFSA guideline breakpoints). Four isolates such as, *L. paracasei* NAST82, *L. helveticus* NAST103, *L. fermentum* NAST2 and *L. coryniformis* NAST94 were found to be susceptible towards the nine tested antibiotics. Three isolates, two of *L. parabuchneri* and one isolate of *L. brevis* NAST92 were found to be highly resistant towards the tested antibiotics. However, other isolates showed susceptible results to some antibiotics and resistant to few other ones (Table 4.13). Four isolates of this study can be used as future probiotic candidate which are within the European food safety authority (EFSA) guidelines. These isolates may not have the transmissible antibiotic genes and may be safe. (EFSA, 2008). However, genetic study regarding antibiotic resistance transmissible genes may be useful before using them as commercial probiotics (Bongaerts et al., 2005; Kirtzalidou et al., 2011).

4.2.3. Preparation of Probiotic Curd (*Dahi*) and Sweet Curd (*Dahi*) using novel lactic cultures (*Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103)

Fermented milk products are part of the diet in many parts of the world and are consumed on a regular basis. The international Dairy Federation (1997) defined fermented milk as "a milk product fermented by the action of specific microorganisms resulting pH reduction and leading to coagulation". These specific microorganisms shall be viable, active, and abundant (at least 10^7CFU/g) in the product to the date of minimum durability (Varadaraj et al., 1993). The starters used for fermentation consist of both homofermentative and heterfermentative mesophilic lactic cultures which produce aroma (Driessen & Puhan, 1988) and other properties required for a curd. Enhancing the nutritional and therapeutic properties of traditional fermented milk can improve the health and physiology of consumers (Varadaraj et al., 1993; Vijayendra & Gupta, 2012).

Dahi, which has an appearance similar to that of yogurt, is a popular traditional fermented dairy product in Nepal and other neighboring countries such as India. It is prepared by fermenting milk from cows, buffalos, chauri, yak, goats etc. with mesophilic lactic cultures and its method of preparation and physicochemical characteristics are well documented (Rati Rao et al., 2006; Dahal et al., 2005). However, the microbiological aspects of *dahi* with respect to probiotics (biodiversity identification and characterization of beneficial attributes of lactic cultures involved in fermentation) and therapeutic values are yet to be explored in Nepalese context (Dahal et al., 2005). Traditional probiotic dairy strains of LAB have a long history of safe use (Fernandez et al., 2015). There is a growing interest in extending the range of foods incorporating probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice based and cereal based products and pharmaceuticals (Lee & Salminen, 1995). Therefore, it is in need to explore the potential lactobacilli, characterize its beneficial attributes and develop a probiotic product which would have nutritive as well as therapeutic values.

At present, there is increased demand of the high quality natural food which is free of artificial ingredients and the contaminants (Varalakshmi et al., 2014). Dairy foods are more likely to be contaminated with a variety of food borne pathogens (Altekruse & Street, 1995; Knabel, 1995; Cullor, 1997). *Bacillus cereus* produces toxins when it

contaminates food products such as dairy items, which can cause either diarrhoea or nausea and vomiting due to emetic toxin production. The presence of *Escherichia coli* in milk products indicates the presence of enteropathogenic microorganism, which constitutes a public health hazard causing bloody diarrhoea and several other disorders. *Listeria monocytogenes* is a food contaminant particularly in raw milk can even grow in the cold temperature of refrigerator which can be killed during cooking and pasteurization. It also causes symptoms such as diarrhoea, vomiting and others. The contamination of *Staphyococcus aureus* in the milk products causes minor skin infection to life threatening diseases such as pneumonia, meningitis, endocarditis and septicemia (Internet visit, 2015. http://www.foodsafety.gov/index.html). Antimicrobial activity is very important criteria for the selection of probiotic microbes as probiotic microbes are natural antagonists of potentially harmful bacteria (Varadaraj et al., 1993; Arques et al., 2014; Nami et al., 2015). In the current study, antagonistic property of lactic cultures (with tolerance to gastrointestinal transit resistance, adhesive to Caco-2 cells and lie within the EFSA guideline during antibiotic resistance profile) was studied and those lactobacilli (*L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103) showing antimicrobial activity to four different food borne pathogens were further studied and a probiotic curd (*dahi*) product was developed.

In view of the fact that fermented products are very popular in Nepalese diet, the use of a starter culture that can ferment as well as check the growth of pathogenic organisms would be very ideal. Hence, novel antagonistic strains (such as *L. helveticus* NAST-RHL103) of lactobacillus (with other probiotic properties) was identified from traditionally prepared *dahi* and used successfully as starter culture production for probiotic curd (*dahi*) as well as Sweet curd (*dahi*) preparation. To the best of our knowledge, this is first attempt for systematic study for characterizing the Nepalese *dahi* isolates at molecular and probiotic level with the aim of preparing probiotic *dahi* emphasizing on antagonistic activity towards various foods borne pathogens.

4.2.3.1. Pre-assessment of antimicrobial substance from indigenous lactic cultures prior to product development

Lactobacillus strains as probiotic are being increasingly examined for their ability to inhibit the pathogenic bacteria. In present investigation, six lactic cultures were used

for antimicrobial study where three showed inhibition to the indicator pathogens (Table 4.14 and Figure 4.37). Out of three positive strains, two strains *viz. Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103 were selected for further study. These lactic cultures exhibited significant *in vitro* probiotic properties such as gastrointestinal transit resistant (acid and bile tolerant), adhesion and also safe for use since they lie within EFSA guideline intended for antibiotic susceptibility test (mentioned in section 4.1.2.4.). Hoover & Steenson (1993); Jacobsen et al. (1999); Shah (2000b); Bao et al. (2010); Kirtazalidou et al. (2011) have also previously reported that lactic cultures such as *L. paracasei* and *L. helveticus* having could be used as the potential probiotic strains. The antimicrobial substances produced by these two cultures demonstrated their bacteriostatic as well as bactericidal properties both in agar plate and broth culture (Table 4.15 and Figure 4.38, 4.39). The similar kinds of results were presented in previous research findings (Jacobsen et al., 1999; Bao et al., 2010; Kirtazalidou et al., 2011).

The antimicrobial activity was found to be considerably stronger when reconstituted skimmed milk broth was used for lactobacilli growth. Serna-Cock et al. (2012) reported that the production of metabolite differs depending on the composition of the growth media. Zalan et al. (2010) also reported that metabolites production may vary in MRS broth and skimmed milk broth which also depends on bacterial strains. While Chumchalova et al. (1998) reported that inhibitory activity of acidocin was higher in MRS than skimmed milk broth. For instance, CFSs of the strain *L. helveticus* NAST-RHL103 cultured from MRS and skimmed milk culture broth separately showed different zone of inhibition (ZOI) against *B. cereus*. The ZOI of CFSs obtained from MRS broth using *L. helveticus* NAST-RHL103 showed 19 mm while it was 24 mm of CFSs from skimmed milk broth. Our results (Table 4 and Figure 1, 4) suggest that this strain and its metabolites produce better antimicrobial substance in reconstituted skimmed milk broth (Hoover & Steenson, 1993). Mostly, antibiotics are used as positive control during antimicrobial assay (Dixit & Kailasapathy, 2012), however, relative inhibitions exhibited by CFSs derived from two lactobacilli were considered as the constructive control in each experiment. The positive control inhibition is generally found to be superior to the crude extracts antimicrobial compound derived from LAB (Acharya, 2005). The cell free supernatants (CFSs) from skimmed milk broth were concentrated and further purified partially using methanol followed by

acetone. The strength of inhibition was found to be increased with the partial purification (Table 4.16 and Figure 4.40) using two different solvents (Chamundeeswari et al., 2012). Further, consistent result was obtained for arbitrary unit per mL (against 2×10^6 CFU/mL of indicator strains in plate assay) and minimum inhibitory concentration based on arbitrary unit per mL (against 2×10^3 CFU/mL indicator strains in broth assay) for *Lactobacillus helveticus* NAST-RHL103 (Table 4.18, 4.19) (Suma et al., 1998; Jena et al., 2013). Our result is supported by the findings of Griffiths & Tellez (2013) where various peptides with physiological functions, such as immunstimulating peptides, antimicrobial peptides, opioid peptides, mineral binding peptides and antihypertensive peptides have been isolated from products fermented with *L. helveticus*. Comparatively, weaker activity was observed for *Lactobacillus paracasei* NAST-RHM82. This weaker inhibition activity result for *L. paracasei* differs from the findings of Ashokkumar et al. (2011). However, the zone of inhibition is comparatively higher (ZOI of the CFSs cultured in MRS broth against *B. cereus, E. coli, L. monocytogenes* and *S. aureus* was found to be 15, 15, 16 & 13 mm respectively and the ZOI increased relatively in Skimmed milk broth culture) (Table 4.14 and Figure 4.37) than reported by Lozo et al. (2004) and Geria et al., (2014).

The antimicrobial property exhibited by both the cultures against four indicator strains is also supported by the scanning electron microscope photographs (Figure 4.40) where the change in the cell morphology of indicator strains after killing effect can be observed. Hence, both the cultures with probiotic properties as well as strong antimicrobial activity against enteric pathogens were used for product development.

4.2.3.2. Preparation of Probiotic Curd (*Dahi*) and Sweet Curd (*Dahi*)

Probiotic bacteria can be found worldwide in a variety of products, including conventional food products, dietary supplements and medical foods. In the United States, the main outlets for probiotic bacteria are dairy foods and dietary supplements and medical foods (Cullor, 1997). Dairy foods containing probiotic bacteria include most major brands of yogurt, culture containing fluid milks, such as “Sweet Acidophilus Milk” and a few brands of cottage cheese. Dairy foods seem to fit naturally with probiotics because of the traditional association of beneficial fermentation bacteria and fermented dairy products (Fernandez et al., 2014). Consumers naturally associate fermented dairy products with live cultures and

perceive a benefit in the presence of these cultures (Sanders, 2000). Therefore this experiment challenges to prepare a probiotic curd (*dahi*) using two indigenous lactic cultures.

Two lactic cultures *viz. Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103 were used to prepared probiotic curd (*dahi*) separately. The optimum conditions used for preparing probiotic curd revealed the products acceptability by their texture, aroma, titratable acidity, pH and viable count which are very important parameters for consumers' acceptance (Masamba & Ali, 2013). The viable count was maintained more than 10×10^{11} for both the products (FAO/WHO, 1997).

Milk fat has more than 400 different fatty acids (Jensen, 2002), though the main composition is covered by 14 to 27 fatty acids. Milk fat has an average of 60% saturated fat, 24% MUFA and 0.5% PUFA (Walther et al., 2008). Milk fat is the richest natural source of conjugated linoleic acids (CLAs), the main isomer being C18:2n-7 cis9, trans11 (Collomb et al., 2006; Park, 2009). Milk fat has a lot of short-chain fatty acids, and in addition, various rare branched, odd-numbered and oxo fatty acids in small amounts. Milk fat has ruminant trans-fatty acids because of the microbial activity in rumen. Free fatty acids (FFA) are generated by both lipolytic processes (C4-C20) and bacterial fermentation (C2-C4). Analysis of various short-chains FFA would be important and their presence can cause flavor changes and other health benefits (Fernandez et al., 2014). The results of our study showed various 14 fatty acids present in the probiotic curd products using two lactic cultures separately (Table 4.22). This outcome is similar to the previous research findings (Yadav et al., 2007; Guler & Park, 2011). Obesity is a growing problem all around the world. The fat reduction in total diet has been considered one of the main issues and it can be achieved by choosing food items with lower fat content and by reducing the fat content of particular food. Dairy products are the most popular reduced fat choices. (Stewart Knox et al., 2005). Astrup et al. (2011) reported that low-fat dairy products are part of a healthy diet.

The sensory evaluation helps defining the product characteristics which are important with respect to the acceptability by the consumers. It represents an important step while developing a new food product (Cruz et al., 2010; Arques et al., 2014). It involves the analysis and interpretation of the responses to different sensory attributes

such as texture, aroma, colour, taste and mouth feel etc. The sensory characteristics of probiotic curd have substantial effect on consumer acceptance (Masamba & Ali, 2013). Through the organoleptic analysis of the probiotic curd (*dahi*) samples, it was established that the *dahi* prepared with *L. paracasei* NAST-RHM82 was found to be the best followed by the curd prepared with *L. helveticus* NAST-RHL103 and the control curd was found to be the last choice. However, the preparation of curd using *L. helveticus* NAST-RHL103 would be appropriate based on the incubation time (only 8 hours), antimicrobial activity and other probiotic properties as compared to the *L. paracasei* NAST-RHM82 (30 hours of incubation time, less adhesive, low strength in antimicrobial activity). This kind of probiotic curd (*dahi*) could be useful for controlling the growth of harmful bacteria and in curing intestinal disease like constipation, diarrhoea, dysentery (Shahani & Chandan, 1979).

Similarly, a sweet curd (*dahi*) was prepared (with evaporated or concentrated milk) using probiotic culture; *Lactobacillus helveticus* NAST-RHL103 (which could grow at high sugar concentration of up to 20%) with optimum growth conditions (Table 4.26). From the biochemical and organoleptic properties; Sweet curd with 20% sugar was found to be the best which had titratable acidity 0.44 %, pH 4.62 and the sugar percentage was reduced by nearly 6% (Figure 4.47). The quality of Sweet curd of Nepalese origin was found to meet the prescribed standard and falls under the grade of good quality (Ghosh & Rajorhia, 1990; Cruz et al., 2010). This probiotic Sweet Curd (*Dahi*) product may be the substitute of the commercial sweet foods (Chethana et al., 2014).

4.2.3.2.1. Effect of Probiotic Curd (*Dahi*) towards Pre-Processing and Post-Processing contaminants

The possibility of contamination is very common during the preparation of fermented foods including dairy products. The contaminant may enter before or during the preparation of indigenous *dahi* which may be due to unclean utensils or poor hygiene of the worker or environment which leads to the spoilage of the product. This examination was performed to validate the antibacterial action of indigenous lactic cultures in the formulated *dahi* as well as to screen them for future bio-preservative agent in foods. Dairy products are prone to the enteric pathogens (Masamba & Ali, 2013). For this, probiotic curd was added with indicator pathogens (*B. cereus, E. coli, L. monocytogenes* and *S. aureus*) before product formation (indicator strains added

together with inoculums i.e. pre-processing contaminants) and after product formation (i.e. indicator strains added after product formation i.e. post-processing contaminants) separately. The results showed that the numbers of indicator strains were slightly lowered during pre-processing contamination (Table 4.23) while completely killed during post-processing contamination after 24 hours storage in refrigerator (Table 4.24) (in case of product prepared using *L. helveticus* NAST-RHL103). Although, almost complete reduction of the pathogens was possible for the product prepared using *L. paracasei* NAST-RHM82 after 12 hours of storage in refrigerator, this strain may not be suitable commercial strain since it took 30 hours (22 hours more time than *L. helveticus* NAST-RHL103) for product formation. The results obtained in case of *L. helveticus* NAST-RHL103 are as good as the findings reported by (Trias Mansilla, 2008), who observed that the use of LAB as potential biopreservative strains of fresh fruit and vegetables provided encouraging results especially for the inhibition of pathogenic *L. monocytogenes* in fresh products and fermenting foods and beverages (Gilliland et al., 1975; Schillinger & Lucke 1989). The absence of enteric pathogens was due to bactericidal activity of the probiotic cultures and their metabolites in both the products. From this study, we can conclude that infections caused by contaminated foods or other sources may be cured by consuming such probiotic curd (*dahi*). Hence the probiotic curd (*dahi*) prepared using *Lactobacillus helveticus* NAST-RHL103 can be the alternative therapeutic application which may protect against gastrointestinal pathogenic infections would be of great importance for future medical use.

5.0. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

5.1.1. Isolation, biochemical and molecular characterization of *Lactobacillus* spp. from traditionally prepared curd (*Dahi*) at different altitudes in Nepal

The main aim of this study was to isolate and genetically characterize the dominant lactobacilli in traditionally prepared curd or *dahi* at different geo-climatic conditions of Nepal. The results obtained during this study highlighted that rod-shaped bacteria were the dominant Lactic Acid Bacteria (LAB) population in traditional Nepalese *dahi* samples (62% over 38% cocci). Within rod-shaped LAB, a wide diversity of *Lactobacillus* species was found confirming that home-made traditional fermented products are naturally rich source of novel *Lactobacillus* strains. In total, 205 bacterial isolates were obtained from 64 curd (*dahi*) samples, of which, 193 were LAB isolates. Later, 120 isolates were identified as lactobacilli belonging to ten different *Lactobacillus* species were identified by species specific PCR. And intra-species genetic diversity analysis (phylogeny) was performed using various molecular marker tools for major groups such as *L. delbrueckii*, *L. paracasei*, *L. rhamnosus, L. fermentum* and *L. parabuchneri*. Intra-speceis genetic diversity study involving number of random primers revealed rich genetic diversity within different species except *L. rhamnosus*. Among the identified isolates, species such as *L. paracasei* and *L. rhamnosus* have a known history of safe use and health-promoting properties,. The new Nepalese isolates of these species may provide useful resources for further studies aiming at the selection of new cultures with potential novel probiotic characteristics. Furthermore, the strain collection obtained from this study represents a first step towards the preservation of the natural biodiversity of bacterial population of the traditionally prepared Nepalese curd or *dahi*.

5.1.2. *In vitro* probiotic properties of *Lactobacillus* spp. isolated from traditionally prepared curd (*Dahi*) at different geo-climatic conditions of Nepal

The lactobacilli obtained from curd (*dahi*) collected in Hilly and Himalayan regions of Nepal could be a potential source of biological materials for dairy industries so these cultures are widely studied in search of novel probiotic candidate. All the lactobacilli obtained from various sources may not possess probiotic properties and hence it is necessary to screen and characterize for probiotic properties for future

food, medical and industrial applications. Viability and survivbility of probiotic bacteria to tolerate pH and bile environment of human GIT are important characteristics in order to provide health benefits. Besides colonization of the GIT, there are several factors including binding capacity to intestinal cells and mucus, antimicrobial inhibition and antibiotic resistance profile are often considered as the main criteria in order to be a putative probiotic candidate. However, a single probiotic candidate may not exhibit all the probiotic properties. In the present study, 24 lactobacilli obtained from *Dahi* collected at various geo-climatic conditions of Nepal were examined for *in vitro* probiotic properties. Of the 24 isolates studied, the four *Lactobacillus* strains *viz. L. paracasei* NAST-RHM82, *L. fermentum* NAST-GHM2, *L. helveticus* NAST-RHL103, and *L. coryniformis* NAST-RHM94 showed considerable *in vitro* probiotic properties (such as tolerance to acid and bile conditions, adhesion to Caco-2 cells monolayer, and antimicrobial activity) as compared to other lactic isolates studied as well as the standard probiotic cultures. Also, only these four lactic isolates completely follow the EFSA guidelines hence these are absolutely safe for use as Probiotics in future. However, additional *in vivo* study may be performed in future to use these cultures as the potential probiotic candidates.

5.1.3. Preparation of Indigenous Probiotic Curd (*Dahi*) and Sweet Curd (*Dahi*) using novel lactic cultures (*Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103) having probiotic properties

Fermented milk products such as curd (*dahi*) containing LAB are traditionally used every day as food in Nepal. The development of starter culture having fermentative as well as probiotic properties would hold a great promise for dairy industries. Hence, present study was undertaken to examine and validate antimicrobial properties of lactic cultures having some probiotic activities to develop probiotic curd (*dahi*) and sweet curd (*dahi*) in laboratory scale. For these reasons, this study was focused in preparing indigenous *dahi* using probiotic cultures of *L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103. Also, sweet curd was prepared using *L. helveticus* NAST-RHL103. Based on the *in vitro* probiotic properties results, antibiotic susceptibility test and antimicrobial activity, these two cultures were used for product development. Additional experiments were performed to understand the antimicrobial properties of these strains. Antimicrobial substance produced from these two cultures

(*L. paracasei* NAST-RHM82 and *L. helveticus* NAST-RHL103) were found to be bacteriostatic as well as bactericidal towards four different food borne pathogens (*Bacillus cereus* F 4810, *Escherichia coli*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* FRI 722).

These two probiotic strains were found to be successful starter culture to prepare curd or *dahi*. The chemical (pH, titratable acidity, percentage fat and fatty acid compositioin), microbiological (viable count in the product) and organoleptic properties (sensory analysis of the product) were also found to be high-quality by chemical, microbiological and sensory analysis respectively. However, the fermentation time to produce *dahi* using *L. paracasei* NAST-RHM82 strain was longer than *L. helveticus* NAST-RHL103 strain. The results obtained reveal that *L. helveticus* NAST-RHL103 could be a potential probiotic candidate because of its strong *in vitro* probiotic properties including antimicrobial activity against food borne pathogens and is safe for use as per EFSA guidelines for antibiotic resistance. Also, incubation time for product development is almost four times shorter as compared to the *L. paracasei* NAST-RHM82 with good sensory characteristics. The experiments performed for pre-processing contaminant and post processing contaminant effects proved that *L. helveticus* NAST-RHL103 produces strong extracellular antimicrobial substance and could also be used as natural preservative agent in foods in beverages. Besides these, *L. helveticus* NAST-RHL103 was able to produce sweet curd (best was to be the product with 20% sugar content). Both the cultures showed its good effect against pre-processing and post-processing contaminants i.e. the food borne pathogens. These findings suggest that these cultures can also act as bio-preservatives replacing the chemical preservatives used in various food industries including dairy sectors. Hence, among several lactic isolates investigated, the lactobacilli isolated from the traditionally prepared *dahi* collected from Hilly region of Rasuwa District *viz. L. helveticus* NAST-RHL103 with good probiotic properties and also being safe for use can be exploited as marker probiotic candidate for industrial production of probiotic curd as well as to develop pharmaceutical probiotic product for future therapeutic and medical uses.

5.2. Recommendations

The diversified geo-climatic conditions, ethnic communities, culture and heritage dictate the various methods of traditional food preparation and their consumption pattern in the Himalayan country Nepal. However, complete scientific information on the various aspects food products, their traditional ethics, production and preparation methods, health benefits, microbiological and biotechnological aspects and mode of consumption of these products are still lacking. These days, use of lactic acid bacteria mainly associated with fermented dairy products such as *Dahi* (curd), cheese, buttermilk, yoghurt etc. is rapidly expanding not only as a starter culture for various dairy products but also for other food based industries (such as Confectionaries, Pharmaceuticals etc.) due to their beneficial health effects. An increasing number of health foods or so called functional foods as well as pharmaceutical preparations are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Knowledge of probiotic bacteria used with different prebiotics increases globally, the demand for products containing probiotic bacteria have increased significantly. The exploration of the various biotechnological aspects of the lactic acid bacteria from the traditional foods such as dairy products of different geo-climatic conditions of Nepal with technological properties (functional, probiotics, pharmaceuticals and medical) have a great importance in biotechnological industry and food science. Following actions in biotechnology area are in need with respect to research and development, industrilization, employment, improved agriculture economy and sustainable development of the country.

1. Investigation on various traditional fermented foods for the isolation, molecular characterization, *in vitro* and *in vivo* probiotic properties are very important things and should be encouraged by the government.
2. Research into mechanisms of probiotics using microbes obtained from traditional fermented foods are also very important, which would, not only improve the knowledge of the mode of action of probiotics into the gastrointestinal tract, but also will provide the appropriate probiotic candiate for future biotechnological applications.
3. While the knowledge of indigenous lactic cultures for their biochemical & molecular characterization, acid & bile tolerance, antimicrobial and antibiotic sensitivity properties has been ascertained as well as two cultures were established

as the starter as well as probiotic cultures, further work is still needed for understanding the *in vivo* properties focusing on the particular mechanisms of probiotics.

4. The inhibitory ability of the indigenous probiotic bacteria especially lactobacilli towards the food borne enteric pathogens has important food and clinical implications. Due to contamination, the spoilage and occurrence of food borne illness is common in developing countries (such as Nepal, India, Bangladesh etc.), the continuation of this research may address the problem of chemical preservative which could be replaced by the lactic culture as bio-preservative agent.
5. Our work has discovered some potential candidate (such as *L. helveticus* NAST-RHL103 which are successfully able to inhibit food borne pathogens such as *B. cereus, E. coli, L. monocytogenes* and *S. aureus in vitro* experiments. These strains need to be further assessed and critically evaluated by *in vivo* and clinical research on patients colonized and infected with the above mentioned pathogens. In addition, it may be useful to study which antimicrobial compound with what mechanisms of actions, the pathogens are being successfully eliminated. Also, the product Curd or *Dahi* prepared with these probiotic lactic cultures could be used to know whether it could be a good substrate to carry the probiotic cultures into the gastrointestinal tract or not. If this study could be investigated and developed a suitable substrate to carry these probiotic bacteria, the food borne pathogens could be eliminated without the use of antibiotics.
6. Finally, future research must investigate the mechanisms by which gut microflora interacts with the intestinal epithelium in health and disease. With this knowledge, optimal probiotic strains can be developed. The viability of probiotics is a key parameter for developing probiotic food products. New technologies should be developed to enable high cell yield in large scale and ensure probiotic stability for a long period in food. Various food matrices, dairy and non-dairy, can be used with probiotics. With different technologies, such as microencapsulation, cell immobilization and continuous fermentation, the probiotics will become an important and viable ingredient in the functional foods, expanding the probiotic application outside the pharmaceutical and supplement industries.

6.0. SUMMARY

A healthy human GIT is a quite complex microbial ecosystem that facilitates normal physiological functions of the host organisms unless harmful and potentially pathogenic bacteria dominate it. In order for the intestine to function optimally, the 'balance' of the bacteria must be maintained and it seems to be increasingly difficult due to change in way of living. At present, study of the traditional fermented foods and later development of functional foods such as dairy foods containing probiotics is one of the key priority areas of the food and medical biotechnological sciences. Dairy foods naturally fit with probiotic organisms for their survival and may play a potential role as a vehicle to transport them through the harsh conditions of GIT and get implanted into the consumer gut to exert various health benefits. It has been widely accepted that consumption of probiotic enriched foods play a preventive and sometimes curative effects against certain diseases like cancer, allergy, diarrhea, bowel diseases, diabetes etc (Dellaglio & Felis, 2005). However, scientific research pertaining to characterization of probiotic microbes of Nepalese dairy origin is still scarce. Therefore, this entire investigation was planned or designed in order to isolate such beneficial lactic acid bacteria from traditionally prepared Curd or *Dahi* from different geographical regions of Nepal, characterize at phenotypic, biochemical and molecular levels, study their probiotic properties using standard techniques and finally select few promising strains for the preparation of probiotic *dahi* for commercial application. The salient findings of the present investigation encompasses the isolation, biochemical and molecular characterization of the *Lactobacillus* species obtained from traditionally prepared *dahi* collected at different geo-climatic conditions of Nepal; intra-species genetic diversity study of the major groups of lactobacilli isolates using various PCR based methods; 16S rRNA sequence based phylogenetic study at species level; screening for *in vitro* probiotic properties; partial purification and characterization of the antimicrobial compounds; the development of the probiotic curd (*dahi*) and sweet curd (*dahi*) chemical, microbiological and organoleptic properties of the product; pre-processing and post-processing contaminant effect towards the enteric food borne pathogens.

The major research works carried out and their findings are summarized in following section.

1. Total of 64 Curd (*Dahi*) samples were collected from four districts (Gorkha, Rasuwa, Bhaktapur and Lalipur) belonging to two geographical regions (Hilly and Himalayan region) of Nepal.
2. Of the total 205 isolates obtained, 193 isolates were preliminary confirmed as lactic acid bacteria where 120 isolates (68%) were found to be rod shaped and 73 isolates were found to be cocci (32%).
3. The rod shaped lactic acid bacteria i.e. lactobacilli were further characterized at phenotypic, biochemical and molecular level.
4. Then, phenotypic and biochemical characterization such as colony morphology, microscopy (grams staining, negative staining), motility test and catalase test were performed for presumptive confirmation of the lactic acid bacteria (*Lactobacillus* species). 120 presumptive lactobacilli isolates were further used for molecular characterization.
5. For, molecular identification and characterization, DNA extraction was carried out by phenol chloroform method.
6. First step was achieved using Polymerase Chain Reaction (PCR) based Internal Transcribed Spacer (ITS) profiling which allowed the identification of lactic isolates at genus level as well as grouping of the similar isolates.
7. The 16S rRNA sequencing of representative strains from each group were allowed their identification.
8. Further molecular identification was also achieved using a number of species specific PCR assays.
9. Based on all these molecular analyses, the 120 isolates were classified as belonging to ten different *Lactobacillus* species *viz.* (1) *L. delbrueckii* subsp. *bulgaricus* (46 isolates), (2) *L. paracasei* (30 isolates), (3) *L. rhamnosus* (21 isolates), (4) *L. fermentum* (12 isolates), (5) *L. parabuchneri* (5 isolates), (6) *L. helveticus* (2 isolates), (7) *L. coryniformis* (1 isolate), (8) *L. harbinensis* (1 isolate), (9) *L. brevis* (1 isolate) and (10) *L. plantarum* (1 isolate).
10. *L. delbureckii* was identified up to subspecies i.e. as *L. delbrueckii* subsp. *bulgaricus* by species specific PCR.
11. Also, representative isolates of *L. paracasei* were identified up to subspecies level as *L. paracasei* subsp. *paracasei* using High Resolution Melt Curve Analysis.

12. Intra-species genetic diversity analysis was studied using various markers for *L. delbrueckii subsp. bulgaricus, L. paracasei, L. rhamnosus, L. fermentum* and *L. parabuchneri.*
13. A high degree of intra-species genetic diversity was revealed for all *Lactobacillus* species, except for *L. rhamnosus* isolates, which produced a single typing profile with all primers studied.
14. Of the 120 *Lactobacillus* isolates, 24 isolates were further selected for probiotic properties analysis based on the intra-species genetic diversity study and the geo-climatic origin of the isolates.
15. Probiotic properties such as gastrointestinal transit resistance (acid and bile tolerance), adhesion, antimicrobial activity and antibiotic sensitivity tests were also carried out.
16. All the 24 isolates studied were able to survive the gastrointestinal transit resistance at pH 3 and 0.3% bile while only 15 isolates were able to survive the gastrointestinal transit resistance at pH 2 and 0.3% bile.
17. Seventeen lactic isolates were found to be adhesive while seven were non-adhesive in Caco-2 cell monolayer.
18. The antimicrobial activity tested against the enteric pathogens by paper disc method was found to show weak inhibition.
19. According to European food safety authority (EFSA) guidelines, antibiotic sensitivity test was carried out for all the 24 isolates. Among them, only four isolates *viz. Lactobacillus paracasei* NAST82, *Lactobacillus fermentum* NAST2, *Lactobacillus helviticus* NAST103 and *Lactobacillus corniformis* NAST94 were found to be safe for different nine antibiotics (Ampicillin, Vancomycin, Gentamycin, Kanamycin, Streptomycin, Erythromycin, Clindamycin, Tetracycline and Chloramphenicol).
20. Based on the *in vitro* probiotic attributes test, six lactic cultures *Lactobacillus fermentum* NAST-GHM2, *Lactobacillus rhamnosus* NAST-GHM25, *Lactobacillus delbrueckii* subsp. *bulgaricus* NAST-RHL74, *Lactobacillus paracasei* NAST-RHM82, *Lactobacillus delbreuckii* subsp. *bulgaricus* NAST-RHL101 and *Lactobacillus helveticus* NAST-RHL103 were selected for further antimicrobial, sugar fermentation and product development studies.

21. Well diffusion method was used to screen the antimicrobial activity against enteric pathogens *viz. Bacillus cereus* F 4810, *Escherichia coli*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* FRI 722.
22. Of the six, three isolates were found to inhibit all the four enteric pathogenic strains and only two strains *viz. Lactobacillus paracasei* NAST-RHM82 and *Lactobacillus helveticus* NAST-RHL103 were further selected for partial purification and characterization of the antimicrobial compound produced by these lactic cultures.
23. These two lactic cultures were further cultured in skimmed milk broth and concentrated and antimicrobial activity (both bacteriostatic and bactericidal) were observed and the strength of the inhibition was found to be higher in cell free supernatants derived from skimmed milk broth as compared to the MRS broth.
24. The ten times concentrated cell free supernatants derived from skimmed milk culture broth of the respective cultures were further purified by using methanol and later methanol concentrate was purified (concentrated) using acetone. Antimicrobial compound during the purification was concentrated so that the strength of inhibition increased considerably against all the four indicator pathogens. However, the strength of inhibition by skimmed milk broth, methanol concentrate and methanol-acetone concentrate of *L. helveticus* NAST-RHL103 was very high as compared to the inhibition by the *L. paracasei* NAST-RHM82 against all the pathogens. And the strength of the inhibition increased relatively following different purification steps.
25. Following the partial purification and its inhibition results, probiotic (*dahi*) curd and sweet curd (*dahi*) were prepared and the effects of pre-processing and post-processing contaminants (four indicator pathogenic strains were used as contaminants) were analyzed for probiotic *dahi*.
26. Product formation conditions such as incoculum size, incubation conditions (time, temperature) were optimized and the product was formed with the optimized conditions.
27. Two products were formed using separate lactic cultures i.e. *L. helveticus* NAST-RHL103 formed the product with 1% inoculums (in the whole milk) at 37 ºC in 8 hours while *L. paracasei* NAST-RHM82 formed the product 1% inoculums at 37 ºC in 30 hours.

28. Microbiological, chemical and organoleptic (sensory analysis) properties were analyzed and were found to be of high quality for both the products. However, from the results it has been confirmed that the product formed with *L. helveticus* NAST-RHL103 is better due to its better probiotic attributes, antimicrobial activity and the short incubation time for product formation.
29. Also, pre-processing and post-processing contaminant effects towards the pathogens were carried out separately and results obtained showed that the contaminants could not grow in the probiotic *dahi* entered before or during the handling (as pre-processing contaminants) but the post contaminants numbers decreased drastically (for all the pathogens) after 24 hours of storage in refrigerator. In addition, the sweet *dahi* was prepared using *L. helveticus* NAST-RHL103. This culture is also able to ferment the milk containing up to 20% sugar and able to produce sweet *dahi*. These results also highlighted the possibilities of using this culture as the biopreservative agent in the food products.
30. Because of the innate properties of the lactic culture *L. helveticus* NAST-RHL103 could be a probable probiotic candidate and the bio-preservative lactic culture in future.
31. Hence, isolation, biochemical and molecular characterization was carried out for the *Lactobacillus* species found in the traditionally prepared curd or *dahi* obtained from different geo-climatic conditions of Nepal. Also, characterization of the *in vitro* probiotic properties and the selection of the potent probiotic cultures with further authentication for antimicrobial activity directed the development of the probiotic curd (antibacterial *dahi*).
32. In conclusion, the major findings of the project "Molecular and Probiotic characterization of *Lactobacillus* spp. isolated from traditionally prepared curd or *Dahi* collected at different geo-climatic conditions of Nepal" helps in providing a potential technology with a probiotic lactic culture *L. helveticus* NAST-RHL103 for preparation of the indigenous probiotic *dahi* having beneficial attributes. Besides the beneficial attributes such as nutritional and functional characteristics, this culture may also be developed as the bio-preservative agent in functional foods to increase the lifespan and replace the unwanted uses of the chemical preservatives.

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AP.1.0 MICROBIOLOGICAL MEDIA, REAGENTS AND BUFFERS

Composition of different types of Media and Buffers, used in the study, are given below. Sterilization of media and buffers were performed at 121 ºC for 20 minutes under 15 psi pressure**.**

AP.1.1 deMann Rogosa Sharpe medium (MRS) (pH 6.5 ± 0.2)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Dipotassium hydrogen phosphate	2.0 g
Sodium acetate	5.0 g
Ammonium citrate	2.0 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Tween 80	1.0 g
Distilled water	1000 ml

AP.1.2 M-17 Medium (pH 7.0 ± 0.1)

Tryptone	5.0 g
Peptone	5.0 g
Yeast extract	2.5 g
Beef extract	5.0 g
Lactose	5.0 g
Sodium-β-glycerophosphate	19.0 g
Ascorbic acid	0.5 g
Magnesium sulfate	0.25 g
Distilled water	1000 ml

AP.1.3 Skimmed Milk Broth (pH 6.8 ± 0.2 at 25 ºC)

Skimmed milk powder	100.0 g
Yeast extract	5.0 g
Glucose	5.0 g
Distilled water	1000 mL

Skimmed milk powder was dissolved in lukewarm water and filtered through moist cotton and later other ingredients were added and made up to 1000 mL

AP.1.4 Brain Heart Infusion (BHI) Medium (pH 7.4 ± 0.2)

Infusion from Beef heart	250.0 g
Infusion from Calf brain	200.0 g
Dextrose	2.0 g
Dipotassium hydrogen phosphate	2.5 g
Sodium chloride	5.0 g
Peptone	10.0 g
Distilled water	1000 ml

AP.1.5 MYP Agar Base (pH 7.2 ± 0.2 at 25 ºC)

Beef Extract	1.0 g
Peptone	10.0 g
D-Mannitol	10.0 g
Sodium Chloride	10.0 g
Phenol Red	25.0 mg
Agar	15.0 g
Distilled water	1000 ml

Supplements: Egg Yolk Emulsion, 100 mL

Polymyxin B (50,000 Iu), 5 mL

AP.1.6 Macconkey Agar (pH 7.1 ± 0.2 at 25 ºC**)**

Enzymatic digest of gelatin	17.0 g
Enzymatic digest of casein	1.5 g
Enzymatic digest of animal tissue	1.5 g
Sorbitol	10.0 g
Bile salts mixture	1.5 g
Sodium chloride	5 g
Neutral red	0.003 g
Crystal voilet	0.001 g
Agar	13.5 g
Distilled water	1000 ml

AP.1.7 Listeria Selective Agar (pH 7.3 ± 0.2 at 25 ºC)

Casein enzymic hydrolysate	17.0 g
Papaic digest of soyabean meal	3.0 g
Yeast extract	6.0 g
Sodium Chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Dextrose	2.5 g
Agar	15.0 g
Distilled water	1000 ml

AP.1.8 Baird Parker Agar Base (pH 7.0 ± 0.2 at 25 ºC**)**

Casein enzymic hydrolysate	10.0 g
Meat extract B	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lighium chloride	2.5 g
Agar	20.0 g
Distilled water	1000 ml

AP.1.9 Phosphate Buffer (0.1 M PH 7)

0.5L OF 1M K2HPO4 AT 174.18G MOL-1 = 87.09G

0.5L OF 1M KH2PO4 AT 136.09G MOL-1 = 68.045G

Volume of 1 M K2HPO4	Volume of 1 M KH2PO4
61.5 mL	38.5 mL

Total volume was made up to 1000 mL with distilled water (pH 7.0)

AP.1.10 Phosphate Buffered Saline (PBS) (pH 7.0)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Distilled Water	1000 ml

AP.1.11 Peptone Water (pH 7.0)

Peptone	1g
Distilled Water	1000 ml

AP.1.12 Glycerol Stock Medium

Glycerol	40.0 ml
Distilled Water	60.0 ml

Sterilized by autoclaving. Glycerol stock added in equal amount to the culture in each cryovial, at the time of preservation.

AP.1.13 Gram Stain (Hucker's)

Crystal Reagents:

Solution A	Solution B
Crystal violet (90%) (2 g)	Ammonium oxalate (0.8 g)
Ethyl alcohol (95%) (20 mL)	Distilled water (80 mL)

Mix solution A and B before using.

Gram's Iodine:

Iodine	1 g
Potassium	2 g
Distilled Water	300 mL

Ethyl Alcohol (95%)

Ethyl alcohol (100%)	95 mL
Distilled Water	5 mL

Safranin:

Safaranin (2.5%)	2.5 g
Ethyl alcohol (95%)	100 mL

10 mL of above solution was dissolved in 100 mL of distilled water.

AP.1.14 Hydrogen Peroxide (3%)

Hydrogen peroxide (30%)	10 mL
Distilled Water	90 mL

AP.1.15 Sodium Hydroxide (0.1N)

Sodium hydroxide (NaOH)	4 g
Distilled Water	1000 mL

AP.1.16 Phenolphthalein Solution (0.5%)

Phenolpthalein	0.5 g
Ethanol (50%)	100 mL

Normal Saline solution (0.85% Sodium Chloride)

Sodium Chloride (NaCl)	0.85 g
Distilled Water	100 mL

Above solution is autoclaved at 15lbs for 20 minutes.

AP.2.0 MOLECULAR REAGENTS AND BUFFERS

AP.2.1.0 Reagents for genomic DNA extraction

AP.2.1.1. TE Buffer

Tris (10mM)	0.1212 g
Di-Sodium EDTA (1mM)	0.0372 g
Deionized water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 (with 6N NaOH) and volume was made to 100 ml. Buffer was autoclaved (121 ºC /15 min) after preparation.

AP.2.1.2 Proteinase 'K'

Weigh empty eppendrof, add approximately 20 mg of Proteinase 'K' and add 1000 µl (1 ml) of TDW. Store in freezer after proper mixing.

(1) Stock sol. – 20 mg/ ml
(2) Storage Tmp. – 20^{o}C
(3) Concentration in reaction – 50 µg/ ml
(4) Reaction buffer – 0.01 M Tris (pH – 7.8) 0.05 M EDTA, 0.5% SDS
(5) Temperature – 37-56^{o}C.

AP.2.1.3 SDS Solution (20%)

SDS	20 g
Deionized water	100 ml

Solution was autoclaved before use

AP.1.2.4 Chloroform: Isoamyl alchol (24:1)

Chloroform	96 mL
Isoamylalcohol	4 mL

Dissolve the solutions and store at 4 ^{o}C in brown bottle.

AP.1.2.5 70% ethanol

Ethanol	70 mL

Distilled Water	30 mL

Dissolve the solutions and store at 4 ^{o}C.

AP.1.2.6 95% ethanol

Ethanol	95 mL
Distilled Water	5 mL

Dissolve the solutions and store at 4 ^{o}C.

AP.1.2.7 Sodium Acetate (3M)

Sodium acetate	24.20 g
Deionized Water	500 mL

Sodium acetate was dissolved in deionized water (400 mL) and pH was adjusted with glacial acetic acid and final volume adjusted to 500 mL with deionized water. The solution was autoclaved before use.

AP.2.2.0 Reagents for PCR

AP.2.2.1 Reconstitution of dNTP's

Working solution of dNTP mix with 10mM of each dNTP from 100mM of each dNTP stock

10µl of each dNTP is taken and the volume is made up to 100 µl i.e., 10µl of dATP + 10 µl of dGTP + 10 µl dCTP + 60 µl of tdH_2O. The effective concentration of each dNTP becomes 10 mM in the mix.

$V_1N_1 = V_2N_2$

10 mM (10,000 µM) X V_1 = 200 µM X 25 µl (Final concentration should be 200 µM of each dNTP in 50 µl)

X = 0.5 µl

200 µM of each dNTP was used in a X 25 µl PCR reaction final volume.

AP.2.2.2 Primer reconstitution

From the stock of 100 µM, working solution of 10 µM is prepared by taking 5 µl of stock in an eppendorf and making up the volume to 50 µl. In a PCR reaction of 25 µl reaction 0.5 µM of primer is used.

AP.2.3.0 Reagents for agarose gel electrophoresis of DNA

AP.2.3.1 TAE Buffer (50 X) (Stock Solution)

Tris	24.20 g
0.5 M Di-Sodium EDTA (pH 8.0)	10.00 ml
Glacial Acetic Acid	5.71 ml
Deionized Water	100 ml

pH of this solution was not adjusted. The solution was autoclaved at 121 ºC /15min

Working Solution (1X)

Working solution was prepared by diluting 1 ml of stock solution to 50 ml with distilled water.

AP.2.3.2 Gel Loading Solution

Bromophenol Blue	0.05 g
Sucrose	40.00 g
Di-Sodium EDTA	3.72 g
SDS	0.50 g
Deionized Water	100 ml

AP.2.3.3 2.0% Agarose

Dissolve 2.0 g in 100 ml of TAE

AP.2.3.4 Ethidium bromide (EtBr):

Stock Solution (10 mg ml^{-1}):

Ethidium bromide (EtBr) was prepared as a stock solution of 10 mg/ml in water, stored at room temperature in screw cap tubes wrapped in aluminium foil.

Working Solution (0.2 µg ml^{-1}):

The working solution was prepared by adding 20 µl of stock solution to 1 L of distilled water.

AP.3.0. DETAILS OF 120 LACTOBACILLI ISOLATES OBTAINED FROM TRADITIONALLY PREPARED CURD (*DAHI*) AT DIFFERENT GEO-CLIMATIC CONDITIONS OF NEPAL.

Table AP.3.1 List of all the isolates, method of identification and name of the identified species

S. N.	Bio - Code	Sampling site	Preliminary identification using	Molecular identification by	Name of identified isolate
1	1	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus parabuchneri*
2	2	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
3	3	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
4	4	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
5	5	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
6	6	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
7	7	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
8	8	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
9	9	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
10	10	GHL	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
11	11	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
12	12	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
13	13	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
14	14	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
15	15	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
16	16	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
17	17	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
18	19	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
19	20	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
20	22	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
21	24	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
22	25	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
23	26	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
24	27	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
25	28	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
26	29	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
27	30	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus*

					rhamnosus
28	31	GHM	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus parabuchneri*
29	32	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
30	33	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
31	34	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
32	36	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
33	37	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
34	38	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
35	39	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
36	40	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
37	48	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
38	51	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
39	53	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
40	54	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
41	57	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
42	58	BHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus parabuchneri*
43	62	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
44	64	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus parabuchneri*
45	65	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
46	66	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
47	67	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
48	68	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
49	69	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
50	70	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
51	71	GHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
52	72	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus rhamnosus*
53	74	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
54	75	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
55	76	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
56	77	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
57	79	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
58	80	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
59	81	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*

60	82	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
61	83	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
62	84	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
63	85	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
64	86	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
65	87	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
66	88	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
67	89	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
68	90	RHM	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus delbrueckii*
69	91	RHM	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
70	92	RHM	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus brevis*
71	93	RHM	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus plantarum*
72	94	RHM	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus coryniformis*
73	95	RHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactibacillus harbinensis*
74	98	RHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus fermentum*
75	99	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
76	100	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
77	101	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
78	102	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
79	103	RHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus helveticus*
80	104	RHL	RSA/ITS	Species Specific PCR	*Lactobacillus helveticus*
81	126	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
82	128	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
83	129	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
84	130	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
85	131	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
86	132	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
87	133	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus paracasei*
88	134	LHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus paracasei*
89	135	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
90	136	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
91	138	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
92	140	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus*

					fermentum
93	141	BHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus delbrueckii*
94	142	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
95	143	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
96	144	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
97	145	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
98	147	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
99	148	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
100	149	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
101	150	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
102	151	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
103	152	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
104	154	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
105	155	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
106	157	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
107	158	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
108	159	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
109	160	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
110	161	BHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus fermentum*
111	162	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus fermentum*
112	127 a	LHL	RSA/ITS	16 S Sequencing & Species Specific PCR	*Lactobacillus delbrueckii*
113	127b	LHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
114	139	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
115	146	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
116	153 a	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
117	153b	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
118	156a	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
119	156b	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus delbrueckii*
120	52	BHL	RSA/ITS	Species Specific PCR	*Lactobacillus parabuchneri*

NOTE: GHM = Gorkha district (Hilly region), BHL = Bhaktapur district (Hilly region), RHM = Rasuwa district (Himalayan region), RHL = Rasuwa district (Himalayan region), LHL = Lalitpur district (Hilly region), RSA = Ribosomal spacer analysis, ITS = Internal transcribed spacer region, PCR = Polymerase chain reaction.

AP.4.0. NULEOTIDE SEQUENCE AND GENBANK ACCESSION NUMBER

AP.4.1. 16S rRNA SEQUENCE and PHYLOGETIC TREE BASED ON 16S rRNA SEQUENCE

>Seq_1 [Lactobacillus parabuchneri NAST-GHM31, 16S ribosomal RNA gene, partial sequence

TGCAGTCGAGCGAGCAGAACCAGCAGATTTACTTCGGTAATGACGCTGGGGACGCGAGC
GGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAA
ACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGT
AAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTAC
AGGCAATTGTTGCATAGT

>Seq_2 [Lactobacilllus parabuchneri NAST-BHL58], 16S ribosomal RNA gene, partial sequence

TGCAGTCGaACGCGTCTTGGTTATTGATGTTAAGTGCTTGCATTTAACTGATTTAACATT

GAGACGAGTGGCGAACTGGTGAGTAACACGTGGGTAACCTGCCCTTGAAGTAGGGGATA
ACACTTGGAAACAGGTGCTAATACCGTATAACAACCAAAACCACCTGGTTTTGGTTTAAA
AGATGGCTTCGGCTATCACTTTAGGATGGACCCGCGGCGTATTAGCTTGTTGGTAAGGTA
ACGGCCTACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGA
CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGT
TGTTGGAGAAGAACAGGTGTGAGAGTAACTGTTCACATCTTGACGGTATCCAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG
GATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAGGTCTGATGTGAAAGCCTTCGG
CTTAACCGGAGAAGTGCATCGGAAACCAGGAGAC

>Seq_3 [Lactobacillus brevis NAST-RHM92], 16S ribosomal RNA gene, partial sequence

TGCAAGTCGAACGAGCTTCCGTTGAATGACGTGCTTGCACTGATTTCAACAATGAAGCGA

GTGGCGAACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGG
AAACAGGTGCTAATACCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCT
TCGGCTATCACTTCTGGATGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCA
CCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
ATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAG
AAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTG
GGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGG
AGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGA

>Seq_4 [Lactobacillus coryniformis NAST-RHM94], 16S ribosomal RNA gene, partial sequence

TGCAGTCGACGCACTGACGTCGACCGAAGCTGCTTGCAGTGGACGTTGATTGACGTGAGT

GGCGGACGGGTGAGTAACACGTGGGTAACCTACCCTTAAGTGGGGGATAACATTTGGAA
ACAGATGCTAATACCGCATAACCATTCAGACCCATGGTCTGAATGAAAAAGGCTTTGGG
TGGCACTTTTGAACGGACCCGCGGCGTATTATTTAGTTGGTAAGGTAACGGCTTACCAAG
ACAATGAT

>Seq_5 [Lactobacillus harbinensis NAST-RHL95], 16S ribosomal RNA gene, partial sequence

TGCAGTCGAACGAGGTTTGGTCAGTTTGCGGTGGTGCTTGCATCACCAATTACCGATCAA
ACCGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTCAGCAGGGGATAACA
TTTGGAAACAGATGCTAATACCGTATAACCACGGAGACCGCATGGTCTCCGGGTAAAAG
ATGGCGCAAGCTATCACTGAAGGATGGACCCGCGGCGTATTAGCCAGTTGGTGGGGTAA
CGGCCTACCAAAGCGATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGC
AAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTT
ATTGAAGAAGAACGTGTGTGACAGTAACTGGTCATGCAGTGACGGTATTCAATCAGAAA
GTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
ATTTATTGGGCGTAAAGCGAGTGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCTTCGGC
TTAACCGAAGAAGGGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAA
CTCCATGTGTAGCGGTG

>Seq_6 [Lactobacillus fermentum NAST-RHL98], 16S ribosomal RNA gene, partial sequence

TGCAGTCGACGCGTTGGCCCAATTGATTGATGGTGCTTGCACCTGATTGATTTTGGTCGC
CAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAA
CATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGCTTAAA
AGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGT
AACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGG
ACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGC
GCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTG
TTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAA
AGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCG
GATTTATTGGGCGTAAAGAGAGTGCAGGCG

>Seq_7 [Lactobacillus helveticus NAST-RHL103], 16S ribosomal RNA gene, partial sequence

TGCAGTCGAGCGAGCAGAACCAGCAGATTTACTTCGGTAATGACGCTGGGGACGCGAGC
GGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAA
ACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGT

AAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTAC
AGGCAATTGTTGCATAGT

>Seq_8 [Lactobacillus delbrueckii subsp. bulgaricus NAST-LHL127], 16S ribosomal RNA gene, partial sequence

TGCAGTCGAGCGAGCTGAATTCAAAGATCCCTTCGGGGTGATTTGTTGGACGCTAGCGGC
GGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAACA
GGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGCAAG
CTGTCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAA
GGCAATGATGCGTAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGG
AGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGA
AGGATAGAGGCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCG
TAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA
ACTGCATCGGAAACTGTCATTCTTGAGT

>Seq_9 [Lactobacillus delbrueckii subsp. bulgaricus NAST- BHL141], 16S ribosomal RNA gene, partial sequence

TGCAGTCGAGCGAGCTGATTCAAAGATCCCTTCGGGGTGATTTGTTGGACGCTAGCGGCG
GATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAACAG
GTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGCAAGC
TGTCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAA
GGCAATGATGCGTAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGG
AGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGA
AGGATAGAGGCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCG
TAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA
ACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAG
CGGTGGAATGCGTAGATATATGGAA

>Seq_10 [Lactobacillus fermentum NAST-BHL161], 16S ribosomal RNA gene, partial sequence

TGCAGTCGACGCGTTGGCCCAATTGATTGATGGTGCTTGCACCTGATTGATTTTGGTCGC
CAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAA
CATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGCTTAAA
AGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGT
AACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGG

ACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGC
GCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTG
TTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAA
AGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCG
GATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGG
CTTAACCGGAGAAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAG

>Seq_11 [Lactobacillus delbrueckii subsp. bulgaricus NAST-RHM91, 16S ribosomal RNA gene, partial sequence

TGCAaGTCGAGCGAGCTGAATTCAAAGATCCCTTCGGGGTGATTTGTTGGACGCTAGCGG
CGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAAC
AGGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGCAA
GCTGTCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCA
AGGCAATGATGCGTAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGG
CCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATG
GAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTGGTGAAG
AAGGATAGAGGCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGC
GTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTA
GCGGTGGAATGCGTAGATATATGGAA

>Seq_12 [Lactobacillus plantarum NAST- RHM93, 16S ribosomal RNA gene, partial sequence

TGCaAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAG
TGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGA
AACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCT
TCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCTCA
CCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAG
AAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTG
GGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCG
AAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGT
GTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGT
CTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTA
GTCCATAC

>Seq_13 [Lactobacillus paracasei NAST-LHL134, 16S ribosomal RNA gene, partial sequence

TGCaAGTCGAACGAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAACATGGAACG
AGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTG
GAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGG
CGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCT
CACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGA
CACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTC
TGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGG
AGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACC
GAGGAAGCGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTG
GTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGG
TAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGA

AP.4.2. GENBANK ACCESSION NUMBERS FOR NUCLEOTIDE SEQUENCES

BankIt1665181 Seq_1	KM009071 *Lactobacillus parabuchneri* strain NAST-GHM31
BankIt1665181 Seq_2	KM009072 *Lactobacillus parabuchneri* strain NAST-BHL58
BankIt1665181 Seq_3	KM009073 *Lactobacillus brevis* strain NAST-RHM92
BankIt1665181 Seq_4	KM009074 *Lactobacillus coryniformis* strain NAST-RHM94
BankIt1665181 Seq_5	KM009075 *Lactobacillus harbinensis* strain NAST-RHL95
BankIt1665181 Seq_6	KM009076 *Lactobacillus fermentum* strain NAST-RHL98
BankIt1665181 Seq_7	KM009077 *Lactobacillus helveticus* strain NAST-RHL103
BankIt1665181 Seq_8	KM009078 *Lactobacillus delbrueckii* strain NAST-LHL127
BankIt1665181 Seq_9	KM009079 *Lactobacillus delbrueckii* strain NAST-BHL141
BankIt1665181 Seq_10	KM009080 *Lactobacillus fermentum* strain NAST-BHL161
BankIt1665181 Seq_11	KM009081 *Lactobacillus delbrueckii* strain NAST-RHM91
BankIt1665181 Seq_12	KM009082 *Lactobacillus plantarum* strain NAST-RHM93
BankIt1665181 Seq_13	KM009083 *Lactobacillus paracasei* strain NAST-LHL134

AP.5.0 QUESTIONNAIRE DURING SAMPLE COLLECTION

KNOWLEDGE, ATTITUDES AND USAGE PATTERNS OF PROCESSED AND FERMENTED DAIRY FOODS IN NEPAL

I. PERSONNEL INFORMATION OF THE RESPONDENT INSTITUTION

1.1 Name of the District & VDC (Ward No. & House No.):

…….

1.2 Name of Respondent: (Male/Female)

…….

1.3 Designation (Education/others):

…….

1.4 Contact Address:

……

❖ Telephone/Fax number:

…….

❖ E-mail address:

…….

II. USES AND CHOICE OF FOOD

2.1 Do you think food and nutrition play great role for maintaining or improving the human health?

i. YES ii) NO

2.2 Do you know that certain foods are health benefits?

i. YES ii) NO

2.3 If yes what are they and what are health benefits:

What you prefer more in your regular diet?

i. Cereals

ii. Vegetables
iii. Fruits
iv. Herbal Drinks
v. Functional Foods
vi. Others :

2.4 What is the general trend you include fermented food in your dairy habit/you use prefer fermented food during:

i) Breakfast ii) Launch iii) Dinner iv) Others:

2.5 How long have you been using fermented food?

…… (Tentative years ago or around).

2.6 Do the health workers/persons (medical person; doctors) suggest/recommend you to use probiotic foods (dairy products) after antibiotic treatment or antibiotic associated diarrhea?

i. YES ii) NO

III. INDIGENOUS BIOTECHNOLOGICAL PRODUCTS BEING DEVELOPED/USED IN YOUR HOUSE/VILLAGE/CITY.

3.1 List of traditional fermented products:

S.N.	Types of products being developed	Traditional Name of the Products		Source of organisms/starter culture	Technology Local/National/Foreign
		Local Name	General Name		
1.	Gundruk				
2.	Masaura				
3.	Aachar(Pickles)				
4.	Sukuti				
5.	Mushroom				
6.	Raksi (Alcoholic)				
7.	Jand (Alcoholic)				
8.	Milk				
9.	Curd				
10.	Butter (Nauni)				
11.	Ghee				
12.	Yogurt (Mohi)				
13.	Cheese				
14.	Churpi				
15.	Chheau (Mushroom)				
16.	Others				

3.2 Please list the Biotechnology related facilities in your VDC/House for production of the fermented foods.

S.N.	Infrastructure (Laboratory space, growth rooms, glass house etc.)	Equipment (please mention the list)	Information/Library (Internet, books, journal facilities)
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			

IV. IMPACTS OF FERMENTED FOODS ON HUMAN HEALTH:

4.1 Do you know about the positive impacts of the fermented foods?

i) YES ii) NO

4.2 If yes what is your opinion:

In your opinion, do you think fermented foods are important?

i. YES ii) NO

What is the importance of the fermented foods?

i. Nutritious
ii. Medical values
iii. Taste
iv. Refreshing
v. Storage for future use
vi. Source of Income
vii. Others:

4.3 DO Most of the family members prefer using fermented foods?

i. YES ii) NO

4.3.1 IF yes what sort of the fermented food they prefer more:

i. Dairy
ii. Vegetables/Leaf
iii. Cereal grains (alcoholic)
iv. Fruits (alcoholic)

v. Meat
vi. Others:

4.3.2 In dairy fermented food what do you prefer more?

i. Milk
ii. Curd
iii. Whey (Yoghurt/Mohi)
iv. Butter
v. Cheese
vi. Khuwa
vii. Others:

4.4 Who usually is involved in processing/making/preparing the fermented foods?

i) House wife ii) House guardian/man iii) Children iv) Other

4.5 Do guardian advice the use of dairy fermented foods?

4.5.1 What, usually, a guardian (mothers) advice for the use of the fermented foods?

i. YES ii) NO

4.5.1.1 IF YES what are the dairy foods suggested to use:

i. Milk
ii. Curd/Yogurt/Mohi
iii. Cheese
iv. Panir
v. Butter
vi. Sweets
vii. Others:

4.6 The dairy fermented food you use ……?

i) Homemade ii) Commercial

4.6.1 If commercial what is the monthly expense you make for the consumption of the dairy fermented foods?

……

4.7 Do you know about the negative impacts of the fermented foods?

i. YES ii) NO

4.7.1 If yes, please specify from following options:

i. Bacterial (please specify the origin)
……..
ii. Fungal (please specify the origin)
……
iii. Viral (please specify the origin)
……...
iv. Hazardous chemicals (please specify the chemical used) …….
v. Epidemiological studies carried out by your institution (please specify the field) ……..
vi. Others if any
……

4.8 Are you incorporating Good Handling Practice (GHP/GMP) in your house/industry for the production of the fermented foods?

i) YES…….ii) NO ……..

4.9 What safety measures are being undertaken to deal with above mentioned infectious or hazardous chemicals in your house/organization?

i. Boiling
ii. Drying
iii. Smoking
iv. Incineration
v. Autoclaving
vi. Hazardous waste disposal
vii. Laboratory isolation
viii. Activities for human safety
……..
ix. Activities for environmental safety
……
x. Any other (please specify):
……

V. PRIORITY AREAS FOR YOUR VDC IS FERMENTED PRODUCTS OR NOT

5.1 In your opinion, which factors are responsible for least development and commercialization of traditional fermented products (Biotechnology/modern biotechnology in Food/Industrial sector) of Nepal? Please tick appropriate opinion from following:

i.Government least priority
ii.Lack of adequate funding in training and R&D
iii.Lack of skilled human resources
iv.Inadequate Government budget in Food and Industrial Biotechnology R&D
v.Lack of national commitment
vi.Lack of adequate training opportunities

vii. Biotechnology being ignored field in Nepal
viii. Highly sophisticated and advanced technology but least paid human resources
ix. Lack of Business Investment
x. Lack of Information Resources/Documentation system
xi. Others if any:
……

5.2 State the most relevant steps to be taken to address the concerns you described above (What you think should be done to enhance the commercialization of the fermented foods).

i. ……
ii. …….

5.3 What information regarding Biotechnology has your own house/VDC/institution developed?

i. Techniques
ii. Modified products
iii. Publications
iv. Website
v. Databases
vi. Newsletters
vii. Others if any: ……..

VI. EXISTING BIOTECHNOLOGY RELATED EXPERTS/EXPERTISE

6.1 Total number of Employees (Technical)…….

6.2 Number of Expert in food/industrial biotechnological fields: (Please further specify subject qualification and expertise).

A. Human Resource (HR) available in the Institute/Industry

S.N.	Training/Degree	Specialization	Degree (National/International)
1.			
2.			

6.3 Is your House/VDC/Industry currently provided helps/funds from various Organizations?

i) Yes …… or ii) No……

If yes please provide details:

A. Current/On-going commercial producer/industries in the field of Fermented food production

S.N.	Name of the Product	In house Project/Collaborative	Duration of the Project	Incharge/Supervisor	Technology Developed for transfer
1.					
2.					
3.					
4.					

6.4 Please provide the list of present/past projects for fermented foods being produced:

……
…
……
……
……
……
……

6.5 Do you have action plans developed for any of your biotechnological projects/or action plans developed at national level?

i) YES …… or ii) NO ……

If yes, could you provide a copy of it for our reference?

…….

6.6 Please name relevant experts and expertise if any in the field of socio-economic impact assessment of Biotechnology/modern biotechnology related projects in Food/Industrial Sector.

i. ……
ii. ……..
iii. ……..
iv. ……..

VII. BIOTECHNOLOGY/MODERN BIOTECHNOLOGY INFRASTRUCTURE FACILITIES

7.1 Characterization of Institution: Please tick the appropriate option:

i. Food Biotechnological R&D
ii. Modern Food Biotechnological R&D (Genetic Engineering, Molecular Diagnostic, Genomics, Proteomics, Bioinformatics)
iii. Food Biotechnological Business (raw material or processed)
iv. Pharmaceutical Biotechnological Business (raw material or processed)
v. Industrial Biotechnological Business

vi. Any other, please specify:

7.2 Biotechnological products being produced /techniques/products being used in your Institution:

S.N.	Biotechnological Industry	Types of products being developed	Annual production	Business Status (Annual Transaction)
1.				
2.				
3.				
4.				
5.				

VIII. ANYTHING YOU WOULD LIKE TO COMMENT/ADD/SUGGEST/HIGHLIGHT

……
……
……
……
……
……
……

AP.6.0 PUBLICATIONS, CONFERENCES AND ACHIEVEMENTS

International Journal Articles (published and under publication)

1. **Koirala R**., Ricci G., Taverniti V., Ferrario C., Malla R., Shrestha S., Fortina M.G. & Guglielmetti S. (2014). Isolation and molecular characterization of lactobacilli from traditional fermented Dahi produced at different altitudes in Nepal. *Dairy Sci Technol*. **94**, 397-408.
2. **Koirala R**., Shrestha S., Guglielmetti S., Ricci G., Vijayendra S.V.N., Varadaraj M.C. & Malla R. (2014). Prevalence of probiotic lactobacilli in *dahi* prepared at different geo-climatic conditions of Nepal. *Indian Food Industry Mag*. Vol. **33** (2): 15 (*Best Poster Abstract Publication*)
3. **Koirala R**., Taverniti V., Balazaretti S., Ricci G., Fortina M.G. & Guglielmetti S. (2015) Melting curve analysis of a groEL PCR fragment for the rapid genotyping of strains belonging to the *Lactobacillus casei* group of species. *Microbiological Research*. **173**: 50-58.
4. *In vitro* probiotic properties of lactobacilli isolated from traditional Nepalese fermented *Dahi*. (*Manuscript in Preparation*).
5. Optimization of culture conditions and sugar utilization study for indigenous lactic cultures. (*Manuscript in Preparation*).
6. Preparation of indigenous fermented probiotic *Dahi* using a novel lactic culture: *Lactobacillus helveticus* NAST-RHL103. (*Manuscript in Preparation*).

International Conferences/Posters/Proceedings

1. **Koirala R**., Shrestha S & Malla R., (2012). Isolation and Molecular characterization of Probiotic Microorganisms from Dairy Products of Nepal. Poster Presentation and Abstract Publication in Proceedings of The Sixth National Conference on Science and Technology, Sept 25 - 27, 2012
2. **Koirala R**., Shrestha S., Guglielmetti S., Ricci G., Vijayendra S.V.N., Varadaraj M.C. & Malla R. (2013). Prevalence of probiotic lactobacilli in dahi prepared at different geo-climatic conditions of Nepal. In: Proceedings of 7th International Food Convention NSuRE-Healthy Foods, December 18-21, 2013 CSIR-CFTRI, Mysore, India.

3. **Koirala R**., Shrestha S & Malla R. (2015). Probiotic attributes of lactobacilli isolated from Nepalese *Dahi*. Poster presentation during World DNA 25th May 2015 at Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur, Nepal.

Awards and Achievements

1. NAST-Universities Collaborative PhD Fellowship (20th May 2010 – 20th May 2015).
2. Indian National Science Academy (INSA) Fellow under INSA-NAST Exchange of Scientist Programme, India (6th November 2011 to 5th February 2013).
3. Erasmus Mundus Europe Asia (EMEA) Doctoral Fellowship, University of Milan coordinated by Lund University Sweden (14th September 2012 to 14th June 2013).
4. CSIR-TWAS Postgraduate Fellowship Award (12th September 2013 to 11th December 2014).
5. Best Poster Award during 7th International Food Convention NSuRE-Healthy Foods, December 18-21, 2013 CSIR-CFTRI, Mysore, India

PhD coursework/Trainings/Participation in Conferences

1. Participation in International Conference Biodiversity. Livelyhood and Climate Change in the Himalayas, 12-14 December 2010, Kathmandu, Nepal.
2. Participation in International Conference on Functional Dairy Foods (ICFDC), Nov. 16-19, 2011, Karnal, India.
3. Participation in R&D Project Formulatin and Evaluation. October 5-7, 2012 in Nepal Academy of Science and Technology, Khumaltar Lalitpur.
4. Participation in 2nd International Symposium on Biotechnology Education, Research & Industrialization, 29th & 30th June, 2010 in WhiteHouse Institute of Science and Technology, Khumaltar, Lalitpur.
5. Training Course on Design and Analysis of Experiments: Using Statistics Wisely organized by the Faculty of Sciences of NAST. 2 August 2010.
6. Successfully participated and passed the Pre-requisite Course Designated for Ph.D. Fellowship by the Faculty of Science at NAST. (Dec. 2010 to Aug. 2011).
7. Research cum Training at National Dairy Research Institute (NDRI), Karnal, India under INSA-NAST Exchange of Scientist Programme. (6th November 2011 to 5th February 2013).

Dairy Sci. & Technol. (2014) 94:397–408
DOI 10.1007/s13594-014-0167-4

NOTE

Isolation and molecular characterization of lactobacilli from traditional fermented Dahi produced at different altitudes in Nepal

Ranjan Koirala · Giovanni Ricci · Valentina Taverniti · Chiara Ferrario · Rajani Malla · Sangita Shrestha · Maria Grazia Fortina · Simone Guglielmetti

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Abstract Dairy products are an important part of daily food in the Himalayan country of Nepal. A wide variety of traditional fermented milk products are obtained in relation to different geo-climatic conditions of the country and different ethnic groups. Locally called Dahi is the most popular product, but little data are available on the autochthonous lactic acid bacteria (LAB) characterizing this yogurt-like product. Thirty-two replicate samples of indigenous Dahi were collected from four districts of Nepal at different altitude. In total of 193 isolates were obtained. Of these, the 120 rod-shaped isolates representing the dominant LAB population (62%) were further identified at the species level by using internal transcribed spacers (ITS) amplification, 16S rRNA gene sequence determination, and species-specific PCR. A further characterization at the strain level was carried out by combining analysis of repetitive elements and randomly amplified polymorphic DNA (RAPD) typing. Based on these analyses, the isolates were grouped in ten different species, among which *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. paracasei*, and *L. rhamnosus* represented the dominant species. A high degree of intraspecies diversity was also observed for all *Lactobacillus* species, except for *L. rhamnosus isolates*, which proved to give a single typing profile. Bacterial isolates represent a source of novel potential probiotics and starter cultures. The strain collection obtained from this study is a first step in the preservation of the natural biodiversity of bacterial population of the traditional Nepalese fermented Dahi.

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1 Introduction

Over the past few years, the microbiological characteristics of traditional fermented milk products have been studied in many countries, and original collections of lactic acid bacteria (LAB) have been constituted (Yu et al. 2011; Bao et al. 2012). They represent an important tool both for preserving the rich microbial biodiversity that characterizes naturally fermented food and for obtaining new cultures. It is known that traditional fermented foods have unique and different microbial populations dependent on the production technology as well as on the environmental characteristics of the localities where they have been produced (Leroy and DeVuyst 2004; Colombo et al. 2009). Moreover, many reports have shown that artisanal dairy products can represent interesting sources for the isolation of bacterial strains with useful probiotic traits (Heller et al. 2003; Taverniti and Guglielmetti 2010).

Dairy products are an important part of daily food intake in the Himalayan country of Nepal. As a consequence of different geo-climatic conditions of the country and of the diversity of ethnic groups, a wide variety of traditional fermented milk products are produced and consumed. Their local names are Dahi (curd), Mohi (buttermilk), Ghiu (butter), and Chhurpi (dried cheese). These products are made with milk from different animals that are reared in the different geographical regions for dairy production, such as cow and buffalo are reared in the Terai and Hilly regions (from 100 to 3,000 m) whereas yak and chauri at high altitudes (>3,000 m) in the Himalayan region.

Locally called Dahi (curd) is the most indigenous and popular product of Nepal. It is a yogurt-like product prepared in different parts of the country and used as either nutritional food, appetizer, or dessert, as well as for the preparation of other ethnic dairy fermented products, as Ghiu, Mohi, and soft Chhurpi (Tamang 2010). There are different conventional methods for the preparation of Dahi, comprising the use of starter cultures, even though this is not a routine practice. In most cases, Dahi is traditionally made at household level, without starter cultures but using a portion of previously produced Dahi or Mahi or cream as inoculum. Usually, after heating or boiling, the milk is cooled to 30–40 °C and then transferred to a wooden (locally named "Theki") vessel, where it is left overnight at 25–30 °C. Fermentation is carried out spontaneously by natural microbiota of the milk, along with the microorganisms that persist on the surface of vessels and in the processing environment. In the Terai region and some Hilly regions, earthenware pots (natural red clay pot locally called "maato ko kataaro") are more common. These pots have a porous surface, so moisture is absorbed by the container especially when the Dahi tends to exude some whey and also gives a "muddy" flavor and a thicker texture. This vessel is wrapped in cloth, in hay, in sawdust, or put in a straw box to maintain the suitable temperature for the souring and coagulation processes. This step is the most difficult to achieve in the regions of Nepal at high altitudes (>3,500 m), with consequent problems related to slow acidification and delay in coagulation. In Nepal, the traditional Dahi can also be obtained by a semi-continuous method. Boiled and cooled milk, inoculated with an indigenous natural starter culture (in many cases, previously made Dahi), is put in the container,

which is covered with clothes and kept warm. The next day, a further quantity of cooled boiled milk is added and the fermentation goes on. This topping up with cooled boiled milk is repeated daily until the container is full. After the last incubation, the Dahi may be either used for consumption or churned to obtain butter or other dairy products.

The present study aimed to isolate and identify the natural dominant lactobacilli in traditional Dahi of the Hilly and Himalayan regions of Nepal, by using phenotypic and molecular methods, with the final purpose of obtaining novel probiotic or starter culture candidates. To our knowledge, no similar investigation on this ethnic fermented product has been carried out so far.

2 Materials and methods

2.1 Collection of samples

A total of 32 replicate samples (altogether 64) of traditional fermented Dahi were collected from four districts, viz. Bhaktapur, Gorkha, Lalitpur, and Rasuwa. These districts lie in the Hilly (HL) and Himalayan (HM) regions, according to the geographical map of Nepal. Stratification of sampling sites were done based on total number of districts in each region, and proportional random sampling method was followed for sample collection. The collected samples were transported to the laboratory under refrigerated conditions (4 °C) and subjected to microbiological analysis within the following 24 h.

2.2 Isolation, enumeration, and phenotypic characterization

Samples (10 g each) were homogenized in 90 mL of 0.85% (*w*/*v*) sterile saline solution in a Vortex (Rexmed, Taiwan); serially diluted in the same diluents; plated in duplicate on de Man, Rogosa, and Sharpe (MRS) (Difco, Detroit, MI, USA); and incubated at 37 °C for 48 h. After incubation and counting, colonies were all picked up when they not exceeded the number of ten colonies per plate, otherwise they were selected randomly. Purity of the isolates was checked by streaking several times and subculturing on fresh MRS agar, as well as MRS broth, followed by microscopic examination. Isolates were Gram-stained and tested for catalase production. Identified isolates of LAB were preserved in MRS broth containing 25% (*w*/*v*) glycerol, at −20 °C.

2.3 Genotypic identification

Genomic DNA for all PCR reactions was extracted from a 100 μL of an overnight culture diluted with 300 μL of TE buffer (10 mmol.L^{-1} Tris–HCl, 1 mmol.L^{-1} Na_2EDTA, pH 8.0) as previously described (Mora et al. 2000). The DNA sequences for the primers used in this study, their corresponding specificities, and the thermal cycle parameters employed are reported in Table 1. PCR reactions were performed in a 25 μL reaction mixture containing 100 ng bacterial DNA, 2.5 μL 10×reaction buffer Dream Taq™ (Fermentas, Vilnius, Lithuania), 200 μmol.L^{-1} of each dNTP,

Table 1 PCR primers and conditions used for isolates identification

Primer specificity and reference	Primer pair (5′ to 3′)	Thermal conditions	Amplicon (bp)
L. delbrueckii subsp. *bulgaricus* (Torriani et al. 1999)	Fw: GTGCTGCAGAGAGAGTTTGATCCTGGCTCAG Rev: ACCTATCTCTAGGTGTAGCGCA	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 1 min at 57 °C, 1 min at 72 °C	1,030
L. paracasei (Ward and Timmins 1999)	Fw: CACCGAGATTCAACATGG Rev: CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 54 °C, 45 s at 72 °C	290
L. rhamnosus (Ward and Timmins 1999)	Fw: TGCATCTTGATTTAATTTTG Rev: CCCACTGCTGCCTCCCGTAGGAGT	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 54 °C 45 s at 72 °C	290
L. fermentum (Coton et al. 2008)	Fw: TGTACACACCGCCCGTC Rev: TTTTCTTGATTTTATTAG	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	460; 270
L. parabuchneri (Coton et al. 2008)	Fw: TGTACACACCGCCCGTC Rev: TGTTACTCCGGTCTGTGC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	330
L. helveticus (Fortina et al. 2001)	Fw: CTGTTTTCAATGTTGCAAGTC Rev: TTTGCCAGCATTAACAAGTCT Fw: CGCTGATTCTAAGTCAAGCT Rev: CGACTAAGAAGTGGAACATTA Fw: TCTTATTACGCAATGGACCAA Rev: AATACCGTTCTTGAGGTTAGA	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C	918; 726; 524
L. brevis (Coton et al. 2008)	Fw: TGTACACACCGCCCGTC Rev: TAATGATGACCTTGCGGTC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 45 s at 72 °C	330
L. plantarum (Torriani et al. 2001)	Fw: CCGTTTATGCGGAACACC Rev: TCGGGATTACCAAACATCAC	2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 56 °C, 45 s at 72 °C	318
Internal Transcribed Spacer region (ITS) (Jensen et al. 1993)	Fw: GAAGTCGTAACAAGG Rev: CAAGGCATCCACCGT	2 min at 94 °C, 5 cycles of 45 s at 94 °C, 1 min at 55 °C; 1 min at 72 °C and 30 cycles of: 45 s at 94 °C; 45 s at 60 °C, 2 min at 72 °C	

0.5 mmol.L^{-1} $MgCl_2$, 0.5 μmol.L^{-1} each primer, and 0.5 U Dream Taq™ DNA polymerase. Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf, Milan, Italy). Amplification products were electrophoresed in 1.5–2.5% (*w*/*v*) agarose gel (with 0.2 μg.mL^{-1} of ethidium bromide) in 1× TAE buffer (40 mmol.L^{-1} Tris-acetate, 1 mmol.L^{-1} EDTA, pH 8.0) and photographed. A GeneRuler DNA ladder mix (Fermentas) was used as a size marker.

The 16S ribosomal RNA (rRNA) gene was amplified by PCR, using primers P0 (5′-GAAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′). The PCR mixtures were subjected to the following thermal cycling: 2 min at 94 °C, then 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, followed by a 7-min final extension at 72 °C. UltraClean PCR Clean-Up Kit (MoBio, Cabru s.a.s, Arcore, Italy) was used to purify PCR products that were sequenced. A 500 bp portion of the 16S rRNA gene was sequenced for representative isolates. Sequencing reactions were performed using primer pA (5′-AGAGTTTGATCCTGGCTCAG; nucleotides eight to 28 of the 16S rRNA gene of *Escherichia coli*), which allowed to obtain the sequence of variable regions V1, V2, and V3 (Edwards et al. 1989). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/blast). The taxonomic differentiation of *L. paracasei* and *L. rhamnosus* was obtained employing species-specific probes as described by Ward and Timmins (1999) (Table 1). The assignment of all *Lactobacillus delbrueckii* isolates to a precise subspecies was obtained through an intraspecies-specific PCR analysis according to Torriani et al. (1999) (Table 1).

2.4 Genetic typing of bacterial isolates

Genetic fingerprinting was carried out by combined repetitive extragenic palyndromic PCR typing technique (REP-PCR) using primers (GTG)5 (5′-GTGGTGGTGGTGGT G-3′; annealing temperature (T_a)=42 °C) and BOXA1R (5′-CTACGGCAAGGCGA CGCTGACG-3′; T_a=48 °C) (Guglielmetti et al. 2008) and random amplification of polymorphic DNA-PCR (RAPD) typing with primers M13 (5′-GAGGGTGGCGGT TCT-3′; T_a=38 °C), AP02 (5′-AGTCAGCCAC-3′; T_a=32 °C), OPI17 (5′-CGAGGG TGGTGATG-3′; T_a=46 °C), OPI02 (5′-GCTCGGAGGAGAGG-3′; T_a=48 °C), and 1254 (5′-CCGCAGCCAA-3′; T_a=33 °C) (Torriani et al. 1999; Mora et al. 2000; Rossetti and Giraffa 2005). An amplification protocol of 35 cycles was used. The PCR products were separated by electrophoresis and photographed. Banding pattern similarity was evaluated by construction of dendrograms using the NTSYSpc software, version 2.11 (Applied Biostatics Inc., Port Jefferson, NY, USA), employing the Jaccard similarity coefficient. A dendrogram was deduced from a similarity matrix using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm. The faithfulness of the cluster analysis was estimated by calculating the coefficient correlation value for each dendrogram.

3 Results and discussion

All samples were from indigenous Dahi products obtained by traditional methods from different geographical regions. The viable mesophilic counts of LAB on MRS varied in

the range of 6.3 to 10.4 $\log_{10}$CFU g^{-1} (Table 2). The samples of Dahi produced in the Lalitpur and Bhaktapur districts showed higher LAB count than the samples of Dahi produced in Gorkha district. Two hundred and five bacterial isolates were collected; among them, 193 isolates were considered as presumptive LAB by their positive Gram reaction, absence of catalase, and lack of mobility. The majority of isolates were rod shaped (120 isolates) and, therefore, plausibly belonging to the genus *Lactobacillus*; the remaining were cocci (73 isolates). Other reports also showed the dominance of lactobacilli in traditional dairy products of the Himalayan region (Tamang et al. 2000; Dewan and Tamang 2007).

The identification and characterization of autochthonous *Lactobacillus* strains are important for understanding their contribution to the sensorial characteristics of the final product and for providing new strains to be used as industrial starters. The genus *Lactobacillus*, in fact, has a dominant role over other bacterial genera in food fermentation technologies (e.g., *L. delbrueckii*, *L. helveticus*, *L. plantarum*) and probiotic applications (e.g., *L. paracasei*, *L. rhamnosus*). For these reasons, the 120 rod-shaped isolates were selected for further studies.

At first, the 120 *Lactobacillus* isolates were submitted to molecular analysis for their identification at species level. To this aim, the isolates were clustered in nine groups according to electrophoretic profiles obtained by PCR amplification of the 16S–23S rRNA spacer region (ITS) (Fig. 1). The taxonomic identification was then reached by 16S rRNA gene sequencing of representative isolates for each cluster and confirmed by species-specific amplifications of all strains belonging to the same cluster. For the majority of the groups, the ITS profile was characteristic of one *Lactobacillus* species with the exception of cluster 2, which included two related species, *L. paracasei* and *L. rhamnosus*. The taxonomic differentiation of *L. paracasei* and *L. rhamnosus* was obtained employing species-specific probes. Finally, an intraspecies-specific PCR analysis (Torriani et al. 1999) allowed the assignment of all *L. delbrueckii* isolates (cluster 1) to a precise subspecies.

Based on these analyses, the 120 isolates were classified as belonging to ten different species (Table 3): *L. delbrueckii* subsp. *bulgaricus* (46 isolates, ITS cluster 1), *L. paracasei* (30 isolates, cluster 2), *L. rhamnosus* (21 isolates, cluster 2), *L. fermentum* (12 isolates, cluster 3), *L. parabuchneri* (5 isolates, cluster 4), *L. helveticus* (2 isolates, cluster 5), *L. coryniformis* (1 isolate, cluster 6),

Table 2 Sampling location and enumeration of lactic acid bacteria (LAB) isolated from indigenous Dahi

No. of samples	Sampling location in Nepal				LAB counts on MRS at 37 °C [$\log_{10}$CFU g^{-1}]	
	District	Region	Altitude (m)	Milk from	Range	Average
8	Gorkha	Himalayan	3,615–3,791	Cow	6.3–8.1	7.94±0.90
4	Rasuwa	Himalayan	3,017–3,977	Chauri	6.9–8.2	7.85±0.72
7	Rasuwa	Hilly	1,717–1,731	Buffalo	7.2–9.4	7.88±0.35
4	Lalitpur	Hilly	1,330–1,366	Buffalo	9.2–10.4	9.25±1.20
9	Bhaktapur	Hilly	1,342–1,357	Cow and buffalo	7.3–8.4	8.10±0.80

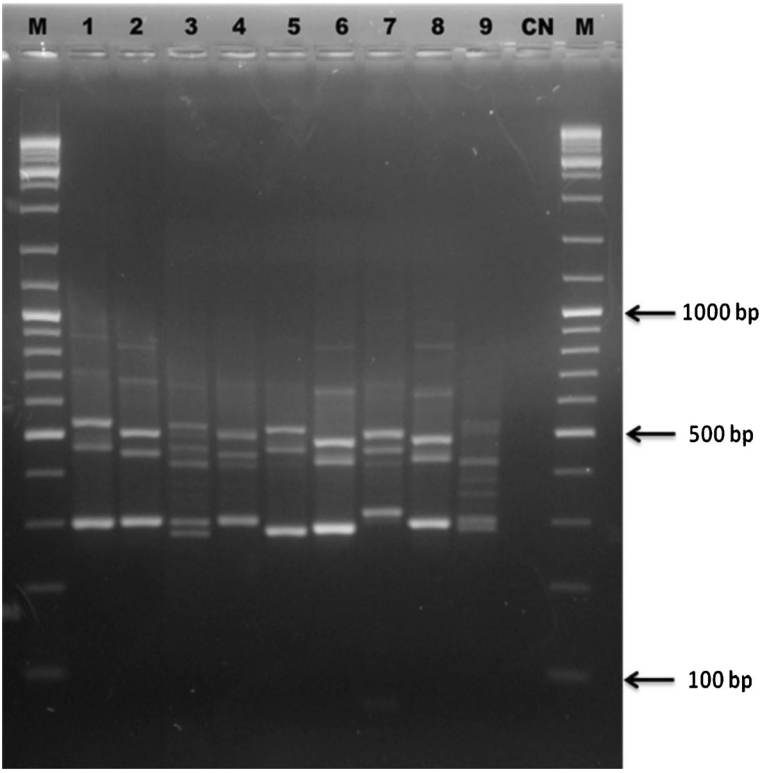


Fig. 1 ITS profiles obtained from representative lactobacilli isolates from traditional Dahi samples. *M* molecular weight

Table 3 Distribution of lactobacilli isolated from indigenous Dahi samples

Species	No. of isolates (%)	Samples				
		Gorkha-HM	Rasuwa-HM	Rasuwa-HL	Lalitpur-HL	Bhaktapur-HL
L. delbrueckii subsp. *bulgaricus*	46 (38)	–	7	10	6	23
L. paracasei	30 (25)	23	4	–	3	–
L. rhamnosus	21 (18)	12	–	–	–	9
L. fermentum	12 (10)	4	–	1	1	6
L. parabuchneri	5 (4)	3	–	–	–	2
L. helveticus	2 (1.7)	–	–	2	–	–
L. brevis	1 (0.8)	–	1	–	–	–
L. coryniformis	1 (0.8)	–	1	–	–	–
L. harbinensis	1 (0.8)	–	–	1	–	–
L. plantarum	1 (0.8)	–	1	–	–	–
Total	120	42	14	14	10	40

HM Himalayan region, *HL* Hilly region

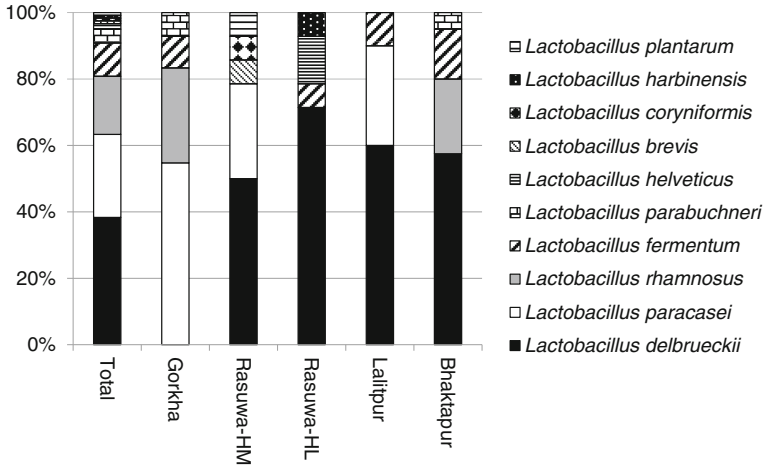


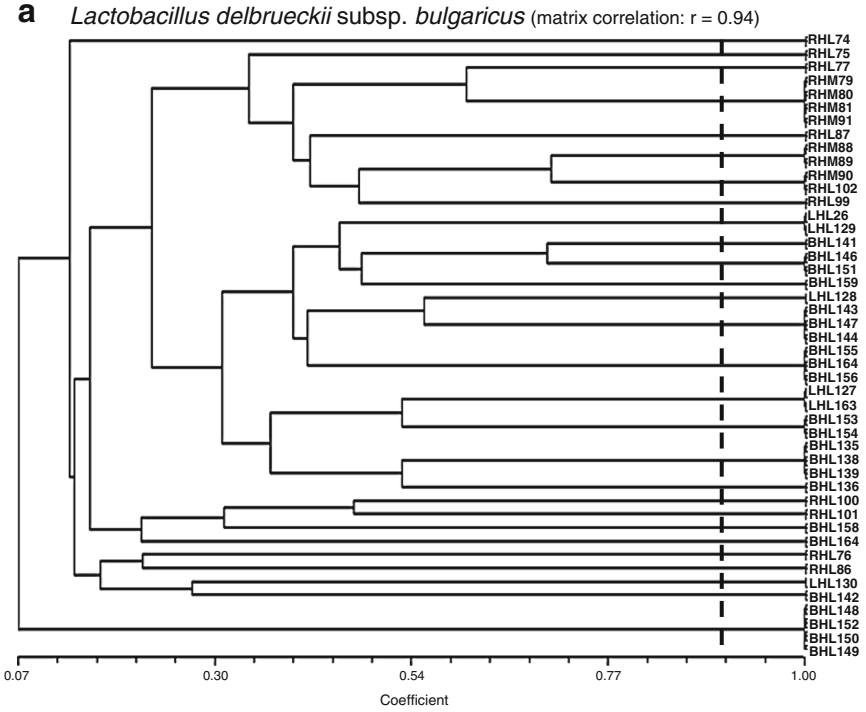
Fig. 2 Relative distribution of the *Lactobacillus* species identified in Dahi samples according to the geographical origin

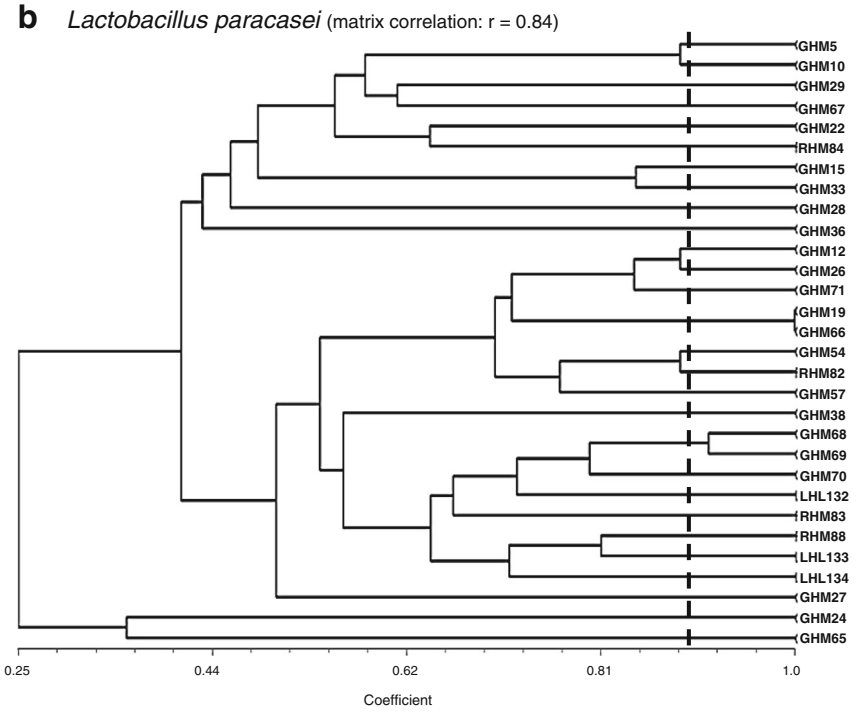
L. harbinensis (1 isolate, cluster 7), *L. brevis* (1 isolate, cluster 8), and *L. plantarum* (1 isolate, cluster 9).

According to our data, the homofermentative *L. delbrueckii* subsp. *bulgaricus* can be considered as one of the dominant lactobacilli in Dahi products. It accounted for 38% of the total isolates and was the predominant *Lactobacillus* population in the majority of the samples. The isolation of *L. delbrueckii* from the traditional Dahi was previously reported (Dellaglio et al., 2005; Watanabe et al., 2009). Particularly, *L. delbrueckii* subsp. *bulgaricus* was shown as the most abundant LAB species also in market Dahi products from Pakistan (Soomro and Masud 2007). Also the species *L. casei* or *L. paracasei* was already reported as a dominant group of lactobacilli in Dahi (Dewan and Tamang 2007; Soomro and Masud 2007). Particularly, we observed that the facultative heterofermentative *L. paracasei* (accounting for 25% of the total isolates) characterizes samples of Dahi produced in the Gorkha district. In general, we found a clearly different distribution of lactobacilli in the diverse dairy samples, which can be explained by the well-known importance of the environment on the relative distribution of different bacterial groups in the microbial ecosystems. Thus, animal origin of the milk, altitude, different technical conditions of product preparation, and any potential factor determining temperature shifts could have affected the bacterial competition during fermentation process.

The other most abundant lactobacilli resulted *L. rhamnosus* (18% of isolates) and *L. fermentum* (10%), two species that were reported to be isolated from Dahi only in

Fig. 3 Unweighted pair group method using average linkages (UPGMA) dendrogram derived from similarity ▶ coefficients calculated by the Jaccard method (simple Jaccard (Sj) coefficients; shown on the scale at the bottom), showing the relationship among isolates, analyzed by RAPD and REP-PCR. Samples with a similarity coefficient higher than 0.9 (*vertical line*) have been included in the same genotype. **a** *Lactobacillus delbrueckii* subsp. *bulgaricus* isolates using combined OPI17, OPI02, and M13 fingerprints. **b** *Lactobacillus paracasei* isolates using combined 1254, M13, BOXA1R, and (GTG)5 fingerprints. **c** *Lactobacillus fermentum* isolates using combined OPI17, 1254, and M13 fingerprints. Origin of the isolates: LHL Lalitpur, HL; *BHL* Bhaktapur, HL; *RHL* Rasuwa, HL; *RHM* Rasuwa, HM; *GHM* Gorkha, HM


a
Lactobacillus delbrueckii subsp. bulgaricus (matrix correlation: r = 0.94)
RHL74
RHL75
RHL77
RHM79
RHM80
RHM81
RHM91
RHL87
RHM88
RHM89
RHM90
RHL102
RHL99
LHL26
LHL129
BHL141
BHL146
BHL151
BHL159
LHL128
BHL143
BHL147
BHL144
BHL155
BHL164
BHL156
LHL127
LHL163
BHL153
BHL154
BHL135
BHL138
BHL139
BHL136
RHL100
RHL101
BHL158
BHL164
RHL76
RHL86
LHL130
BHL142
BHL148
BHL152
BHL150
BHL149
0.07
0.30
0.54
0.77
1.00
Coefficient


b
Lactobacillus paracasei (matrix correlation: r = 0.84)
GHM5
GHM10
GHM29
GHM67
GHM22
RHM84
GHM15
GHM33
GHM28
GHM36
GHM12
GHM26
GHM71
GHM19
GHM66
GHM54
RHM82
GHM57
GHM38
GHM68
GHM69
GHM70
LHL132
RHM83
RHM88
LHL133
LHL134
GHM27
GHM24
GHM65
0.25
0.44
0.62
0.81
1.0
Coefficient

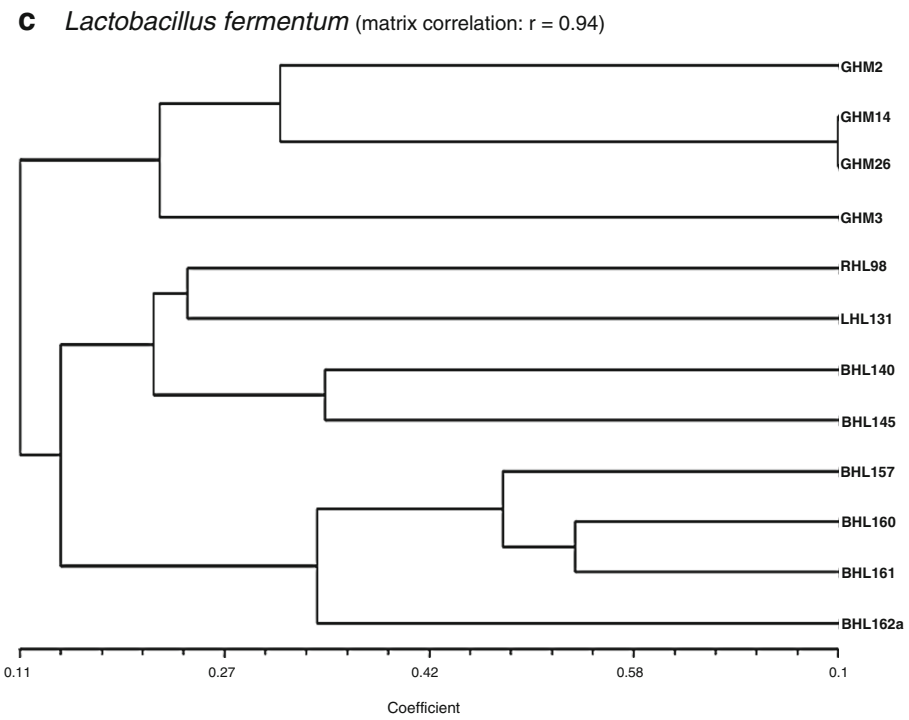


Fig. 3 (continued)

another published study, in which, however, the identification was based exclusively on sugar fermentation patterns (Bhardwaj et al., 2012).

In addition, a few other *Lactobacillus* species were found at very low frequency in some samples (Table 3; Fig. 2). Reportedly, also several other *Lactobacillus* species have been identified in traditional fermented Dahi, such as *L. bifermentans* and *L. alimentarius* (Dewan and Tamang 2007). Our study indicates that the Himalayan ethnic fermented milk products are a rich source of different lactobacilli confirming previous results (Satish Kumar et al., 2013).

The technological properties and the probiotic potential of microorganisms are markedly strain specific; for this reason, we undertook the molecular fingerprinting of the main *Lactobacillus* species characterizing the traditional Dahi samples using RAPD and REP-PCR, which are techniques possessing intraspecies discriminatory power (Fig. 3). Notably, this analysis revealed that *L. rhamnosus* isolates, although originating from samples collected from different geographical regions, had identical/monomorphic fingerprints with all the tested primers. On the contrary, *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei*, and *L. fermentum* isolates showed a high degree of biodiversity at the strain level. More specifically, the cluster analysis resulting from the combined patterns of the different primer sets revealed that most of the profiles within each species were unique (Fig. 3). Considering that technological and probiotic features of microorganisms are strain specific, this result is of interest in light of the possibility to select new potential probiotic strains or starter cultures to be used at industrial level.

4 Conclusion

The main aim of the present study was to isolate and genetically characterize the dominant lactobacilli in traditional Dahi collected from the different geographical regions of Nepal. The results obtained during this study highlight that rod-shaped bacteria were the dominant LAB population in traditional Nepalese Dahi samples (62% over 38% cocci). Within rod-shaped LAB, a wide diversity of *Lactobacillus* species was found, confirming that home-made traditional fermented products are a natural rich source of novel *Lactobacillus* strains. Among the identified isolates, there are species that have a known history of safe use and health-promoting attitude, such as *L. paracasei* and *L. rhamnosus.* The new Nepalese isolates of these species may provide a useful resource for further studies involving the selection of new cultures with potential novel probiotic characteristics. Furthermore, the strain collection obtained from this study represents a first step in the preservation of the natural biodiversity of bacterial population of the traditional Nepalese fermented Dahi.

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Melting curve analysis of a *groEL* PCR fragment for the rapid genotyping of strains belonging to the *Lactobacillus casei* group of species

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ABSTRACT

Lactobacillus casei group (*Lcs*) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei*, and *L. rhamnosus*), which are widely used in the dairy and probiotic industrial sectors. Strategies to easily and rapidly characterize *Lcs* are therefore of interest. To this aim, we developed a method according to a technique known as high resolution melting analysis (HRMa), which was applied to a 150 bp *groEL* gene fragment. The analysis was performed on 53 *Lcs* strains and 29 strains representatives of species that are commonly present in dairy and probiotic products and can be most probably co-isolated with *Lcs* strains. DNA amplification was obtained only from *Lcs* strains, demonstrating the specificity of the *groEL* primers designed in this study. The HRMa clustered *Lcs* strains in three groups that exactly corresponded to the species of the *L. casei* group. A following HRMa separated the 39 *L. paracasei* strains in two well distinct intraspecific groups, indicating the possible existence of at least two distinct genotypes inside the species. Nonetheless, the phenotypic characterization demonstrated that the genotypes do not correspond to the two *L. paracasei* subspecies, namely *paracasei* and *tolerans*. In conclusion, the melting curve analysis developed in this study is demonstrably a simple, labor-saving, and rapid strategy obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species. The application of this method to a larger collection of strains may validate the possibility to use the proposed HRMa protocol for the taxonomic discrimination of *L. casei* group of species. In general, this study suggests that HRMa can be a suitable technique for the genetic typization of *Lactobacillus* strains.

1. Introduction

Lactobacillus casei, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are three distinct species of phylogenetically closely related facultative heterofermentative lactic acid bacteria (LAB), collectively known as the species of the *L. casei* group (*Lcs*). *Lcs* includes typical non-starter LAB, commonly isolated from human gut (Wall et al. 2007) and vaginal mucosa, and widely found in many traditional fermented foods, where they contribute, through specific biochemical activities, to the quality and, in many cases, to the preservation of finished products. Particularly, they represent the major components of the autochthonous microbial population of many traditional cheeses and are used as subsidiary cultures (or ripening cultures) in cheese production (Colombo et al. 2009; Franciosi et al. 2008; Koirala et al. 2014).

Many studies have also shown the health-promoting potential of *Lcs*, as demonstrated by their extensive use as probiotics (Ferrario et al. 2014), or mixed cultures in dairy products and, as functional component, in a new generation of probiotic food products, including soy yoghurt (Donkor et al. 2005) and table olives (Lavermicocca et al. 2010). In fact, the two most-studied and best-known probiotic microorganisms worldwide are *L. paracasei* Shirota (Yakult Honsha Co., Ltd.) and *L. rhamnosus* GG (Valio Ltd.). However, the high

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relatedness of the members of this bacterial group makes difficult their differentiation. As a consequence, the taxonomic position and nomenclature of this group has been controversial until recently (Collins et al. 1989; Dellaglio et al. 1991; Tindall 2008).

Correct taxonomic assignment of microorganisms deliberately used in food and feed is a mandatory requirement, according to the Joint FAO/WHO Expert Meeting reports on probiotics (FAO/WHO 2002) and the European Food Safety Authority (EFSA) consultations on the Qualified Presumption of Safety (QPS) approach (EFSA 2004). Manufacturers, in fact, must follow the recommendation that each labeled denomination for microbial species conforms to the International Code of Nomenclature of Bacteria (Lapage et al. 1992).

Another important aspect concerning industrial strains refers to the fact that technological or probiotic features are mostly strain dependent and, therefore, genotyping methods are needed to assure quality management (Herbel et al. 2013). Several methods of genetic typization have been used to characterize strains belonging to the *Lcs* (Diancourt et al. 2007; Dimitonova et al. 2008; Capra et al. 2011; Sato et al. 2012); however, these methods have often inherent limitations. For instance, multi-locus sequence typing protocols (Diancourt et al. 2007) need DNA sequencing steps that make the protocol quite expensive and time-intensive. Furthermore, PCR-based fingerprinting techniques such as RAPD, ERIC-PCR, BOX-PCR, and rep-PCR (Capra et al. 2011; Dimitonova et al. 2008) are poorly reproducible and do not allow the confirmation that the isolate under investigation actually belongs to the *L. casei* group of species. Also MALDI-TOF MS of ribosomal proteins (Sato et al. 2012) have been proposed for *Lc*S characterization, but this method imply a quite complex protocol that requires equipment not easily found in the research and clinical laboratories of microbiology.

In this study, we designed a protocol to run a melting curve analysis of a small hyper-variable region that we identified inside the *Lactobacillus groEL* gene. Gene *groEL* codes for the 60-kDa group I chaperonin GrolEL that, because of its essential function, exhibits high sequence conservation across species (Goyal et al. 2006). Our results show that melting curve analysis protocol here presented is a simple and fast strategy to perform the genetic typization of bacterial strains belonging to the species of the *L. casei* group.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Eighty-two bacterial isolates originating from human intestine, vagina, oral cavity, dairy products, and commercial probiotic foods were included in this study (Table 1). These isolates were obtained from the bacterial culture collection of our Department or from international culture collections (American Type Culture Collection, ATCC; Deutsche Sammlung von. Mikroorganismen und Zellkulturen, DSMZ; Belgian Coordinated Collections of Microorganisms BCCM/LMG). Lactobacilli were grown in de Man, Rogosa and Sharpe (MRS) (Fluka, Sigma-Aldrich, St. Louis, MO, USA). Bifidobacteria were cultivated in MRS (Difco Lab., Augsburg, Germany) supplemented with 0.05% cysteine–HCl in anaerobic conditions. Streptococci and enterococci were grown in M17 (Difco Lab.) supplemented with 2% lactose or glucose. *Bacillus* was cultivated in nutrient broth (Difco). All strains were incubated at 30 °C, with the exceptions of *Lactobacillus helveticus*, *Streptococcus thermophilus* and bifidobacteria that were grown at 37 °C.

2.2. DNA extraction, conventional PCR and sequencing

Genomic DNA for all PCR reactions was extracted from 100 μl of an overnight culture diluted with 300 μl of TE 1 X buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) as described by Arioli et al. (2007). End-point PCR was carried out with DreamTaq DNA Polymerase (Fermentas, Vilnius, Lithuania) according to manufacturer instruction. All the others PCR reagents were from Fermentas. Species-specific PCR for *L. casei* species group was performed with conserved primerY2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′) and specific primers CASEI (5′-TGCACTGAGATTCGACTTAA-3′), PARA (5′-CACCGAGATTCAACATGG-3′) or RHAMN (5′-TGCATCTTGATTTAATTTTG-3′), as described by Ward and Timmins (1999). The hyper-variable *groEL* region identified in this study was amplified with primers GroHRM-F (5′-GTTTGATCGCGGCTATCTGA-3′) and GroHRM-R (5′-CCTTGTTGMACGATTTCTTG-3′) through a PCR profile consisting of an initial denaturation time of 2 min at 95 °C followed by an amplification for 35 cycles of denaturation (45 s at 94 °C), annealing (45 s at 59 °C) and extension steps (30 s at 72 °C); the PCR was completed with an elongation period (5 min at 72 °C). Amplicons obtained through end-point PCR with GroHRM primers were purified from reaction master mix with UltraClean PCR Clean-Up Kit (MoBio, Cabru S.a.s., Arcore, Italy) and then sequenced on both strands (Primm srl, Milan, Italy). GenBank accession numbers of the 14 partial *groEL* gene sequences determined in this study are indicated in Table 1.

2.3. Real-time quantitative PCR and high resolution melting analysis

The concentration and purity of the DNA samples were determined spectrophotometrically with a Take3 Micro-Volume Plate in the Eon BioTek Microplate Spectrophotometer (AHSI S.p.A., Bernareggio, Italy). After quantification, DNA samples were diluted with nuclease-free water to reach a concentration of 5 ng μl^{-1} and stored at −20 °C. Quantitative real-time polymerase chain reactions (qPCR) were carried out in a final volume of 15 μl containing 7.5 μl of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories S.r.l., Segrate, Italy), 0.25 μM of each primer and 15 ng of template DNA. During the set-up of the protocol, DNA template concentrations between 50 and 0.08 ng were also tested. qPCR experiments were performed in a CFX96 thermo-cycler (Bio-Rad Laboratories) as follows: initial denaturation at 98 °C for 1 min, followed by 45 cycles at 95 °C for 30 s, 59 °C for 20 s and 72 °C for 1 s; a unique step of 65 °C for 10 s was finally performed. Each DNA sample was analyzed at least in duplicate. At the end of the amplification protocol, melting curves of the amplicons were immediately determined by monitoring fluorescence from 65 to 95 °C, with temperature increments of 0.2 °C.

For high resolution melting curve analysis (HRMa), raw fluorescence data were exported from Bio-Rad CFX-Manager software and a single text (.txt) file for each strain was prepared by indicating data from 70 to 95 °C as depicted below:

Temperature	Fluorescence
70.00	8383.74
70.20	8348.91
70.40	8314.21
70.60	8279.45
...	...

The .txt documents were used as input files for software uAnalyze℠ v. 1.8 (Dwight et al. 2012); available on line at https://www.dna.utah.edu/uv/uv.php), which was employed for the HRMa. The software parameters used for the normalization of raw fluorescence melt data were as follows: horizontal sliders set at temperatures 78 °C and 79.5 °C for the analysis of *L. casei* species, and set at 78 °C and 81 °C for the analysis of *L. paracasei* intra-species genotypes.

Table 1
Bacterial strains included in the study. All strains were commercially available or present in the culture collection of our Department. (A) Strains belonging to the *L. casei* group of species; (B) other species. HMRa, High Resolution Melting analysis.

(A)

Strain	Source	Species identification		HMR genotype	Partial *groEL* GenBank accession numbers[b]
		Specific primers[a]	HRMa		
LMG 23516	Belgian culture collection	*L. casei*	*L. casei*	*III*	
LMG 6904^T (=ATCC 393^T)	Belgian culture collection	*L. casei*	*L. casei*	*III*	
DH 4	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 17	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	HG422840
DH 20	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 40	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	HG422841
DH 62	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 6	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
GG	Commercial strain (Chr. Hansen A/S)	*L. rhamnosus*	*L. rhamnosus*	*I*	
YM 1	Commercial fermented milk	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 4	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 11	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 21	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
DSM 20021^T	German culture collection	*L. rhamnosus*	*L. rhamnosus*	*I*	
ATCC 25302^T	American culture collection	*L. paracasei*	*L. paracasei*	*IIa*	
DH 10	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422838
DH 65	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	
DH 84	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422839
DH 132	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422837
DH 133	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	
LP 3	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIa*	
ATCC 334	American culture collection	*L. paracasei*	*L. paracasei*	*IIb*	
DG	Human gut/commercial strain (Sofar S.p.A.)	*L. paracasei*	*L. paracasei*	*IIb*	HG422829
FMBr3	Raschera D.O.P. Traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422835
I 1	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
I 2	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
I 16	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
IX 1	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422830
IX 2	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 20	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 3	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 4	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 6	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 7	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 8	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422833
L 9	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LB 7	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 14	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 22	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 24	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	HG422832
LMG 9191^T	Belgian culture collection	*L. paracasei*	*L. paracasei*	*IIb*	HG422831
LP 1	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 2	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 6	Bra traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 10	Bra tenero traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP15	Commercial strain(GS in forma)	*L. paracasei*	*L. paracasei*	*IIb*	
LP26	Bra tenero traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422834
LP28	Bra traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LPAB	Cocoa beans fermented	*L. paracasei*	*L. paracasei*	*IIb*	
LPC-S01	Human vagina/commercial strain (Sofar S.p.A.)	*L. paracasei*	*L. paracasei*	*IIb*	HG422836
LPDT	Cocoa beans fermented	*L. paracasei*	*L. paracasei*	*IIb*	
LPT34	Fruit kefir	*L. paracasei*	*L. paracasei*	*IIb*	HG422842
Shirota	Commercial probiotic strain (Yakult Co., Ltd)	*L. paracasei*	*L. paracasei*	*IIb*	

(B)

Strain	Species	Source
LVN 4	*Bacillus coagulans*	Commercial probiotic product
BB-12	*Bifidobacterium animalis* subsp. *lactis*	Commercial probiotic strain (Chr. Hansen A/S)
MIMBb75	*Bifidobacterium bifidum*	Human feces (Guglielmetti et al., 2009)
BBR09	*Bifidobacterium breve*	Commercial probiotic strain (Sacco S.r.l.)
BNF09	*Bifidobacterium longum* subsp. *infantis*	Commercial probiotic strain (Sacco S.r.l.)
F1	*Enterococcus faecalis*	Toma del Mottarone traditional Italian cheese
YVS3	*Enterococcus faecium*	Commercial probiotic product
EIP9	*Enterococcus italicus*	Pannerone traditional Italian cheese

Table 1 (*Continued*)

(B)

Strain	Species	Source
LA5	*Lactobacillus acidophilus*	Commercial probiotic strain (Chr. Hansen A/S)
DH 92	*Lactobacillus brevis*	Dahi traditional Nepalese dairy product
DH 94	*Lactobacillus coryniformis*	Dahi traditional Nepalese dairy product
RoCa	*Lactobacillus curvatus*	Raschera traditional Italian cheese
DH 147	*Lactobacillus delbrueckii*	Dahi traditional Nepalese dairy product
DH 98	*Lactobacillus fermentum*	Dahi traditional Nepalese dairy product
DH 95	*Lactobacillus harbinensis*	Dahi traditional Nepalese dairy product
DH 103	*Lactobacillus helveticus*	Dahi traditional Nepalese dairy product
MIMLh5	*Lactobacillus helveticus*	Grana Padano whey starter (Guglielmetti et al., 2010b)
LJHO7	*Lactobacillus johnsonii*	Human feces
DH 31	*Lactobacillus parabuchneri*	Dahi traditional Nepalese dairy product
NF1	*Lactobacillus pentosus*	Commercial probiotic product
YVS2	*Lactobacillus plantarum*	Commercial probiotic product
PRE2	*Lactobacillus salivarius*	Commercial probiotic product
V32	*Lactococcus garvieae*	Rainbow trout filet
LGB3	*Lactococcus garvieae*	Cabbage
E1	*Lactococcus lactic* subsp. *lactis*	Toma del Mottarone traditional Italian cheese
E7	*Lactococcus lactic* subsp. *lactis*	Toma del Mottarone traditional Italian cheese
K12	*Streptococcus salivarius*	Commercial probiotic product
ST3	*Streptococcus salivarius*	Human pharyngeal mucosa (Guglielmetti et al., 2010a)
N4	*Streptoccus thermophilus*	Toma del Mottarone traditional Italian cheese

[a] According to Ward and Timmins 1999.
[b] GenBank accession numbers of the 14 partial *groEL* gene sequences determined in this study.

2.4. Phenotypical tests

Carbon source fermentation was determined by using a microtitre plate assay and a basal CHL medium at pH 6.3 (Bio-Merieux, Montelieu-Vercieu, France) containing bromocresol purple as indicator, and the desired filter-sterilized carbohydrate at a final concentration of 0.5% (w/v). The plates were incubated at 30 °C and were examined for color change after 24 and 48 h of incubation. Survival assay at 72 °C was carried out by sinking for 40 s in a water bath a glass capillary tube containing the bacterial suspension. Growth at 40 °C and survival at 72 °C were determined in MRS broth. The residual viable population was determined by plate counting on MRS agar after 48–72 h of incubation at 30 °C.

3. Results and discussion

Lactobacilli are one of the most important taxonomic groups of microorganisms involved in food microbiology and human nutrition, for which genetic typization and unambiguous identification must be considered a prerequisite for their correct use. In the last decade, a high number of systematic studies of lactobacilli have been carried out using molecular approaches targeting several genes, such as *16s rRNA*, *tuf*, *recA* or *rpoB* genes (Chavagnat et al. 2002; Felis et al. 2001; Piwat and Teanpaisan 2013; Rantsiou et al. 2004; Ventura et al. 2003). A few years ago, Blaiotta et al. proposed a partial DNA sequence (499-bp) of the gene *hsp60* (*groEL*, encoding a 60-kDa heat shock protein) as a useful molecular marker for the genetic typing of *Lactobacillus* strains isolated from food samples (Blaiotta et al. 2008). Therefore, we analyzed this genetic target with the aim to identify an internal region with a potential discriminative power sufficiently high to genetically characterize strains belonging to the *L. casei* group (*Lcs*). To this aim, we performed a ClustalW alignment of the *groEL* gene sequences from the type strains of the species *L. casei*, *L. paracasei* and *L. rhamnosus*. Consequently, we identified a 110 bp region with relatively high sequence variability inside the *groEL* gene (Fig. 1). Then, we designed a pair of primers (named GroHMR) in the conserved flanking regions (Fig. 1), which allowed obtaining a 150 bp PCR amplicon from *Lcs* strains. Subsequently, we generated a dendrogram using the Neighbor Joining algorithm based on 13 *groEL* sequences available in GenBank and 14 sequences obtained during this study from *Lcs* strains available in our laboratory (Table 1; Fig. 2). The resulting dendrogram separated the bacterial strains into three clusters, which exactly corresponded to the species of the *L. casei* group. The type strain of the species *L. casei* clustered with strain *L. zeae* ATCC 15820 (Fig. 2). Accordingly, the International Committee on Systematics of Bacteria rejected the species *L. zeae* and included it in the species *L. casei* (Tindall 2008). Furthermore, several strains (viz. ATCC 334, ATCC 25598, DSA-15, IMAU60056 and DSM 5622^T), though annotated in GenBank as *L. casei*, resulted to belong to the species *L. paracasei*. A possible explanation for these misidentifications can be found in the fact that until quite recently the type strain of the species *L. casei* was ATCC 334, which now has been reclassified as a *L. paracasei* (Tindall 2008). Therefore, our results showed that the selected 150 bp *groEL* region can be potentially useful in distinguishing among *L. casei*, *L. paracasei*, and *L. rhamnosus*.

We then tested in real-time quantitative PCR (qPCR) the specificity of GroHM primers on 53 strains belonging to the *L. casei* group (Table 1). The taxonomic identification of these strains was confirmed by PCR amplification with species-specific primers (Ward and Timmins 1999). In addition, we also included in the analysis strains belonging to 25 different bacterial species (including 13 *Lactobacillus* species besides *Lcs*), which are commonly present in dairy and probiotic products and can be most probably co-isolated with *Lcs* strains. Among the tested bacteria, we included strains isolated from commercial probiotic products that claimed to contain a *Lcs* bacterium. Our experiments with species-specific primers (Ward and Timmins 1999) confirmed the presence of *L. rhamnosus* in all products that labeled this species. In contrast, we found *L. paracasei* in all products asserting to include a *L. casei* strain. We never found *L. casei* in them according to PCR with species specific primers. Furthermore, in accordance with a technical opinion by EFSA (2010), our analyses confirm that the well-known bacterial strain Shirota, differently from what is claimed on the label, belongs to the species *L. paracasei* and not to *L. casei*. Though widespread use of misclassified microbial species or species with fictitious names in commercial probiotic products have been reported (Aureli et al. 2010), we believe that what we observed in this study should not be attributed to poor quality control by manufacturers but rather to the confusion that has persisted until recently about

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ATCC 393T   AAGGCAGCGGTTGATGAACTGCACAAGATCAGCCACAAGGTTAACGGCAAGAAGGAAATC
ATCC 7469T  AAGGCTGCAGTTGACGAATTGCACAAGATCAGCCACAAGGTTAACGGCAAGAAGGAAATT
DSM 5622T   AAGGCTGCCGTTGACGAATTGCACAAGATTAGCCACAAAGTTAATGGTAAGAAAGAAATC
            ***** ** ***** *** ********** ******** ***** ** ***** *****

            GCCCAGGTTGCTTCGGTTTCATCTTCCAATGAAGAAGTCGGCAATCTGATTGCGGACGCT
            GCCCAAGTTGCTTCCGTTTCCTCTTCTAATGAAGAAGTCGGCAACCTGATTGCTGACGCG
            GCGCAGGTTGCGTCCGTTTCTTCCTCAAATACAGAAGTTGGTAGTCTGATTGCCGACGCC
            ** ** ***** ** ***** ** ** ***  ****** ** *  ******** *****

            ATGGAAAAAGTTGGCCATGATGGTGTTATTACCATCGAAGAAAGCAAAGGGATCGACACT
            ATGGAAAAAGTTGGCCATGATGGTGTGATTACCATTGAAGAAAGCAAAGGGATTGATACT
            ATGGAAAAAGTTGGCCACGATGGTGTGATTACCATTGAAGAAAGCAAAGGGATTGACACT
            ***************** ******** ******** ****************** ** ***

                                        GroHRM-F →
            GAACTTTCCGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGTCAATACATGGTA
            GAACTCTCTGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGCCAGTACATGGTT
            GAACTCTCTGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGCCAGTACATGGTC
            ***** ** ********************************** ** ********

            ACCGACAATGATAAAATGGAAGCTGATCTGGACGATCCATATATCCTGATTACCGATAAG
            ACCGACAACGACAAGATGGAAGCTGATCTGGATGATCCATATATCCTGATTACCGACAAG
            ACTGATAATGATAAGATGGAAGCTGACCTTGACGATCCTTATATCTTGATCACCGACAAA
            ** ** ** ** ** ********** ** ** ***** ****** **** ***** **

                                                  ← GroHRM-R
            AAGATTTCCAACATCCAGGACATTCTTCCACTATTACAAGAAATCGTTCAACAAGGTAAG
            AAGATCTCCAATATTCAAGATATTCTGCCACTCTTGCAAGAAATCGTGCAACAAGGCAAG
            AAGATTTCCAATATTCAGGACATTTTGCCGCTGTTACAAGAAATCGTTCAACAAGGTAAG
            ***** ***** ** ** ** *** * ** ** ** ********** ******** ***

            GCACTGTTAATCATTGCTGACGATGTTGCCGGCGAAGCATTGCCAACCCTGGTTCTTAAC
            GCACTGTTGATCATTGCTGACGATGTTGCCGGTGAAGCACTGCCGACCTTGGTTCTGAAC
            GCACTGTTGATCATTGCTGACGACGTTGCTGGTGAAGCATTGCCAACCTTAGTTCTGAAC
            ******** ************** ***** ** ****** **** *** * ***** ***
```

Fig. 1. ClustalW alignment of 480 bp from the sequence of gene *groEL* of strains *Lactobacillus casei* ATCC393^T (GenBank accession number AY424336), *Lactobacillus rhamnosus* ATCC 7469^T (AF429659) and *Lactobacillus paracasei* DSM 5622^T (AY424339). Gray areas indicate the target regions of primers GroHRM-F and GroHRM-R.

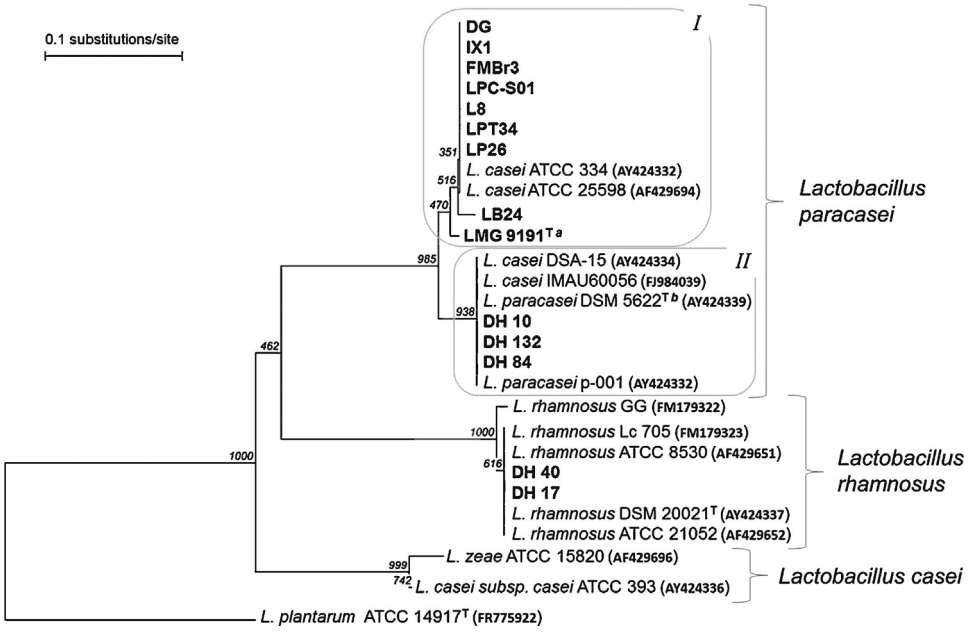

Fig. 2. Neighbor-joining dendrogram of aligned *groEL* gene sequences from *L. casei* group of species. Alignments of sequences were made considering the 150 bp fragment that is amplified with primers GroHRM-F and GroHRM-R (Fig. 1). Bootstrap values (1000 replicates) are shown close to the considered node. Sample indicated in bold refers to bacterial strains whose *groEL* gene sequence has been determined in this study. The *groEL* gene sequences obtained from GenBank are shown with the database accession number between brackets. Taxonomic names for database sequences are according to GenBank annotation. *Lactobacillus plantarum* ATCC 14917^T has been included as outgroup. [a], type strain of *L. paracasei* subsp. *tolerans*; [b], type strain of *L. paracasei* subsp. *paracasei*.

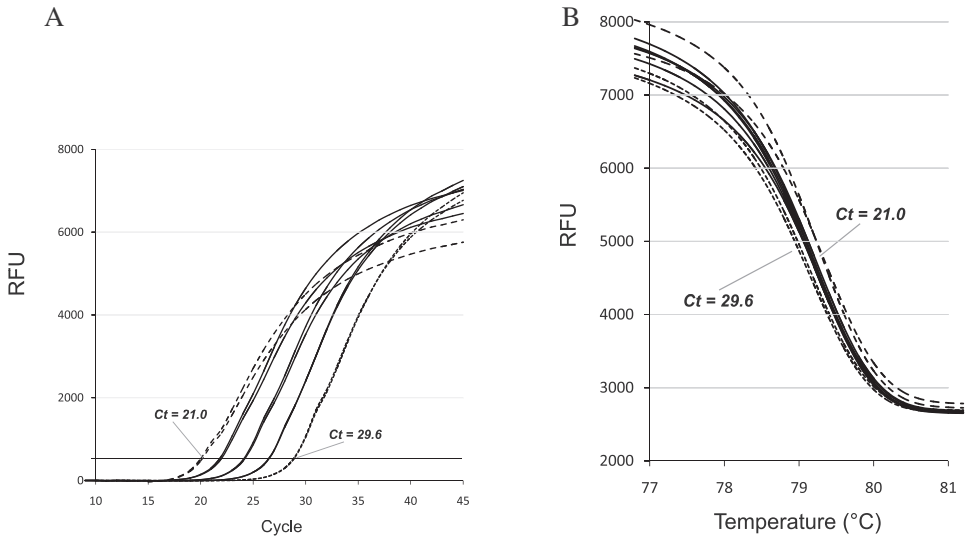

Fig. 3. Real time quantitative PCR (qPCR) experiment performed with the total DNA isolated from *L. paracasei* DSM 5633^T. DNA was serially 1:5 diluted from 50 ng per reaction. (A) qPCR amplification curves. (B) Melting curves of the qPCR amplicons. Ct, real time PCR threshold cycle. RFU, relative fluorescence units. Duplicates for each condition are shown.

distinguishing between *L. casei* and *L. paracasei* (Dellaglio et al. 2002; Tindall 2008).

After PCR with GroHRM primers according to the proposed protocol, we obtained an amplification curve with a Ct between 21 and 27 exclusively from strains that were previously identified as *L. casei*, *L. paracasei*, or *L. rhamnosus*, whereas the strains belonging to other taxa resulted in a Ct over 35 (Data not shown 1, only for referees). Therefore, the proposed real time PCR with GroHMR primers allowed the discrimination between *Lcs* strains and strains belonging to other taxa. However, the analysis of a larger collection of strains is needed to demonstrate the ability of GroHRM primers to amplify exclusively *Lcs* bacteria.

Since real time PCR amplification curves did not allow the discrimination among the *Lcs* strains included in our study, we developed a strategy based on melting curve analysis of the 150 bp *groEL* gene fragment amplified with GroHMR primers. We based our protocol on a technique known as high resolution melting analysis (HRMa). HRMa protocols, developed to detect small variations in DNA sequences, have found important applications in single nucleotide polymorphism (SNP) and methylation studies (Messiaen et al. 2012; Pistek et al. 2012) and in clinical microbiology (Ruskova and Raclavsky 2011) for identifying, detecting, and typing several pathogens, such as *Mycobacterium tuberculosis* (Lee and Ong 2012), *Staphylococcus aureus* (Chen et al. 2012), *Listeria* spp. (Jin et al. 2012), *Clostridium difficile* (Pecavar et al. 2012), *Salmonella* serovars (Zeinzinger et al. 2012), *Enterococcus faecium* (Tong et al. 2011), and fungal pathogens (Somogyvari et al. 2012). Using HRMa to study food associated lactic acid bacteria is much more limited. To the best of our knowledge, only one study employed HRM for the characterization of lactic acid bacteria; particularly, in this study, HRM was applied to analyze denaturing gradient gel electrophoresis (DGGE) bands obtained from dairy products (Porcellato et al. 2012). In addition, recently, the use of HRMa targeting genetic regions different from *groEL* was also proposed for the taxonomic identification of species belonging to the *L. casei* group (Iacumin et al. 2015).

According to the conventional HRMa protocols, we used in this study a PCR reaction solution including a saturating fluorescent intercalating agent and collected melt data in 0.2 °C increments in a Real-Time PCR thermal cycler. Initially, we tested the effect of DNA template concentration on melting curve profiles and accordingly ran melting analyses of PCR amplicons obtained with 5 different concentrations (from 50 to 0.08 ng per reaction) of the DNA isolated from *L. paracasei* DSM 5633^T, which was arbitrarily chosen as reference strain. We found that DNA concentrations producing Ct values of less than 20 induced a significant shift in the melting curves, which could affect the result of the analysis. In contrast, template concentrations corresponding to Ct values of up to 28 did not affect melting curves (Fig. 3A and B). Similar results were obtained with DNA extracted from strains *L. casei* ATCC 339^T and *L. rhamnosus* GG, which were selected to represent the other two species constituting the *Lcs* group. Consequently, we used in the following experiments a DNA concentration of 5 ng μl^{-1} since it produced Ct values between 21 and 28. Specifically, we ran the assay with DNA extracted from the 53 *Lcs* strains under study (Table 1). The amplicons that we obtained from these experiments had an average melting temperature (Tm) of 79.12 ± 0.45 °C. The melting curves obtained were then analyzed in order to verify the possibility to cluster the different *Lcs* strains. To this aim, unlike other studies, we used no commercial software specifically designed for HRMa; instead, raw fluorescence melt data were normalized by means of freeware on-line software developed at the University of Utah (uAnalyze^{SM} v. 1.8; Dwight et al. 2012). In a first analysis, the 53 *Lcs* strains were clustered in 3 well distinguished groups (Fig. 4A), which had distinct average Tm values (Fig. 4). This result was obtained by setting the two horizontal sliders of the software at the temperature of 78 and 79.5 °C. Notably, the resulting clusters of curves exactly divided strains according to the species (Fig. 4A).

The 39 strains belonging to *L. paracasei* were further analyzed by setting horizontal parameters at 78 and 81 °C. In this way, we were able to separate *L. paracasei* strains in two well distinct genotypic groups. Strain clustering through melting curve analysis was in accordance with the dendrogram based on the 150 bp corresponding to the region amplified for melting analysis (Fig. 2). In fact, not only *L. casei*, *L. paracasei* and *L. rhamnsosus* strains included in the study were well distinguished by the NJ tree, but, similarly to melting analysis, the dendrogram also divided *L. paracasei* strains in two distinct genetic clusters.

Collins et al. (1989) described two subtaxa in the species *L. paracasei*, which correspond to the subspecies *paracasei* and *tolerans* (Tindall 2008). Melting curves obtained after HRMa analysis of *L. paracasei* strains are shown in Fig. 4B. Group "a" included the type strain of the species *L. paracasei*, DSM 5622^T, which

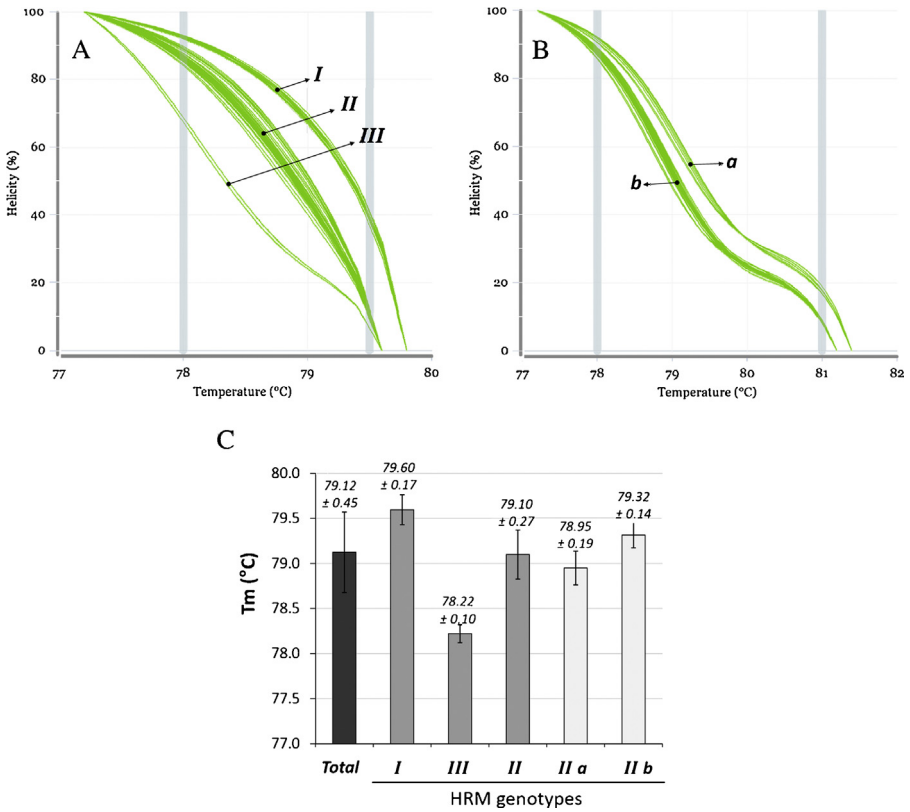

Fig. 4. High resolution melting curve analysis (HRMa) of the 150 bp *groEL* DNA fragment amplified by qPCR from strains belonging to species of the *Lactobacillus casei* group (*Lcs*, Table 1). Panel A, HRMa performed on 53 *Lcs* strains; the group of curves I includes 12 *L. rhamnosus* strains; group II includes 39 *L. paracasei* strains; group III includes 2 *L. casei* strains. Panel B, HRMa performed on 39 *L. paracasei* strains; the cluster of curves "a" includes *L. paracasei* subsp. *paracasei* ATCC 25302^T and other 6 strains; the cluster of curves "b" includes *L. paracasei* subsp. *tolerans* LMG 9191^T and other 31 strains. Panel C, amplicons average melting temperature (Tm) of the gentypes obtained through HRMa. Data for each bar of the histogram are reported as mean value of the Tm ± standard deviation.

is also the reference strain for the subspecies *paracasei*. On the contrary, the reference strain *L. paracasei* subsp. *tolerans* LMG 9191^T clustered in group "*b*". We therefore hypothesized that the two genotypes observed inside *L. paracasei* could correspond to the subspecies *L. paracasei* subsp. *paracasei* (group "a") and *L. paracasei* subsp. *tolerans* (group "b"). To corroborate this hypothesis, a number of *L. paracasei* strains were characterized to verify some of the phenotypic features which have been proposed. Specifically, the subspecies *tolerans* has been described as a facultative heterofermentative bacterium that produces acid

Table 2
Phenotypic characterization of *Lactobacillus paracasei* strains based on acid production from 13 carbon sources and growth at 40 °C (A), and resistance to pasteurization at 72 °C for 40 s (B). HRM groups *a* and *b* refer to Fig. 4B. + positive; −, negative; ±, weak activity.

(A)

	HRM group *a*				HRM group *b*							
	ATCC25302^T	LP3	DH10	DH133	LMG9191^T	ATCC334	SHIROTA	LPC-S01	DG	LP1	LPAB	FMBr3
Amygdalin	±	−	±	±	−	+	+	+	±	±	+	+
Arbutin	+	−	+	±	−	+	+	+	±	±	+	+
Cellobiose	+	±	±	±	−	+	±	+	+	+	+	+
Esculin	±	−	±	±	−	±	±	±	±	±	±	±
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	−	+	+	+	+	+	+	±	±	+	+
Maltose	±	−	+	+	−	±	+	±	+	+	+	+
Mannitol	+	−	+	+	−	+	+	+	+	+	+	+
Melezitose	+	−	+	±	−	+	±	+	+	±	+	+
Ribose	+	±	+	+	−	+	+	+	+	+	+	+
Sucrose	±	±	+	+	−	±	±	+	+	+	+	+
Salicin	+	−	+	±	−	+	+	+	+	+	+	+
Trehalose	+	−	+	+	−	+	+	+	+	+	+	+
Growth at 40 °C	+	−	+	+	−	+	+	+	+	+	+	+

(B)

Pasteurization resistance (CFU ml^{-1})	ATCC25302^T	LP3	LMG9191^T	ATCC334
Before treatment	3×10^7	1×10^7	2×10^7	3×10^7
After treatment	<10	<10	10^6	<10

from lactose, but (differently from *L. paracasei* subsp. *paracasei*) not from amygdalin, arbutin, cellobiose, maltose, mannitol, melezitose, salicin and trehalose (Abo-Elnaga and Kandler 1965; Collins et al. 1989). Furthermore, *L. paracasei* subsp. *tolerans* can be distinguished from *L. paracasei* subsp. *paracasei* by the fact that does not hydrolyze esculin, does not grow at 40 °C, and survive heating at 72 °C for 40 s (Abo-Elnaga and Kandler 1965; Collins et al. 1989). More recent data concerning sugars fermentation abilities in *Lcs*, however, indicate that the above mentioned grouping is too restrictive. An enormous diversity of sugar utilization gene cassettes were recently identified, in fact, in the genome of several strains (Smokvina et al. 2013; Douillard et al. 2013). Accordingly, the characterization performed in our study revealed that none of the phenotypes mentioned above can univocally distinguish strains of HRM group "*a*" from strains of group "*b*" (Table 2). Therefore, we cannot conclude that the two genotypes identified inside the species *L. paracasei* by HRM correspond to the subspecies *tolerans* and *paracasei*. Nonetheless, it should be also mentioned that the phenotypic features describing *L. paracasei* subsp. *tolerans* have been based on a very limited number of strains; precisely, 6 strains isolated from pasteurized milk were considered by Abo-Elnaga and Kandler (1965) and only two strains were reported by Collins et al. (Collins et al. 1989); no other studies focusing on the phenotypic characterization of *L. paracasei* subspecies are available in literature. As a consequence, we believe that the opportunity to maintain the two subspecies, *tolerans* and *paracasei*, inside the species *L. paracasei* should be reconsidered.

4. Conclusions

The *L. casei* group of species (*Lcs*) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei*, and *L. rhamnosus*), which are widely used in the dairy and probiotic industrial sectors. Strategies to easily, unambiguously, and rapidly genetically characterize the bacteria of the *L. casei* group are therefore of interest. To this aim, we developed a method based on analysis of the melting curve of a 150 bp DNA fragment obtained by PCR from gene *groEL*. The experiments carried out during this study showed that melting curve analysis is a simple and labor-saving strategy that can be used for the initial rapid clustering of *Lactobacillus* isolates from food samples or biologic specimens. Specifically, the proposed method can represent a novel convenient molecular tool to obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species. In addition, the analysis of a significantly larger number of strains may also corroborate the hypothesis that the experimental protocol here presented, besides genotyping, is also suitable for the taxonomic discrimination of the species in the *L. casei* group.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2015.01.001.

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Title : Prevalence of probiotic lactobacilli in dahi prepared at different geo-climatic conditions of Nepal

Authors : Ranjan Koirala, Sangita Shrestha, Simone Guglielmetti, Giovanni Ricci, Vijayendra SVN, Varadaraj MC & Rajani Malla

Affiliation : Nepal Academy of Science and Technology, Nepal

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loading the sample, there was a 5 min waiting period to allow the sample to recover and achieve temperature equilibrium and samples were subjected to a programmed shear rate linearly increasing from 0–300 s^{-1} in 3 min and successive linearly decreasing shear rate from 300 s^{-1} to 0 in 3 min. Three models were used to predict the flow behaviour namely, the power law, Herschel–Bulkley, and Casson models.

Results and Conclusion: The prepared fenugreek fraction and whole grain flour (WGF) pastes exhibited non Newtonian pseudoplastic behaviour at different temperature tested. For the same temperature, differences in shear stress and hysteresis loop magnitudes were observed among fractions, being more noticeable at lower temperatures. Among all evaluated model, the power law model adequately described well the flow behaviour of the different fractions and WGF at different temperatures. The fenugreek roller milled fractions exhibited rheopectic behaviour that increased viscosity with increasing the shear speed and the temperature.

Effect of probiotic cultures on fermentation of moringa leaves extract

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Objectives: To determine the effect of *Lactobacillus plantarum* and *Enterococcus hirae* on the fermentation of moringa leaves extract (MLE).

Methodology: Washed moringa leaves were ground to paste by adding equal volume of water and sterilized. This was diluted by adding 2 volumes of sterile distilled water and inoculated with *Lactobacillus plantarum* and *Enterococcus hirae*, at 1% level with different cell counts and incubated at 37°C for 48 h. This was used to know the effect of fermentation of MLE on raffinose content and antimicrobial activity against food borne pathogens like *Bacillus cereus*, *Listeria monocytogenes* and *E. coli*, which was determined by HPLC and well assay methods, respectively. The total carbohydrate content of the fermented product was determined by phenol-sulfuric acid method. The viable count of probiotic cultures was also estimated by standard plate count method.

Results and Conclusion: The fermented MLE found to reduce the raffinose content considerably. It has shown antimicrobial activity against all pathogenic bacteria tested. The total carbohydrate content was reduced by 40%. The product indicated reduction in raffinose content by 60%. Increase in viable count by 1-2 log was noticed at all levels of the inoculum used. In conclusion, the cultures used in this study can be used to reduce flatulence factor (raffinose) with added antimicrobial property in MLE.

Prevalence of probiotic lactobacilli in *dahi* prepared at different geo-climatic conditions of Nepal

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Objective: To find the dominant natural lactic acid bacteria (LAB) in traditionally fermented Nepalese *dahi*, study genetic diversity and determine the probiotic properties of selected isolates.

Methodology: Altogether, 64 traditional fermented *dahi* samples were collected from different altitudes of Nepal. Isolates of *Lactobacillus* to an extent of 62% were obtained and further identified to species level by Ribosomal Spacer Analysis (RSA), 16S rRNA sequencing and species-specific attributes. Intra species genetic diversity analysis was carried out by combined use of repetitive element and Randoml Amplified Polymorphic (RAPD) DNA. Identified cultures of *Lactobacillus* species were evaluated for probiotic properties such as gastrointestinal transit resistance, adhesion using Caco-2 cell, antibacterial activity, antibiotic resistance and sugar fermentation pattern.

Results and Conclusion: Among the presumptive isolates of LAB, 120 isolates were identified to 10 different species of *Lactobacillus* based on PCR-based methods. The intra specific genetic diversity revealed that the biodiversity of the isolated cultures correlated with *dahi* samples of different altitudes. Two isolates of *Lactobacillus delbrueckii* subsp. *bulgaricus* were found to be more prominent among the tested isolates. Both the isolates were able to survive and grow at pH 2 and 0.3% bile, positive for adhesion to Caco-2 cells and exhibited inhibitory activity against *Pseudomonas aeruginosa*. Besides, they were able

to grow in presence of galactose, fructose, sucrose, raffinose and xylose. The study indicated the genetic diversity among LAB in various dahi samples of Nepal.

Genetic regulation of banana fruit ripening by non-genetic approach

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Objectives: Banana is a climacteric fruit which ripens rapidly. Many studies on fruit ripening addressed this problem through genetic engineering where suppression of one or two enzymes concomitantly retarded ripening. Contrarily, the present study demonstrates the possible genetic regulation through simple formulations composed of nature-identical chemicals which effectively impart genetic control by up- or down-regulation of ripening genes.

Methodology: Effects of various growth regulators and signaling compounds (Gibberellic acid, Auxin, Cytokinin, Abscisic acid, Salicylic acid, Methyl jasmonate, and Nitric oxide) for inhibiting/ delaying ripening were evaluated. Expression pattern of genes involved in diverse class of biochemical and physiological functions such as cell wall hydrolysis (Polygalacturonase, Pectate lyase, Pectin methyl esterase, β-galacturonase, Expansins and Chitinase), carbohydrate metabolism (Sucrose synthase, Sucrose phosphate synthase, α-amylase, β-amylase), ethylene pathway (1-aminocyclopropane-1-carboxylic acid synthase, 1-aminocyclopropane-1-carboxylic acid oxidase), ethylene signal perception and transduction (CTR1, ERS-1, ERS-2, EIL-1, EIL-2, EIL-3, EIL-4, MADS-1, MADS-2, MADS-4 and MADS-6), senescence (Catalase, Peroxidase, SOD, PAL) was studied. Effects of individual growth regulator/ signaling compounds on the expression patterns of individual class of genes were evaluated. Based on genetic expression data, various formulations were developed and were analyzed for their efficiency to delay fruit ripening.

Results and Conclusion: Different growth regulators imparted diverse effects on different classes of ripening-related genes. Though the maximum inhibition of ripening was imparted by Gibberellic acid, it could not down-regulate all class of genes. The information generated through this study was useful for developing treatments for precise ripening and extended shelf life, allowing extensive marketability of banana fruits.

Abelmoschus esculentus (okra) extract may provide protection against oxidative stress in type 2 diabetic erythrocytes.

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Objectives: Oxidative stress is believed to be a major contributing factor in the development of late complications of diabetes. Plant polyphenols are reported to exert many biological effects due to their antioxidant property. *Abelmoschus esculentus* commonly known as okra is a plant with surplus medicinal potential, bioactive principles of which may provide defence against oxidative damage. The present study was undertaken to evaluate the protective effects of okra on markers of oxidative stress in diabetic rats.

Methodology: 35 Male Wistar strain rats were induced with alloxan to generate experimental diabetes. Okra was suspended in 0.2% sodium carboxy methyl cellulose and fed to rats upto 35 days. Biomarkers of oxidative stress including erythrocyte plasma membrane redox system (PMRS), lipid peroxidation, advanced oxidation protein products (AOPP), intracellular glutathione, and plasma antioxidant capacity as FRAP values were measured after okra supplementation in diabetic rats.

Results and Conclusion: Alloxan induced rats showed increased oxidative stress as evidenced by increased activity of erythrocyte PMRS (153%), lipid peroxidation (31%) and AOPP (290%). A signifcant ($p < 0.01$) decrease in plasma antioxidant potential (22%) and intracellular glutathione (75%) has also been observed. Supplementation with okra protected rats from alloxan induced oxidative damage. However, supplementation of okra to control rats showed increased antioxidant defense.

"Part of the secret of success in life is to eat what you like and let the food fight it out inside."

- Mark Twain