Diversity of Lactic Acid Bacteria and Their Probiotic Properties in Some Naturally Fermented Milk Products of Sikkim

A Thesis Submitted

To

Sikkim University

For the Degree of Doctor of Philosophy

By

Ranjita Rai

Supervisor: Professor Dr. Jyoti Prakash Tamang

Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
Gangtok 737102, India

JUNE 2020

DEDICATED

TO

MY PARENTS

Shri PAHAL MAN RAI

&

MON MAYA RAI

DECLARATION

I declare that the present Ph.D thesis entitled **"Diversity of Lactic Acid Bacteria and Their Probiotic Properties in Some Naturally Fermented Milk Products of Sikkim"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervision of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Tadong, Sikkim University, is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok. No part thereof has been submitted for any degree or diploma in any University/ Institution.

Ranjita Rai

Ranjita Rai
Registration No: 13/PD/MB/01
Research Scholar
Department of Microbiology
School of Life Sciences
Sikkim University (www.cus.ac.in)
Gangtok

Date: 18th June 2020
Place: Tadong, Gangtok

6 माइल, सामदुर, तादोंग -737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स -251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
(A central university established by an Act of Parliament of India in 2007 and accredited by NAAC in 2015)

CERTIFICATE

This is to certify that the PhD thesis entitled **"Diversity of Lactic Acid Bacteria and Their Probiotic Properties in Some Naturally Fermented Milk Products of Sikkim"** submitted to **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Miss Ranjita Rai** for the award of PhD Degree in Microbiology, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. It is a record of bonafide investigation carried out and completed by her under our supervisions and guidances. She has followed the rules and regulations laid down the University. The results are original and have not been submitted anywhere else for any other degree or diploma.

It is recommended this PhD thesis to be placed before the Examiners for evaluation.

SUPERVISOR

Professor Dr. Jyoti Prakash Tamang
FNAAS, FNABS, FIAMS, FBRS
ICIMOD Mountain Chair (www.icimod.org)
Professor, Department of Microbiology
Sikkim University (www.cus.ac.in)
Science Building, Tadong 737102, Sikkim, INDIA

प्रो. ज्योति प्रकाश तामंग
Professor Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
PROFESSOR
DAICENTRE Laboratory, Department of Microbiology
SIKKIM UNIVERSITY (central university)
Tadong 737102, Gangtok, Sikkim, INDIA

HEAD

Dr. Hare Krishna Tiwari
Associate Professor
Department of Microbiology
Sikkim University, Tadong

अध्यक्ष
Head
सूक्ष्मजीव विज्ञान विभाग
Department of Microbiology
सिक्किम विश्वविद्यालय
Sikkim University

Place: Tadong, Gangtok
Date: 18th June 2020

6 माइल, सामदुर, तादोंग -737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स -251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
(A central university established by an Act of Parliament of India in 2007 and accredited by NAAC in 2015)

Date: 15th June 2020

PLAGIARISM TEST CERTIFICATE

This is to certify that the plagiarism check has been carried out for the following PhD thesis with the help of **URKUND SOFTWARE** and the result is **1% tolerance rate**, within the permissible limit (below 10% tolerance rate) as per the norm of Sikkim University.

"Diversity of Lactic Acid Bacteria and Their Probiotic Properties in Some Naturally Fermented Milk Products of Sikkim"

Submitted by **Miss Ranjita Rai** under the supervision of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Sikkim University, Tadong 737102, Sikkim, India.

Ranjita Rai

Ranjita Rai
Signature of the candidate

Professor Dr. Jyoti Prakash Tamang
Supervisor

प्रो. ज्योति प्रकाश तामांग
Professor Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
PROFESSOR
DAICENTRE Laboratory, Department of Microbiology
SIKKIM UNIVERSITY (central university)
Tadong 737102, Gangtok, Sikkim, INDIA

Professor A.S. Chandel
Librarian, Sikkim University

पुस्तकालयाध्यक्ष
Librarian
केन्द्रीय पुस्तकालय Central Library
सिक्किम विश्वविद्यालय
Sikkim University

URKUND

Document Information

Analyzed document	Ranjita PhD Thesis-Similarity test.docx (D74341550)
Submitted	6/8/2020 7:42:00 AM
Submitted by	Prof A S Chandel
Submitter email	librarian@cus.ac.in
Similarity	1%
Analysis address	librarian.sikkim@analysis.urkund.com

Sources included in the report

W	URL: https://www.researchgate.net/publication/240546619_Identification_and_enzymatic_pr ... Fetched: 6/8/2020 7:45:00 AM	2
W	URL: https://www.nature.com/articles/s41598-018-19524-6 Fetched: 4/13/2020 5:57:12 AM	2
W	URL: https://scielo.conicyt.cl/pdf/ciagr/v44n3/0304-5609-ciagr-44-03-0215.pdf Fetched: 6/8/2020 7:45:00 AM	2

Achandel

पुस्तकालयाध्यक्ष
Librarian
केन्द्रीय पुस्तकालय Central Library
सिक्किम विश्वविद्यालय
Sikkim University

ACKNOWLEDGEMENTS

First and foremost, praises and thanks to the God, the Almighty, for his blessings throughout my research work to complete the research successfully.

I would like to express my deep and sincere gratitude to my Supervisor Professor Dr. Jyoti Prakash Tamang for giving me the opportunity to do research and providing invaluable guidance throughout this research. His dynamism, vision, sincerity and motivation have deeply inspired me. He has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was my great privilege and honor to work and study under his guidance.

I would like to acknowledge my sincere gratitude to our former Honorable Vice-Chancellors of Sikkim University Prof. M.P Lama, Prof. T.B Subba and present Vice-Chancellor Prof. Avinash Khare for providing us with the requisite infrastructure.

I would like to extend my sincere gratitude to Dr. H.K Tiwari (Head of Department), Dr. Bimala Singh, Dr. B.M Tamang, Dr. Nagendra Thakur and Dr. Anil Kumar Verma (Assistant Professor), Department of Microbiology for their suggestion and guidance.

I would also like express my gratitude to Mrs. Radha Basnet, (Laboratory Technician) and Mr. Pukar Biswakarma, (Laboratory Assistant), Department of Microbiology for their endless help and providing all the required chemicals while doing my research work.

I expressed my sincere gratitude to Dr. Namrata Thapa, Head, Department of Zoology, Nar Bahadur Degree College, Tadong for her blessings, guidance and support during my entire PhD work.

My sincere acknowledge to the Department of Science and Technology (DST), Govt. of India, New Delhi for research project grant and providing JRF and SRF fellowship till completion of my PhD Thesis.

I would like to express my gratitude to Professor G.B Nair, Director, THSTI, Gurgaon, Haryana and Dr. N.C Talukdar, Director, IASST, Guwahati, Assam for their valuable training during my PhD work.

I would like extend my sincere gratitude to Dr. K. Jeyaram (Scientist E), Dr. Romi and Dr. Santosh Keisam for their technical help and support during my PhD research work.

I would also like to extend my gratitude to Dr. Sudhan Pradhan, Dr. Arun Kumar Rai, and Dr. Rajen Chettri for their constant suggestion and support.

I would also like to thank my seniors Dr. Shankar Pd. Sha, Dr. Pramila Koirala and Dr. Kriti Ghatani for their help and guidance throughout my research work.

I would like to thank my colleagues Dr. Varsha Rani Gajamer, Mrs. Bhumika Poudyali, Mr. Ranjan Kaushal Tirwa and Mr. Ishfaq Nabi for their care and support.

I would also like express my gratitude towards my Bioinformatic Team, Mr. Nakibapher Jones Shangpliang, Ms. Meera Ongmu Bhutia, Mrs. Pooja Pradhan, Mrs. Anu Anupma, Mr. Pynhunlang Kharnoir, Ms. Priyamvada Pariyar and Mr. Souvik Das for their love, care and support; good luck to all.

I would also like to thank my junior colleagues Dr. Mingma Thendup Sherpa, Mr. Sayak Das, Dr. Ashish Kumar Singh, Mr. Lalit Chaurasia, Ms. Suberna Thapa and Mr. Kundhey Hang Subba, Department of Microbiology for their help and support.

I would also like to thank Dr. Mangesh Survavanshi for teaching and helping during molecular data analysis.

My sincere gratitude to the Sikkim villagers, who provided sample, participated in the survey and shared their valuable traditional knowledge about the process of preparing naturally fermented milk product. I had not been able to complete my work without their knowledge and help.

I am extremely grateful to my beloved parents Mr. P.M Rai, Mrs. Mon Maya Rai for their unconditional love, prayers, care and sacrifices for educating and preparing me for my future. Also I express my gratitude to my sisters and brothers for their support and valuable prayers. My Special thanks go to my friend Ms. Laxmi Rai for her love, care and support.

Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

Date: 18th June 2020 **Ranjita Rai**

CONTENTS

INTRODUCTION

Naturally Fermented Milk

Historically and culturally milk of domesticated animal is consumed fresh and also fermented into various milk products during human evolution (Tamang et al. 2020). The constituents in milk that make it nutritious also make milk highly perishable, thus fermentation emerged as the major way of milk preservation with its nutrients in dietary history of human being (Tamang and Samuel 2010). Fermented foods are produced from raw substrates of plant or animal origins (milk/meat) mostly by natural fermentation or few products by back-slopping or additional of traditionally prepared starter culture(s) containing functional microorganisms which modify the raw or cooked substrates organoleptically and biochemically into edible products that are socially acceptable as fermented foods by the consumers (Tamang 2010a; Tamang et al. 2016a). Bio-chemical components of raw or cooked substrates of plant or animal sources are converted by microbiota present therein, during fermentation thereby enhancing the nutritional value of foods with improved flavour and texture, shelf live of the product, promote health-benefits to consumers including vitamins and minerals, antioxidant and probiotic functions (Tamang 2015; Tamang et al. 2016b; Marco et al. 2017). Fermented milk products are classified into two major groups on the basis of dominant functional microorganisms (Mayo et al. 2010; Surono and Hosono 2011): (I) lactic fermentations, dominated by species LAB, and is consisted of thermophilic type (e.g., yogurt, Bulgarian buttermilk), probiotic type (acidophilus milk, bifidus milk), and mesophilic type (e.g., natural fermented milk, cultured milk, cultured cream and buttermilk); and (II) fungal-lactic fermentations, where LAB and yeasts species co-exist to generate the final product and is consisted of alcoholic milks (e.g., *kefir, koumiss*), and mouldy milks (e.g., *viili*). In milk fermentation starter cultures are of two types, depending on the principal function, (a) primary cultures to participate in the

acidification, and (b) secondary cultures for flavour, aroma and maturing activities (Topisirovic et al. 2006; Bintsis 2018; Hutkins et al. 2018; Tavakoli et al. 2019).

Naturally fermented milk (NFM) product is one of the oldest methods of milk fermentation using raw or boiled milk to ferment spontaneously or naturally or by using back-slopping method (Robinson and Tamime 2006; Parker et al. 2018; Tamang et al. 2020). NFM product supplements probiotic bacteria with specific health-promoting benefits to the consumers (Franz et al. 2014; Rezac et al. 2018). NFM products are popular milk items in dietary cultures of many communities in the world such as *kurut* of China (Sun et al. 2010), *aaruul, airag, byasulag, chigee*, *eezgii* and *tarag* of Mongolia (Takeda et al. 2011; Oki et al. 2014; Wang et al. 2016), *ergo* of Ethiopia, *kad, lben, laban, rayeb, zabady* and *zeer* of North Africa and Middle East Asia, *rob* (from camel milk), *biruni* (cow/camel milk), *mish* (cow/camel milk) of Sudan, *amasi* (*hodzeko, mukaka wakakora*) of Zimbabwe, *filmjölk* and *långfil* of Sweden (Mayo et al. 2010; Tamang et al. 2016a), *nunu* of Ghana (Akabanda et al. 2013), *trachanas* of Cyprus (Bozoudi et al. 2017), *koumiss* or *kumis* or *kumys* or *kymys,* and *kefir* of the Caucasian area (Wu et al. 2009; Ahmed et al. 2013), *lait caillé* of Senegal (Parker et al. 2018). NFM products harbour several species of bacteria which include *Lactococcus (Lc.) lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis*, *Leuconostoc (Leuc.) mesenteroides, Lactobacillus (Lb.) casei, Lb. paracasei, Lb. fermentum, Lb. helveticus*, *Lb. plantarum, Lb. acidophilus, Lb. coryniformis, Lb. curvatus*, *Lb. kefiranofaciens, Lb. kefiri, Lb. buchneri*, *Lb. jensenii, Lb. kitasatonis, Enterococcus (E.) faecium*, *Streptococcus (S.) thermophilus* and species of *Pediococcus, Acetobacter*, *Gluconobacter* (Tamang et al. 2000; Mathara et al. 2004; Patrignani et al. 2006; Dewan and Tamang 2006, 2007; Hao et al. 2010; Yu et al. 2011; Oki et al. 2014; Wang et al. 2016; Bozoudi et al. 2017; Colombo et al. 2018; Parker et al. 2018; Shangpliang et al. 2018) and yeasts-

Saccharomyces (Sacch. cerevisiae, *Issatchenkia orientalis, Kazachstania unispora, Pichia mandshurica* (Watanabe et al. 2008; Bozoudi et al. 2017).

Yogurt is a widely consumed highly nutritious fermented milk resulting from fermentation of milk by *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (Tamime and Robinson 2007; Rul 2017), and *Lb. acidophilus, Lb. casei, Lb. rhamnosus, Lb. gasseri*, *Lb. johnsonii*, *Lb. plantarum* and *Bifidobacterium* spp. (Qian et al. 2018). *Kefir* is a viscous, acidic, slightly effervescent, and mildly-alcoholic milk beverage produced by spontaneous fermentation of milk with kefir grains that originated in Europe and Asia and that has spread globally (Ahmed et al. 2013). Bacteria- *Lb. kefiranofaciens*, *Leuc. mesenteroides*, *Acetobacter pasteurianus*, *Lb. helveticus*, *Leuc. citreum*, *Leuc. gelidum*, *Leuc. kimchi,* and yeasts- *Kazachstania* spp. and *Saccharomyces cerevisiae* were also reported from *kefir* (Jianzhonga et al. 2009; Marsh et al. 2013; Walsh et al. 2016, 2018; Seol et al. 2019).

Probiotics

The FAO/WHO defined the probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). Probiotics are one of the functional properties of fermented foods (Tamang 2015), and some lactic acid bacteria (LAB) are used as health-promoting organisms (Hwanhlem et al. 2010; Monteagudo-Mera et al. 2012). Probiotic cultures should demonstrate the following properties to be recognised as functional food components such as acid and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of

bioavailability of food compounds and production of vitamins and enzymes (Ouwehand et al. 1999; Shi et al. 2016; Campana et al. 2017; Ghosh et al. 2019). Bacterial strains belonging to *Lactobacillus* and *Bifidobacterium* are mostly claimed as probiotics (Prasad et al. 1998; Vlasova et al. 2016). However, some species belonging to the genera *Lactococcus, Enterococcus, Pediococcus, Leuconostoc*, *Bacillus* and yeast *Saccharomyces* are also considered as probiotic microorganisms (Sanders and in't Veld 1999; Fijan 2014). Non-LAB *Propionibacterium freudenreichii* has been claimed as probiotic with functionality (Holzapfel et al. 2001, 1998; Huang et al. 2019). Microorganisms, generally categorised as probiotics, are *Lactobacillus acidophilus, Lb. amylovorus, Lb. casei, Lb*. *crispatus*, *Lb. delbrueckii* subsp. *bulgaricus, Lb. gallinarum, Lb. gasseri, Lb. johnsonii, Lb. paracasei, Lb. plantarum, Lb. reuteri, Lb. rhamnosus*, *Enterococcus faecalis, E. faecium, Lactococcus lactis, Leuconostoc mesenteroides, Pediococcus acidilactici, Sporolactobacillus inulinus, Streptococcus thermophilus* and non-LAB- *Bacillus cereus* var. *toyoi, Escherichia coli* strain *nissle*, *Propionibacterium freudenreichii*; and yeasts- *Saccharomyces cerevisiae, Sacch. boulardii* (Holzapfel et al. 2001; Shah 2007; Vlasova et al. 2016; Garcia-Gonzalez et al. 2018; Huang et al. 2019). Fermented milk products are especially considered as ideal vehicle for delivering probiotic bacteria to the human gastrointestinal tract (Shah 2007; Rezac et al. 2018). Incorporation of probiotic cultures in fermented milk are mostly practised by firstly, addition of probiotics together with the starter cultures; secondly, production of two batches separately, one containing the probiotic microorganism in milk with high concentration of viable cells and another with starter cultures, when the fermentation stages are completed the batches are then mixed; and lastly, the use of a probiotic microorganism as a starter culture (Soccol et. al. 2010; Moslemy et al. 2015; Fenster et al. 2019).

Fermented Milk Products in India

Domestication of animals for milk and milk products has been practising in India since the Harappan civilization for last 8000 years (Sarkar et al. 2016). Historical importance of cow and its milk products was mentioned in *Rig Veda*, the oldest sacred book of the Hindus (Jamison and Brereton 2014). *Veda* and *Upanisad* mentioned the origin of *dahi* and fermented milk products in India during 6000-4000 BCE, one of the oldest fermented milk products in the world (Yegna Narayan Aiyar 1953; Achaya 1998). *Dahi* plays an important part in the socio-religious habits of Indians and is considered as sacred item in many of their festivals and religious ceremony both by Hindu and Buddhists (Tamang 2020). India has many varieties of major and minor region-specific naturally fermented milk (cow's/buffalo's/yak's) products (Sarkar 2008). Some of them have been documented and studied: *dahi* (Mohanan et al. 1984; Ghosh and Rajorhia 1990; Rathi et al. 1990; Sharma et al. 1993; Gupta et al. 2000; Agarwal and Bhasin 2002; Shruti and Kansal 2011; Jadhav et al. 2013; Balamurugan et al. 2014; Mohania et al. 2014), *shrikhand* (Boghra and Mathur 2000; Devshete et al. 2013; Singh and Singh 2014; Singh et al. 2014; Karche et al. 2015), *lassi* (Patidar and Prajapati 1988; Padghan et al. 2015; Patel et al. 2015; Sudheendra Ch et al. 2018).

Milk in Sikkim

Sikkim is one of the mountainous states of India located in the Eastern Himalayas with an area of 7096 sq. km and an altitude of 300 m to 8500 m ranging from sub-tropical to alpine zones (Fig. A). Sikkim shares borders with China in the north and northeast, Bhutan in the east, Nepal in the west, and Gorkhaland Territorial Administration (GTA) in the state of West Bengal in the south. The state of Sikkim is administratively divided into four districts viz. North district, East district, South

district and West district. A total population of Sikkim is 6,10577 (www.census2011.co.in).

Figure A. Map of Sikkim.

About 80% ethnic communities in Sikkim are Gorkha or Nepali and 20% are Bhutia and Lepcha (Tamang 2005). The Gorkha or Nepali community has several castes including Limboo, Rai, Tamang, Chettri, Bahun, Magar, Pradhan/Newar, Gurung, Bhujel, Dewan, Sunwar, Khagatey, Sansyasi/Giri, Sherpa, Kami, Damai, Sarki and Maji (Tamang 2010b). Livestock mostly plays a subsidiary role in the mixed farming system in Sikkim which includes cattle, sheep, goats, pigs, yaks, poultry, etc., which is mainly, used for meat, milk and milk products. Yak (*Bos grunniens*) is reared mostly on extensive alpine and sub alpine scrublands between 2100 m to 4500 m altitude for milk, milk products and meat (Balaraman and Golay 1991). Accordingly to National Dairy Development Board, annual milk production in Sikkim during 2018-19 was 61,000 tonnes (www.nddb.coop). Cow milk and milk products are popular in Sikkim. However, yak milk and milk products are also popular in North district and some regions in West district. About 88.3% of people in Sikkim are non-vegetarians and 11.7

% people are vegetarians (Tamang et al. 2007). In Sikkim, per capita consumption of ethnic fermented foods and beverages is 163.8 g/day representing 12.6 % of fermented foods in daily intake of meal (Tamang et al. 2007). Culinary and typical cuisine of different ethic people in Sikkim is unique and unparalleled since Sikkim is wedged in between Nepal and Bhutan with vast expanse of the Tibetan Plateau in north (Tamang 2005). Probably the dietary culture of modern Sikkim is a fusion of the traditional cuisines and culinary of ethnic Gorkha/Nepali, the Tibetan and the Lepcha with modifications based on availability of raw materials, food preference, adaptability, acceptability and other social ethos (Thapa and Tamang 2020). Modern methods of milk processing in Sikkim started in Sikkim since 1980 after establishment of a cooperative-run Sikkim Co-operative Milk Producers' Union Limited, manufacturing pasteurized cow milk and its milk products (www.sikkimilk.coop). However, majority of ethnic people of Sikkim prepare the traditional way of naturally fermented milk products either through natural fermentation or using 'crude back-slopping' method. Dishes prepared from naturally fermented milk products constitute a unique cuisine and represent the traditional culinary and gastronomy of Sikkim (Thapa and Tamang 2020).

Previous Reports on NFM products from Sikkim

Some naturally fermented milk products of Sikkim were studied earlier and few genera/species of LAB were reported based on limited conventional methods including cell morphology, phenotypic and biochemical tests, and also commercial kits Analytical Profile Index (API) for identification based on sugar profiles (Tamang et al. 2000; Dewan and Tamang 2006, 2007). LAB from *dahi* samples of Sikkim reported earlier included *Lactobacillus bifermentans, Lb. alimentarius, Lb. casei* subsp. *pseudoplantarum*, *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris,* and also

few yeasts *Saccharomycopsis* and *Candida* (Dewan and Tamang 2007), from *chhu* - *Lb. farciminis, Lb. brevis, Lb. alimentarius, Lb. salivarius,* and *Leuconostoc lactis* subsp. *cremoris,* and also some yeasts *Saccharomycopsis* and *Candida* (Dewan and Tamang 2006), *philu- Lb. paracasei* subsp. *paracasei, Lb. bifermentans* and *Enterococcus faecium* are (Dewan and Tamang 2007), and from *somar- Lb. paracasei* subsp. *pseudoplantarum* and *Lc. lactis* subsp. *cremoris* (Dewan and Tamang 2007). Tamang et al. (2000) reported few species of LAB isolated from soft variety *chhurpi* of Sikkim based on mol% G+C content of DNA determined spectrophotometrically which included *Lactobacillus plantarum, Lb. curvatus, Lb. fermentum, Lb. paracasei* subsp. *pseudoplantarum* and *Leuconostoc mesenteroides.* Nutritional value of these NFM products of Sikkim was also analysed (Dewan and Tamang 2007; Tamang et al. 2012).

Research Gap

Studies on composition of dominant microbial communities are always useful to control the process that contributes to maintain the quality of the finished product. However, previous reports on identity of bacteria from some naturally fermented milk products of Sikkim was purely tentative (Dewan and Tamang 2006, 2007), no confirmation of their identity upto species level was performed using 16S rRNA gene sequencing tool. Moreover, no studies have been conducted yet on bacterial community in NFM products of Sikkim by using high-throughput sequencing method supported by bioinformatics and also determination of some probiotic attributes supported by gene detection using PCR technique. Hence, the present Thesis emphasizes on bacterial diversity and their probiotic attributes in NFM products of Sikkim. Determination of some probiotic characters of isolated LAB strains from NFM products may provide the

detailed scientific information on NFM for selection of native microbial strains with proper identity for development of starters for functional fermented milk production.

Experimental Designs to Study the Objectives

In the present Thesis, we selected five different types of naturally fermented milk products of Sikkim based on milk of cow and yak viz., *dahi* (cow-milk)*, dahi* (yak-milk)*,* soft *chhurpi* (cow-milk), soft *chhurpi* (yak-milk), *mohi* (cow-milk), hard *chhurpi* (yak-milk) and *philu* (yak-milk). Based on above mentioned research gaps we designed the experiments and conducted our studies to fulfil the following objectives on:

- Isolation of lactic acid bacteria from NFM products.
- Their identity by culture-dependent method (phenotypic and 16S rRNA gene sequence) and culture-independent technique (high-throughput sequencing).
- Screening of probiotic attributes: Acidification and Coagulation; Beta-galactosidase activity; Hydrophobicity assay; Acid Tolerance; Lysozyme and Bile (0.3%); Bile salt hydrolysis activity and Antimicrobial activity.
- Gene detection *in vivo* for presence of different gene encoding enzymes in LAB strains isolated from NFM products.

OBJECTIVES

- To isolate LAB in some common naturally fermented milk (NFM) products of Sikkim.
- To identify LAB by phenotypic and genotypic methods.
- To determine some probiotic properties of dominant LAB from NFM products.

REVIEW OF LITERATURE

The importance of cow rearing and its milk products has been mentioned in the Hindus' oldest sacred book *'Rig Veda'* (Jamison and Brereton 2014) which are originated in Indus valley civilization (Tamang 2020). Veda and Upanishad described the origin and fermentation of the *dahi*, one of the oldest fermented milk products in the world during 6000-4000 BCE (Yegna Narayan Aiyar 1953). There are more than 350 major and region-specific types of ethnic fermented foods produced either naturally or by adding mixed starter culture using indigenous knowledge of fermentation (Tamang 2020). As per the International Dairy Federation (1988), fermented milk is a dairy product made from milk, either skimmed or not, where functional microorganisms are alive with different cultures and are free from pathogenic microorganisms. Generally fermented dairy products are categorized into four types: (1) acid/alcohol eg. *kefir* and *koumiss*, (2) acid-type high eg. Bulgarian sour milk, (3) acid-type medium eg. Acidophilus milk and yogurt, and (4) low acid-type such as buttermilk grown and cream grown (Kosikowski 1977). There are more than 70 bifidus-and acidophilus dairy products produced worldwide according to (Shah 2001). Below are some of the popular fermented milk products that are widely consumed throughout the world (Table A).

Table A. Traditional fermented milk products of the world				
Product	**Milk Source**	**Nature**	**Regions of production**	**References**
Acidophilus milk	Cow milk	Sour milk	Russia, East Europe, Greece, Turkey, North America, Scandinavia	Kurmann et al. (1992); De Roos and Katan (1998); Ozden (2008); Shiby and Mishra (2013)
Airag	unpasteurized mare's milk	Fermented milk	Central Asia, Kazakhstan, Krygyzstan, some parts of Russia and Jinjiang, Inner Mongolia in China	Koroleva (1988); Liu (2011); Mu et al. (2012); Watanabe et al. (2008); Oki et al. (2014); Choi et al. (2016)
Bulgarian buttermilk	Cow milk	Sour milk	Yugoslavia, Bulgaria, Greece, Turkey, Albania, Romania	Kosikowski (1977); Steinkraus (1996); Koleva et al. (2009)
Butter	Milk	Soft past	Algeria, Indian subcontinent	Bettache et al. (2012); Guetouache and Guessas (2015); Deosarkar et al. (2016); Mourad and Bettache (2018)
Buttermilk	Cow milk	Sour milk	USA, Canada, Russia, Scandinavi, Middle East, Egypt, Ethiopia, India, Australia, New Zealand	Mulder and Walstra (1974); Oberman (1985); Corredig and Dalgleish (1997)
Cheese	Cow milk	Soft or hard solid	All parts of the world	Galloway and Crawford (1985); Carr (1981); Grahame (1996)
Chhurpi (hard/soft)	Cow/ yak milk	Hard mass; Smooth white fluffy	Sikkim, Darjeeling hills and Arunachal Pradesh (India)	Katiyar et al. (1991); Pal et al. (1996); Tamang et al. (2000); Dewan and Tamang (2007); Prashant et al. (2009)
Dahi	Cow or buffalo milk	Yoghurt-like fermented milk	Indian subcontinent	Ramakrishnan (1979); Rathi et al. (1990); Misra (1992); Harun-ur-Rashid et al. (2006)
Kefir	Goat, sheep, or cow milk	Acidic, mildly alcoholic, effervescent milk	Russia, Europe, Middle East, North Africa	Garrote et al. (1997); Loretan et al. (2003); Plessas et al. (2007); Sarkar (2007); Kakisu et al. (2011); Miguel et al. (2011); Ahmed et al. (2013)

Kishk	Sheep milk-wheat	Milk-wheat mixture; dried balls	Greece, Turkey, Egypt, Libya, Middle East, Iran	Abd-el-Malek and Demerdash (1977); Basson (1981); Tamime and O'Connor (1995); Gadallah and Hassan (2019)
Koumiss/ *Kumiss*	Horse, donkey or camel milk	Acid/alcoholic milk	Russia, Mongolia, China	Auclair and Accolas (1974); Kosikowski (1977); Tamime (1981); Campbell-Platt (1987); Wu et al. (2009); Yao et al. (2017); Tang et al. (2020)
Kurut	Raw yak milk	Fermented milk	Qinghai Tibet	Cai (1985); Cao et al. (2004); Zhang et al. (2008); Sun et al. (2010); Liu et al. (2015)
Lassi	Milk	Buttermilk or dahi, sometimes sweetened	Indian subcontinent, Mongolia, Middle East, North Africa, West Africa, Europe	Mital (1977); Patidar and Prajapati (1998); Sukumar (2004); Sudheendra Ch et al. (2018)
Laban rayeb	Milk	Yoghurt-like fermented milk	Egypt, Turkey, Middle East	Abd-El-Malek and Demerdash (1970); Morcos (1973); El-Gendy (1983); Oberman (1985); El-Samargy (1997)
Misti dahi	Milk	Sweet yoghurt-like	Eastern India	Ray and Srinivasan (1972); Ghosh and Rajorhia (1990); Rao and Solanki (2007); Raju and Pal (2009)
Nunu	Raw cow milk	Fermented milk	Ghana and other parts of West Africa	Akabanda et al. (2010); Akabanda et al. (2013)
Paneer	Buffalo, cow, milk	Cheese-like solid	Indian subcontinent	Aneja (2007); Shrivastava and Goyal (2007); Bhandekar et al. (2018)
Rabri/Rabdi	Buttermilk, cereals, pulses	Thick slurry-like product	India	Chatterjee et al. (1994); Prasad (1997); Khaskheli et al. (2008); Ghayal et al. (2015)
Shrikhand	Cow or buffalo milk	Sweetened dewatered dahi	Western and southern India	Boghra and Mathur (2000); Swapna and Chavannavar (2013); Mane et al. (2017)
Tarag	Unpasteurized mare's milk	Fermented milk	Central Asia, Kazakhstan, Krygyzstan, some part of Russia and Jinjiang, Inner Mongolia in China	Ishii and Konagaya (2002); Hasisurong and Manglai (2003); Watanabe et al. (2008); Oki et al. (2014)

Trachanas	Sheep milk, wheat	Wheat-fermented milk; sweet-sour soup or biscuit	Cyprus, Greece, Turkey	Economidou (1975); Tamime and O'Connor (1995); Daglioglu et al. (2000); Ozdemir et al. (2007); Georgala et al. (2012)
Yoghurt	Cow, goat, sheep, buffalo or camel milk	Fermented milk	All parts of the world	Oberman (1985); Adams and Moss (1995); Obi et al. (2016); Lisko et al. (2017); Agustinah et al. (2019)

Acidophilus milk

Acidophilus milk is one of the well-known dairy products with sour taste that are used as a nutritional dairy food in many countries (Gomes and Malcata 1999). Acidophilus milk has therapeutic and beneficial properties for the safety. It has also stated that the growth of *Lactobacillus acidophilus* may replace undesirable microorganisms with a beneficial lactic fermentation under the condition in the gastrointestinal tract (De Roos 1998; Amiri et al. 2010). Milk is heated at 95°C during acidophilus milk preparation, homogenized and cooled at 37°C. Pure (2-5%) *Lb. acidophilus* was inoculated and held for 12-24 hours for incubation. Upon incubation, milk is cooled to 5°C and held under cold conditions. However, milk is heated at above 120°C in other acidophilus milk production centres and then cooled and later inoculated with pure culture of *Lb. acidophilus*. The high heating temperature removes unnecessary micro-organisms and promotes enhanced growth of *Lb. acidophilus*. Where as in the processing of unfermented acidophilus milk product, *Lb. acidophilus* is inoculated in cold milk (5-7°C) and place under the cold conditions (Kurmann et al. 1992; Ozden 2008; Shiby and Mishra 2013). Acidophilus milk contains 1.5 to 2.0% lactic acid and does not produce alcohol. It is suitable for consumption due to its chemical characteristics such as acidity and pH-value (Kosikowski 1977; Moayednia and Mazaheri 2012). It has probiotics characters due to its high acridity that makes the intestinal tract acidic environment

inhibiting the growth and proliferation of the gas-forming putrefactive bacteria in the gut (Oberman 1985; Ashar and Prajapati 2001a,b; Shah 2001; Bull et al. 2013; Ghosh et al. 2019).

Airag

Airag is a traditional beverage made by the Mongolian nomads who naturally ferment the milk of the fresh mares (Choi 2016). It is also popular beverage produced in Central Asia such as Kazakhstan, Krygyzstan, a part of Russia and Jinjiang, and China (Koroleva 1988). It is also known as *chigee*, and is mildly alcoholic in taste and sour (Guo et al. 2020). Fresh mare's milk was used during the *airag* preparation. Previously made *airag* is used as an inoculum and properly mixed with milk (Koroleva 1988). After mixing it properly the mixture is put to an animal skin bag and then store at room temperature in order to produce acid, ethanol and flavour. Ethyl alcohol is produced after fermentation as an end product and its pH reaches below 4 (Liu et al. 2011). Lactobacilli and yeast are involved during *airag* fermentation (Watanabe et al. 2008; Wu et al. 2009; Sun et al. 2010; Mu et al. 2012). Yeast plays a vital role in the fermentation of sugar and converts it into ethyl alcohol and carbon dioxide to produce a mildly alcoholic carbonated beverage (Wulijideligen et al. 2013). *Airag* has been considered as a nutritious beverage from ancient times, with health-promoting properties (Donmez et al. 2014); it has a high content of polyunsaturated fatty acids in the milk and probiotic microorganisms of mares as well as basic nutrients such as calcium and protein (Malacarne et al. 2002; Csapo et al. 1995; Csapo-Kiss et al. 1995).

Bulgarian buttermilk

Bulgarian or bulgaricus buttermilk is extremely sour milk; it is prepared from boiled goat or cow's milk, inoculated with a previous fermented milk inoculum (Oberman 1985). Bulgarian buttermilk is believed to have originated from the tradition of Trak that is, from the ancient tradition of the sheep breeders who came from Bulgaria to Asia in the 15th century (Oberman 1985). Bulgarian milk is primarily fermented by *Lactobacillus bulgaricus* and its total acidity (lactic acid) can rise from 2.0 to 4.0% (Kosikowski 1977). Incubation temperature ranges from 38°C to 47°C for bulgaricus buttermilk (Steinkraus 1983). *Lb. bulgaricus* converts the milk lactose into lactic acid during fermentation and produces acetaldehyde as flavor compounds (Marshall 1982).

Buttermilk

Buttermilk is a by-product of sour milk or cream butter making, and/or sweet cream making (Gebreselassie et al. 2016). It has water-soluble cream components such as milk protein, lactose, minerals, and milk-fat globule membrane (MFGM), which is disrupted during churning and transferred to the buttermilk fraction (Corredig and Dalgleish 1997). Due to its high content in MFGM material, Buttermilk contains more phospholipids than milk (Mulder and Walstra 1974). Phospholipid has emulsifying properties and its high buttermilk content makes it suitable for use as a functional ingredient in this milk component (Elling et al. 1996; Corredig and Dalgleish 1998a; Wong and Kitts 2003). Furthermore, phospholipids also appear to have anticarcinogenic effects against colon cancer and to have a defensive function against bacterial toxins and infections (Dillehay et al. 1994; Schmelz et al. 1996; Rueda et al. 1998; Sprong et al. 2002). Buttermilk is made from fresh skim milk, or skimmed milk

that is partially pasteurized. It is commonly fermented with one or more selected strains of *Lactococcus lactis, Lc. cremoris,* and one or more streptococci fermenting citric acid species, *Leuconostoc cremoris*, and occasionally *Lc. lactis* subsp. *diacetylactis* (Tamime and Robinson 1988). Bacterial population is growing very rapidly but the fungal and yeast population are gradually replicating in buttermilk (Vijayalakshmi and Murugesan 2001). Buttermilk is consumed as it is by all members of the household, or as a side dish after processing into other products, such as *ajibo*, a variety of Ethiopian cottage cheese made from heated buttermilk and *hazo*, spiced buttermilk (Negussie et al. 2012). The fermentation is spontaneous as it is fermented in an unregulated manner by lactic acid bacteria (LAB) and yeasts that acquired access to the milk from the atmosphere (Gebreselassie et al. 2016). Microorganisms involved in commercial buttermilk preparation include *Lc. lactis* ssp. *cremoris* and *lactis, Lc. lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp. (Doyle and Beuchat 2007). Apart from LAB, *Saccharomyces cerevisiae* has also role in buttermilk fermentation (Gebreselassie et al. 2016).

Butter

Butter is one of the popular dairy products prepared during churning of curd and a good example of storing milk fat into butter (Guetouache and Guessas 2015). Butter is widely used as a spread and condiment, as well as in cooking applications such as baking, producing sauce, and frying pan and is composed of protein from butterfat, water, and milk (Deosarkar et al. 2016). In eastern Algeria, the traditional butter made from sheep's milk is called *sman*, *dhan* or *zabda* (Idoui et al. 2009). LAB is predominant microflora in Algerian butter, particularly the genera *Lactococcus*, *Leuconostoc* and *Lactobacillus* and *Enterococcus* (Idoui et al. 2009; Bettache et al.

2012; Mourad and Bettache 2018). The predominant species in conventional butter include *Lactococcus lactis* spp. *diacetylactis* (Idoui et al. 2009). Butter mainly consists of saturated fat and is an essential dietary cholesterol source (Deosarkar et al. 2016).

Cheese

Cheese is one of the most important dairy products made from milk used daily in the world. It is prepared by multiple processing techniques based on lactic acid bacteria fermenting the milk. This has a long shelf-life and is rendered to inhibit bacterial growth by reducing water content and adding salts (Grahame 1996). Cheese and cheese products have significant nutritional qualities and market significance worldwide (Galloway and Crawford 1985). The cheese and cheese products were categorized into four major classes according to USDA (1978): very hard (grating), hard, semi-soft, and soft. There are various cheese varieties produced and some of the common cheese varieties are as follows *cheddar, camembert, swiss, cottage, asiago old, romano, sapsago, spalen, caciocavallo, emmentaler, gruyere, brick, munster, limburger, port du salut, trappist, roquefort, gorgonzola, blue stilton, blue wensleydale, brie, neufchatel, parmesan* etc. (Androuet 1976). LAB strains of *Lactococcus* spp. play crucial roles in the conversion of lactose from cheese to lactic acid (Carr 1981). Cheese, fish, wine, beer, dry sausages, and other fermented foods contain essential organic compounds such as biogenic amines (Ten Brink et al. 1990; Halasz et al. 1994). Food associated with histamine poisoning is commonly found in cheese however (Silla-Santos 2001). In 1969, in the Netherlands, the very first recorded case of histamine poisoning occurred, involving *Gouda* cheese (Stratton et al. 1991). So far there have been several studies conducted to assess the amine content of cheese products. Cheese contains a variety of amines, including histamine, tyramine, cadaverine, putrescine, tryptamine and

phenylethylamine (Besancon et al. 1992; Abd-Alla et al. 1996; Schneller et al. 1997; Vale and Gloria 1997). The lactic acid bacteria frequently produce histamine and tyramine in fermented foods and dairy products (Stratton et al. 1991; Leisner et al. 1994; Barbieri et al. 2019). *Enterococcus faecalis* has been reportedly associated with the production of tyramine in cheese and other fermented dairy products (Holt et al. 1994; Celano et al. 1996). Bacterial community study showed that *Firmicutes* was the most dominant phylum in artisan cheese (Falardeau et al. 2019).

Dahi

Dahi also known as curd is a popular ethnic fermented milk product consumed by a large number of the population all over India (Tamang et al. 2016a). The word *dahi* derives from the Sanskrit word "*dadhi*" (Yegna Narayan Aiyar 1953). This is prepared from or from a mixture of cow and buffalo milk using a conventional method using previously produced *dahi*, containing lactic acid bacteria and other fermenting micro-organisms (Harun-ur-Rashid et al. 2006; Ramakrishnan 1979). The fresh cow's or buffalo's milk is boiled in a pot in traditional *dahi* preparation process, cooled to room temperature, and transferred to a hollow wooden pot or container. A small amount of *dahi* previously prepared (back-slopping technique) is applied to the boiled and cooled milk left at room temperature for 1-2 days in the summer and 2-4 days in the winter for natural fermentation. The duration of fermentation depends on the season as well as the place's geographic location (Tamang et al. 2016a). Nutritional value and palatability of *dahi* are well known (Rathi et al. 1990). It is a product similar to plain yoghurt but differing in acidity (Batra and Millner 1975; Mital 1977; Shuaib and Azmey 1977). It is consumed as either plain or flavoured as a dessert, refreshing and savory (Misra 1992; Harun-ur-Rashid 2006; Rai et al. 2016). *Dahi* preparedness and consumption has been

recorded since 2000 BCE (Prakash 1961). According to the Indian traditional medicine system (Ayurveda), *dahi* is useful in treating diarrhoea and other acute/chronic GI disorders from ancient times (Viswanathan et al. 2003). Microbiota present in *dahi* are *Lactobacillus bulgaricus, Lb. acidophilus, Lb. helviticus, Lb. casei, Lb. brevis, Streptococcus thermophilus, Lactococcus lactis, Lactococcus cremoris, Enterococcus faecalis, Lb. bifermentans, Lb. alimentarius, Lb. paracasei, Streptococcus cremoris, Streptococcus lactis, Lb. acidophilus, Lb. cremoris, Ped. pentosaceus, P. acidilactici, W. cibaria, W. paramesenteroides, Lb. fermentum, Lb. delbrueckii* subsp. *indicus*, *Saccharomycopsis* spp. and *Candida* spp. (Laxminarayana et al. 1952; Ranganathan et al. 1964; Ramakrishnan 1979; Mohanan et al. 1984; Dewan and Tamang 2007; Patil et al. 2010; Balamurugan et al. 2014; Joishy et al. 2019). *Dahi* is prepared using starter culture combination of *Lactococcus lactis* subsp. *lactis*, or *Lc. lactis* subsp. *diacetylactis* or *Leuconostoc* spp. for commercial production (Misra 1992; Balamurugan et al. 2014). Based on the BIS (1980a).

Kefir

Kefir is a spontaneously carbonated, slightly acid fermented milk product made from *kefir* grains that contains a complex and unique blend of yeast and bacteria in a polysaccharide matrix (Sarkar 2007; Ahmed et al. 2013). *Kefir* is one of the popular fermented dairy products; it was first eaten in Russia's Caucasus Mountains, many years ago. In Europe, Asia, and South and North America, it has claimed to be a safe food of high nutritional value (Kakisu et al. 2011). This is prepared by fermenting different kinds of milk, such as sheep, cow, goat, etc., often claimed as a relaxing and self-carbonated drink (Miguel et al. 2011). *Kefir* grains are white or yellow in colour, with diameters ranging from 0.3-3.5 cm (Plessas et al. 2007). *Kefir* grains are the main ingredient in water-insoluble fermented *kefir* beverages; their grains appear to settle on

the bottom of the fermentation broth (Garrote et al. 1997; Loretan et al. 2003). *Kefir* is acidic, mildly alcoholic beverage in nature (Hartles et al. 1977), and is composed of water (89-90% w/w), lipids (0.2% w/w), proteins (3.0% w/w), sugar (6.0% w/w), and ash (0.7% w/w) (Garrote et al. 1997). *Kefir* is either eaten as a drink or as a spoon much like yogurt, or can be sweetened with sugar or mixed with fruit or biscuits (Mogilevsky 1977; Hartles et al. 1977). Microbial composition of *kefir* has species of yeasts *Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia*), species of lactic acid bacteria *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and sometimes, acetic acid bacteria (Paraskevopoulou et al. 2003; Witthuhn et al. 2005; Nalbantoglu et al. 2014). The predominant yeast species in *kefir* comprise of *Torulopsis holmii* and *Saccharomyces delbrueckii* (La Rieviere 1963; Hartles et al. 1977; Chin Wen et al. 1999; Diosma et al. 2014). *Lactobacillus brevis* is the predominant bacterium in *kefir* (La Rieviere et al. 1967).

Kishk

Kishk is a fermented dried food made from a mixture of milk and cracked parboiled cereal and is commonly consumed in the eastern Mediterranean and in Egypt (Tamime and O'Connor 1995). It is stored in dried balls form (Abd-El-Malek and Demerdash 1977). It is a well known food among the rural populations in Egypt, Syria, Lebanon, Jordan, Iraq and North Africa (Basson 1981). *Kishk* is a healthy food with an outstanding level of maintenance, high in vitamin B than wheat or milk and a therapeutic benefit (Morcos et al. 1973). *Kishk* contains protein 187-213 g/kg, fat 36-107 g/kg, carbohydrates 639-721 g/kg and ash 33-64 g/kg and has high fiber and β-glucan content (Tamime et al. 2000). The high yeast count in the sample refers to the potential use of starter culture containing yeast fermenting lactose (Tamime and

Robinson 1999). The lactic acid bacterial counts are 10^3 to 10^6 cfu/g (Tamime and McNulty 1999). The primary microorganism during the fermentation of *kishk* is lactobacilli (*Lactobacillus casei, Lb. plantarum, Lb. brevis*) that is involved in the fermentation cycle (Abd-El-Malek and Demerdash 1977). El-Sadek et al. (1958) and Mahmoud (1977), reported that spore formers were predominant microflora from Egyptian *kishk* such as *B. licheniformis, B. subtilis* and *B. megatherium* (57-75%), followed by lactic acid bacteria (25-43%) of the bacterial flora as a whole.

Koumiss

Koumiss, sparkling white or grey fermented milky acid-alcoholic drink, is one of the popular dairy products produced in Russia, China and Mongolia from mare's milk (Kosikowski 1977; Tang et al. 2020). As mentioned by Steinkraus (1996), the traditional processing of *koumiss,* fresh mare or goat milk is stored in a wooden vessel or smoked horse skins, and warm milk of the mare is added with boiling water. Previous old *koumiss* is used as an inoculum and the mixture is adequately covered and preserved for 15 to 24 h. Until the milk taste turns sour, the fermentation is not completed and thick mass is formed to its surface. The mixture is then stirred and crushed until the curd is completely separated, forming a dense liquid. The mixture is wrapped again and fermented for a further 24 h to get *koumiss*. LAB plays a crucial role in processing lactic acid; yeast for ethanol and carbon dioxide production (Surono 2004). The primary fermenting microorganisms during *koumiss* fermentation are *Lactobacillus bulgaricus*, yeasts *Candida kefir*, *Torulopsis* spp. (Kosikowski 1977; Tamime 1981; Tang 2020). *Koumiss* and *kefir* are related foods, both containing alcohol and lactic acid (Steinkraus 1996). The only difference is their starter cultures structure used in producing both types of milk fermentation while *kefir* is granular in

structure (Wang et al. 2008). Based on pH, *koumiss* is categorised into three types: strong *koumiss* (pH 3.3-3.9), medium (pH 3.9-4.5) and mild (pH 4.5-5) (Danova et al. 2005). *Koumiss* has several medicinal values and is used to treat digestive diseases and other chronic diseases such as tuberculosis, bronchitis and anaemia (Auclair and Accolas 1974; Hasisurong and Manglai 2003; Tamang 2015; Tamang et al. 2016b). A novel probiotic strain *Lactobacillus casei Zhang* was identified by screening lactic acid bacteria isolated from *koumiss* samples from Inner Mongolia, China and showed many probiotic properties such as high-level acid and bile stress resistance, antibacterial, antioxidant and immunomodulatory properties (Wu et al. 2009; Ya et al. 2008, Zhang et al. 2010). Consumption of *koumiss* has been known since ancient times and is the primary food of European Russia's migrating tribes and parts of Asia (Auclair and Accolas 1974). The main fermentation products are lactic acid (0.7-1.8%), ethanol (1-2.5%), carbon dioxide and such by products help to retain effervescence and flavour of the product (Yao et al. 2017).

Kurut

Kurut is one of the Tibetan nomadic dairy products fermented naturally from raw yak milk (Sun et al. 2010). It is a stable food of the culture of the Tibetan tin China. The traditional method of preparing fermented milk products is a practice of every Tibetan individual in Qinghai province in China (Sun et al. 2010). Raw milk is fermented at room temperature of 10-25°C in a large jar for one week. *Kurut* is similar to *kefir* and *koumiss*, producing alcohol and lactic acid (Sun et al. 2010). *Kurut* has a pure white colour, viscous in nature with exceptional organoleptic acidity and alcohol sensations. *Kurut* contains milk fat (5.37%), protein (5.44%), calcium (140 mg/100 g), phosphorus (146 mg/100 g), magnesium (154 mg/100 g), zinc (5.74 mg/100 g), and B vitamin (Zhang et al. 2008). The Tibetans of Qinghai region regarded *kurut* as sacred food (Cai

1985). It was one of the significant fermented indigenous milk products that play a vital role in the economic and dietary cuture of the Qinghai population (Cao et al. 2004). Yeast also play key roles during *kurut* fermentation (Ehrmann et al. 2002; Sun et al. 2010), however, LAB such as *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* are also prevalent microorganisms in *kurut* (Zhang et al. 2008; Sun et al. 2010; Liu et al. 2012).

Lassi

According to Mital (1977), *lassi* is made from *dahi*, in reality it is a by-product obtained by indigenous methods during the preparation of butter (*ghee*). During preparation of *lassi*, *dahi* is churned with frequent adding of water until granules of butter are produced. The butter granules are then removed from the liquid component, the remaining liquid is called *lassi* (Laxminarayan and Shankar 1980). *Lassi* is known to have digestive, nutritional value and assists in treating gastrointestinal disorders such as diarrhoea, dysentery, colitis, piles and jaundice (Steinkraus 1996; Anon 2003). *Lassi* composition varies depending on the form of milk used; the manufacturing process, the addition of water during churning and the efficiency of fat granulate separation (Sukumar 2004). *Lassi* includes several ingredients fat, 0.80%; protein, 1.29%; lactose, 1.20%; lactic acid, 0.44%; ash, 0.40%; calcium, 0.60%; and phosphorus, 0.04% with water (Rangappa and Achaya 1974) and has several health promoting benefits (Anon 2003; Rasane et al. 2017). *Lassi* contains a combination of *Lactobacillus acidophilus* and *Streptococcus thermophilus* which is considered to be appropriate and stable organoleptically (Patidar and Prajapati 1998; Padghan et al. 2015).

Laban rayeb

Laban rayeb is a popular Egyptian fermented milk product (Khalafalla et al. 1988). In the preparation, fresh milk is poured into a pot of earthenware and held in a warm position untouched until the cream rises and the lower partly skimmed milk coagulates. The leftover curd called *'laban rayeb'* is either eaten as fermented milk or converted to a soft acid cheese known as *karish* (Abd-El-Malek and Demerdash 1970; El-Gendy 1983), after separating the upper layer of cream which is later turned into butter. *Laban rayeb* is consumed primarily in lower and partially upper parts of Egypt (Abd-El-Malek and Demerdash 1970). It has a mildly acidic taste with buttermilk-like aroma (Morcos et al. 1973; Oberman 1985). *Laban* is used as yogurt cheese and dairy spread (El-Samargy 1997). For acidity, the pH of *laban* ranges from 4.1 to 4.8 varies from 0.8 to 1.3% (Morcos 1973). Studies have confirmed that the predominant species such as *Lactococcus lactis subsp. lactis, Streptococcus thermophilus, Lactobacillus bulgaricus* and lactose fermenting yeasts (Vedamuthu 1982; Ahmed et al. 2016).

Misti dahi

Mishti dahi, also called sweetened yoghurt, is a very popular Indian fermented diary product mostly in Eastern regions of India (Rao and Solanki 2007). In the organized and unorganized dairy sector *misti dahi* is produced in large quantities (Ray and Srinivasan 1972). It has creamy color, smooth texture and appealing aroma (Raju and Pal 2009). *Misti dahi* is usually prepared by milk of cow, buffalo or mixed milk, with a certain amount of sugar added (Ghosh and Rajorhia 1990). The milk is heated over low fire until it is partly condensed and produces a distinctive light cream to light brown caramel. After cooling of milk, lactic acid culture is added, and then the mixture is kept undisturbed for fermentation in earthen pots (De 1980; Aneja et al. 2002). Curdling

occurs overnight at room temperature (Ghosh and Rajorhia 1990). When curd (firm body) has been set, it is kept below 4°C and served chilled (Singh 2007). *Misti dahi* contains fat (1-12%) and cane sugar (6-25%), total solids (27-43%), non-fat milk solids (11-16%) and sucrose (13-19%) (Ghosh and Rajorhia 1987; Sarkar et al. 1992, 1996). Mixture of starter culture is preferred for commercial production of *misti dahi* such as *Lactococcus lactis, Lactococcus diaceytylactis, Lactococcus cremoris* and *Leuconostoc* spp. (Ghosh and Rajorhia 1990; Gupta et al. 2000; Paul et al. 2016).

Nunu

Nunu is a yogurt-like milk product made in Ghana and other West African regions (Akabanda et al. 2013). It is prepared by raw milk by spontaneous fermentation where the first milk is collected and kept for approximately 24 h in clay pot container and left undisturbed to ferment. The product is churned after fermentation, and then the butter is separated, leaving a thick product (Akabanda et al. 2013). *Nunu* is normally eaten alone or often combined with a fermented product called *fura* based on cereal (Akabanda et al. 2013). *Lactobacillus helveticus, Lb. plantarum, Lb. fermentum, and Leuconostoc. mesenteriodes* and some yeasts are involved during *nunu* fermentation (Akabanda et al. 2010; 2014).

Paneer

Paneer is a variety of soft cheese obtained by coagulating milk without fermentation and is consumed mostly in raw forms or other culinary dishes and snacks (Aneja 2007). Consumption and preparation of *paneer* might have originated in 75-300 CE during Kusana and Saka Satavahana periods (Mathur 1991). *Paneer* is defined as milk product obtained from milk of cow or buffalo or both, by precipitation with sour milk, lactic

acid, or citric acid (PFA 2010). *Paneer* has fat content, vitamins and minerals such as calcium and phosphorus (Kanawjia and Singh 1996; Shrivastava and Goyal 2007; Khan and Pal 2011; Bhandekar et al. 2018).

Rabri

Rabri is among the most commonly favoured condensed and sweetened dairy products with creamy white to coloured caramel, chewy texture and a pleasantly sweet flavor (Chatterjee et al. 1994). During *rabdi* preparation, fresh milk is heated to the surface until a thin layer of coagulated cream is formed (Khaskheli et al. 2008). With thin wooden stick the milk is stir occasionally, and creamy layer clotted at the pan's edge is put one over the other. The process is continued until 1/6 of the original milk remains and 5-6% of the sugar is added by weight of the original milk. The clotted cream popularly called *malai* is scraped off from the bottom of the pan and preserved in thick sweetened milk (Prasad 1997). However, *rabri* preparation processes differ from region to region, and vary in concentration as well (Prasad 1997). *Pediococcus acidilactici*, *Bacillus* spp. and *Micrococcus* spp. were reported from *rabri* (Ramakrishnan 1977; Ghayal et al. 2015).

Shrikhand

Shrikhand is an indigenous fermented dairy product prepared by lactic acid bacteria (Swapna and Chavannavar 2013). It is a semi-solid and sweetish-sour milk product traditionally prepared at home in Western India as delicious sweet dish (Mane et al. 2017). The name *Shrikhand* is derived from a "*Shikharini*" Sanskrit word (Swapna and Chavannavar 2013). *Shrikhand* preparation involves fermentation and coagulation and

is generally made from buffalo milk due to higher yields and consumer preferences (BIS 1980b). The essential raw material, *chakka*, is obtained from the acid curd by drainage of whey (Boghra and Mathur 2000). *Lactococci*, *Leuconostocs*, *Streptococci* and *Lactobacilli* have been isolated from *shrikhand* (Karche et al. 2015). The fermented dairy products play an important role in human body vitamin B complex synthesis and in stomach disease prevention, as many lactic species develop natural antibiotics (Mane et al. 2017). Studies have indicated that *shrikhand* possesses antibacterial properties against such pathogenic bacteria (Sarkar et al. 1996).

Tarag

Tarag, a fermented milk product made from cow, yak, goat, or camel milk in Mongolia, Kazakhstan , Kyrgyzstan and other Central Asian regions of Russia times (Watanabe et al. 2008; Sun et al. 2014; Liu et al. 2015). It is commonly produced by natural fermentation without any commercial starter cultures (Liu et al. 2015). To generate acidity, alcohol and flavor up to the desired level, *tarag* will be fermented at an ambient temperature of about 10 to 25°C for at least 5 to 8 d. In this way, together with the traditional *tarag*, a stable microbial phase was naturally formed, maintained, and passed down over generations (Sun et al. 2014). *Tarag* is regarded as a medicinal food (Ishii and Konagaya 2002; Hasisurong and Manglai 2003). LAB species such as *Lactococcus* and *Acetobacter* and yeasts *Saccharomyces*, *Trichosporon*, and *Kluyveromyces* have been reported from *tarag*, however, *Lactobacillus* is the predominant bacterium whereas and *Galactomyces* is the dominant yeast in *tarag* (Sun et al. 2014).

Trachanas

Trachanas is a fermented milk product made from mixture of milk and crushed wheat grain which is cooked together, dried, stored in biscuit form and consumed as thick, sweet-sour soup (Economidou 1975). *Trachanas* and *trachanas*-like products are prepared and eaten in many countries with different local dialects, such as *tarhana* in Turkey, *kishk* in Egypt, Jordan, Lebanon, Palestine and Syria, *kushuk* in Iraq, *tarkhineh* in Iran, *talkuna* in Finland and *thanu* in Hungary (Tamime and O'Connor 1995; Daglioglu 2000; Ozdemir et al. 2007; Georgala 2012), *kapestoes* or *zamplaricos* in Greek (Economidou 1975) and *trachanas* in Cyprus (Bozoudi et al. 2017). *Trachanas* is also commonly used for feeding weaned infants and young children (Economidou and Steinkraus 1977). It is usually prepared during the summertime when there are large amounts of milk and the weather is conducive to proper rapid drying (Gocmen et al. 2004). *Trachanas* has high nutritional value, low pH and a sour taste (Georgala 2012). Main fermenting species in *trahanas* are *Lactobacillus casei* subsp. *paracasei, Lb. bulgaricus Streptococcus thermophilus*, *Lactococcus lactis,* and yeast *Saccharomyces unisporus* (Economidou 1975; Bozoudi et al. 2017). LAB and yeasts play a critical role during milk acidification, and also help to form some volatile odour compounds (Carpino et al. 2010; Bozoudi et al. 2017).

Yoghurt

Yogurt is one of the widely available popular fermented milk products in Europe and America (Adams et al. 1995). Yogurt originated in Asia with name *yoghurut* (Rasic and Kurmann 1978) or *jugurt* (Adams et al. 1995) in Turkey (Oberman 1985). In yoghurt production, milk is heated to a temperature about 85°C and 95°C for 30 min, and cooled and starter couture is inoculated to make viscous flavoured yoghurt (Obi et al.

2016). *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are the predominant species present during yogurt fermentation (Robinson 1990; Sieuwerts 2016). Yeasts spp. (*Candida mycoderma, Candida krusei, Candida tropicalis*) are considered as spoilage organisms (Robinson 1990; Ayşe et al. 2019), while some bacterial strains of the slime producer *Lactococcus lactis, Lactococcus lactis* subsp. *Lactococcus lactis* var. *diacetylactis, Leuconostoc* spp. *taette* are considered as adjunct microflora (Chandan 1989; Robinson 1990; Sert et al. 2017). The pH of yoghurt is 4.2-4.3; it is the end result of a symbiotic crop of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* growing at 40-45°C temperatures (Gilliland 1985). A ratio of 1:1 of the 'rods' and 'cocci' types is considered ideal for the development of flavour and texture during yogurt preparation (Rasic and Kurmann 1978; Vedamuthu 1982; Chen et al. 2017). *Lactobacillus bulgaricus* has a higher proteolytic function than the *Streptococcus thermophilus* (Tamine and Robinson 1985). Yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. is known as probiotic bacteria (Shah 2001; Obi et al. 2016; Lisko et al. 2017). Yogurt promotes several health benefits to the consumers including probiotics and antioxidants (Shah and Jelen 1990; Aukrust and Blom 1992; Harris et al. 1992; Shah 1994; Caplice and Fitzgerald 1999; Obi et al. 2016; Lisko et al. 2017; Agustinah et al. 2019).

MATERIALS AND METHODS

MATERIALS

Media Used

Lactobacillus MRS Agar (de Man et al. 1960)

(Per 1000mL distilled water)

a. Peptone	10g
b. Beef extract	10g
c. Yeast extract	5g
d. Potassium phosphate	2g
e. Tri-sodium citrate	2g
f. Dextrose	20g
g. Tween 80	1mL
h. Sodium acetate	5g
i. Magnesium sulphate	0.58g
j. Manganese sulphate	0.28g
k. Calcium carbonate	1.5%
l. Agar	20g
m. Distilled water	1000mL

MRS broth (M369, HiMedia, Mumbai)

a. Peptone	10g
b. Beef extract	10g
c. Yeast extract	5g
d. Potassium phosphate	2g
e. Tri-sodium citrate	2g
f. Dextrose	20g
g. Tween 80	1mL
h. Sodium acetate	5g
i. Magnesium sulphate	0.58g
j. Manganese sulphate	0.28g
k. Calcium carbonate	1.5%
l. Distilled water	1000mL

Arginine hydrolysis medium (Thornley 1960)

a. Peptone	10g
b. Yeast extract	5g
c. D (+) glucose	0.5g
d. Potassium phosphate	2g

e. Magnesium sulphate	0.1g
f. Manganese sulphate	0.05g
g. Sodium acetate	5.0g
h. Tri-sodium citrate	20g
i. Tween 80	1g
j. Arginine	0.3%
k. Phenol red	0.01g
l. Distilled water	1000mL
m. pH	5

Carbohydrate fermentation medium (Schillinger and Lucke 1987)

a. Peptone	10 g
b. Yeast extract	5g
c. Potassium phosphate	2g
d. Tri-sodium phosphate	2g
e. Carbohydrate	0.5%
f. Tween 80	1mL
g. Sodium acetate	5g
h. Magnesium sulphate	0.58g

i. Manganese sulphate 0.28g

j. Phenol red 0.004%

k. Distilled water 1000ml

Sugars

a. Arabinose (RM 045, HiMedia, Mumbai)

b. Cellobiose (RM 098, HiMedia, Mumbai)

c. Dextrose (Glucose) (RM 077, HiMedia, Mumbai)

d. Glycerol (RM 101, HiMedia, Mumbai)

e. Lactose (RM 565, HiMedia, Mumbai)

f. Maltose (RM 018, HiMedia, Mumbai)

g. Melibiose (RM 106, HiMedia, Mumbai)

h. Mannitol (PT0604, HiMedia, Mumbai)

i. Raffinose (RM 107, HiMedia, Mumbai)

j. Rhamnose (RM 062, HiMedia, Mumbai)

k. Sucrose (RM201, HiMedia, Mumbai)

l. Trehalose (RM 110, HiMedia, Mumbai)

m. Xylose (RM 111, HiMedia, Mumbai)

n. Fructose (DD017, HiMedia, Mumbai)

o. Mannose (DD007, HiMedia, Mumbai)

p. Salicin (DD011, HiMedia, Mumbai)

Nutrient Agar (M012, HiMedia, Mumbai)

Nutrient Broth (M002, HiMedia, Mumbai)

Mueller Hinton Agar (M173, HiMedia, Mumbai)

McFarland Standard Set (R092, HiMedia, Mumbai)

Skim milk (RM1254, HiMedia, Mumbai)

Ringer solution (M525, HiMedia, Mumbai)

Sodium deoxytaurocholate (RM9822, Himedia, Mumbai)

Sodium Cholate (RM202, Himedia, Mumbai)

REAGENTS USED

Gram's Iodine solution	(S013, HiMedia, Mumbai)
Gram's crystal violet	(S012, HiMedia, Mumbai)
Gram's Decolourizer	(S032, HiMedia, Mumbai)
Safranin	(S027, HiMedia, Mumbai)
3% Hydrogen Peroxide Solution	(88597, Merck, New Jersey)
n- hexadecane	(RM 2238 HiMedia, Mumbai)
Nessler's reagent	(Cappuccino and Sherman 2008)
a. Potassium iodide	50g
b. Mercuric chloride (saturated)	35mL
c. Distilled water (ammonia free)	25mL
d. Potassium hydroxide (50%)	400mL
Nitrate Reduction Test Reagent	(M439S, HiMedia, Mumbai)
Barrit Reagent A	(Cappuccino and Sherman 2008)
a. α- Naphthol	5 g
b. Alcohol (absolute)	100 ml
Barrit Reagent B	(Cappuccino and Sherman 2008)
a. Potassium Hydroxide	40 g
b. Distilled water	100 ml

Nitrate Reagent A	(Cappuccino and Sherman 2008)
a. Sulphanilic acid	0.8 g
b. 5 N Acetic acid	100 ml
Nitrate Reagent B	
a. α - Napthylamine	0.6 g
b. N Acetic acid	100 ml
Gram's Crystal Violet	(S012, HiMedia, Mumbai)
Iodine solution	(M425, HiMedia, Mumbai)
a. Iodine	1.0 g
b. Potassium iodide	2.0 g
c. Distilled water	300 ml
Safranin	(RM1315, HiMedia, Mumbai)
a. Safranin	2.5 g
b. 95% ethanol	100 ml
Malachite green (5% solution)	(S020, HiMedia, Mumbai)
a. Malachite green 5.0 g	5.0 g
b. Distilled water	100 ml
Physiological Saline (0.85%)	(Feng et al. 2002)
a. Sodium Chloride	0.85 g
b. Distilled water	1000 ml
Ethanol	(MB106, HiMedia, Mumbai)
Ethidium Bromide	(RM813, HiMedia, Mumbai)
1×TAE buffer	(ML016, HiMedia, Mumbai)

Agarose	(V3125, Promega, US)
Gel loading dye	(G1881, Promega, US)
Nuclease free Water	(P1193, Promega, USA)
Sodium Hydroxide Solution	(MF8D, Merck Millipore, US)
Gotaq green Master Mix	(M7122, Promega, US)
Proteinase K	(V3021, Promega, US)
RNAse	(A7973, Promega, US)
Lysozyme	(MB098, HiMedia, Mumbai)
X-Gal	(MB069, Himedia, Mumbai)
Sodium acetate	(S2889, Merck, US)
DNA ladder (100bp)	(MBT049, HiMedia, Mumbai)
DNA ladder (1kb)	(MBT051, HiMedia, Mumbai)
Polyethylene Glycol (MW400)	(GRM3662, HiMedia, Mumbai)
Phenol:Chloroform:IsoamylAlcohol 25:24:1	(P3803, Merck, USA)
PCR Primers	(ILS, Delhi)

INSTRUMENT USED

1. Biological Incubator (Accumax, CIS-24BL, Kolkata)
2. Stomacher (Seward, United Kingdom)
3. Water Distillation unit (Riviera, 72240020, Kolkata)
4. Water Bath (RIME-1322, Remi, Mumbai)
5. Laminar Air Flow (1386, Thermo Scientific, USA)
6. UV-Transilluminator (MD-25/HD-25, Wealtec, USA)
7. Bio Spectrometer (Eppendorf, Germany)
8. Gel Doc Imaging System (Bio Rad, USA)
9. Freezer (-80^{0}C) (TSE240A, Thermo fisher, USA)
10. Freezer (-20^{0}C) (ROFV-170, Remi, Mumbai)
11. Thermal Cyclers (2720, Applied Biosystems, USA)
12. Electrophoresis Unit (Bio Rad, USA)
13. Orbital Shaker Incubator (RSB-12, Remi)
14. Analytical Weighing Balance (AX 204, Mettler, Kolkata)
15. Microwave Oven (28L, Samsung, Mumbai)
16. Hot Air Oven (Instrumentation India, Kolkata)
17. Digital pH meter (A321, Thermo Scientific, USA)
18. Centrifuge (CL21, Thermo Scientific, USA)
19. Autoclave (Instrumentation India, Kolkata)
20. Compound Microscope (EX1000, Olympus, Japan)
20. Phase contrast microscope (CKX41, Olympus, Japan
22. ABI-DNA- Sequencer (ABI 35600, HITACHI, Japan)
23. NGC Illumina-Miseq (Illumina Platform, USA)
24. Nano-DropND-1000 (Nano Drop Technologies, USA)

25. Qubit Fluorimeter	(Invitrogen, USA)
SOFT WARE USED	
Sequence Scanner	(Applied Biosystems-V1.0, USA)
ChromasPro	(Technelysium-V1.34, Australia)
MEGA 7	(Pennsylvania State University) Vl.7.0.26, USA)
PAST	(Palaeontological Association-V4.0, Norway)
QIIME	(University of Colorado- V2-2019.10, USA)
PICRUST	(Dalhousie University, V2-2019,Canada)
GRAPHPAD PRISM	(GraphPad software, Inc-V8.0.2, USA)
REFERENCE STRAINS	**Accession no.**
Lactobacillus plantarum	MTCC 1407(T)
Lactococcus lactis subsp. *lactis*	MTCC 440
Leuconostc mesenteroides subsp. *mesenteroides*	MTCC 867
Lactobacillus plantarum	MCC 2034
Lactobacillus plantarum subsp. *plantarum*	MTCC 2974
Lactobacillus fermentum	MTCC 2760

Lactobacillus brevis	MTCC 2198(T)
Enterococcus faecium	MTCC 2763
Escherichia coli	MTCC 2413
Staphylococcus aureus subsp. *aureus*	MTCC 740
Bacillus cereus	MTCC 1272
Salmonella enteric subsp. *enterica* ser. *typhimurium*	MTCC 3223

MTCC: Microbial Type Culture Collection, Chandigarh; MCC: Microbial Culture Collection, Pune, India.

METHODOLOGY

Survey

Documentation of the traditional preparation of naturally fermented milk (NFM) products was based on a survey conducted in different households through face to face interactions/interviews with the help of structured questionnaire (Table A) with different ethnic communities of Sikkim from different places viz. Aritar, Tarku, Thingling, Entel, Yumesamdong, Lachung, Chongri, Yangang, Pakyong, Martam and 32 miles of Sikkim. Households were chosen only from locations where established NFM products are known to be available. During survey, various criteria were sought e.g. milk source/origin, temperature and materials used, period of fermentation, sensory properties, culinary, mode of consumption, socio-economy.

Table B: Questionnaire on Naturally Fermented Milk Products in Sikkim

I. General information **Date**:

1. Name of the Informant:
2. Ethnic group:
3. Name of:
    a. Village /Revenue:
    b. Sub-division:
    c. District:
4. Approximate number of house hold:
    a. House hold in village:
    b. Population of village:
5. Distance of the village from
    a. Nearest market (km):
    b. Nearest town (km):

II. Information on products:

6. Local name:
7. Type and source of milk used:
8. Flow sheet of traditional methods of preparation:
9. Taste, texture, colour and nature:
10. Culinary/Mode of consumption:
11. Time of storage:
12. Any socio-ethnical importance of this product?
13. Are you economically dependent on this product? Yes/No
14. What is the approximate amount of monthly/annual production of this product?
15. What is the approximate income from the sale of this product? (monthly)

Remarks:

Name and signature of investigator:

Collection of Samples

A total of 22 different traditionally prepared naturally fermented milk (NFM) products viz. *dahi* (cow-milk), *dahi* (yak-milk), soft *chhurpi* (cow-milk), soft *chhurpi* (yak-milk), *mohi* (cow-milk), hard *chhurpi* (yak-milk), and *philu* (yak-milk) were collected from different places of Sikkim in pre-sterile sample bottles, kept in ice-box carrier and transported to laboratory and stored at -20^{o}C for further analysis.

Determination of pH

Ten g of the sample was combined with 20 ml of carbon-dioxide-free distilled water in a homogenizer for 1 min and the slurry pH was measured directly using an automated pH meter optimized with standard buffer solutions.

CULTURE DEPENDENT ANALYSIS

PHENOTYPIC CHARACTERIZATIONS

Microbiological Analysis

Ten g of sample was homogenized with physiological saline (0.85%) using stomacher at 200 rpm for 3 min. Serial dilution (10^{-1} to 10^{-8}) was made by following the method of (Dewan and Tamang 2007). One ml of homogenized mixture was transferred into MRS agar plate with (1%) $CaCo_3$ by pour plate method and incubated in anaerobic jar for 48 h at 30°C. Selected colonies were further sub-cultured twice in MRS agar. Glycerol (30%) was prepared for the preservation of pure culture and stored at -80°C.

Colony morphology

Initially, all the pure culture plates containing the isolates were tested for their colony's morphology (Harrigan 1998). On a clean, grease-free slide, a loopful of a colony was

then picked from the 24 h old bacterial culture plate and smear was prepared. After air-drying, the smear was stained with safranin, washed with running water, air-dried and examined under the oil immersion.

Gram's staining

A suspension of 24 h old bacterial cultures was taken and smears were made on grease-free slides. The smears were then heat fixed, flooded by crystal violet, allowed to react for 1 min, and washed for 5 sec with water. The smear was then flooded with iodine solution for 1 min, and washed again with water. Holding the slides against a white surface, 95% ethanol was poured drop by drop from the top edge of the slide until no more colour is drained from the lower edge of the slide. After washing with water, the smear was counter-stained with safranin for 45 sec and washed again with water. The slide was then air-dried and observed under oil immersion objective of the bright field microscope (Cappuccino and Sherman 2008).

Catalase production

This test was performed by preparing clean grease free slide in which few drops of 3% H_2O_2 was added. After this, a loopful of the culture was mixed with the previously added hydrogen peroxide (H_2O_2). The production of gas bubbles were observed as described by Schillinger and Lucke (1987). The production of gas bubbles indicated the presence of catalase, while absence of gas bubble indicated absence of catalase.

Gas (CO_2) production from glucose

In order to distinguish whether the lactic acid bacteria (LAB) isolates were homofermentative or heterofermentative, CO_2 production from glucose was checked (Schillinger and Lucke 1987). Tubes of 5 mL MRS broth containing inverted Durham's

tube was inoculated with 24 h old cultures and incubated at 30°C for 24 to 48 h. Accumulation of gas in the inverted Durham's tubes indicated positive result whereas no gas production indicates negative result.

Ammonia from arginine

The arginine hydrolysis test was done following the method of Schillinger and Lucke (1987) and carried out in MRS broth. Ammonia was detected using Nessler's reagent. Appearance of dark orange colour indicated the presence of ammonia.

Growth at different pH

The growth of LAB at different pH was measured by following method of Schillinger and Lucke (1987). The pH of MRS broth was adjusted to 3.9 and 9.6 using 1 N HCl and 10% w/v NaOH, respectively. The broth was filter sterilized, added 5 mL to each tube and inoculated with 24 h old broth culture. The tubes were incubated at 30°C and growth was observed after 48-72 h of incubation.

Growth at different temperatures

The growth of LAB at different pH was measured by following method of Schillinger and Lucke (1987) and Choksi and Desai (2012). Tubes containing MRS broth were inoculated with the isolates from the culture plate, incubated at 10°C, 15°C and 45°C and growth was observed after 48-72 h of incubation.

Growth in NaCl concentrations

Salt tolerance of LAB isolates were tested by inoculating a loop-full of culture in 4 ml MRS broth supplemented with 6.5% NaCl and incubated for 48 h at 30°C. Cultures were observed for growth after incubation (Choksi and Desai 2012).

Fermentation of sugars

Tubes of 4 mL MRS broth without beef extract, containing 0.5% w/v of different sugars instead of glucose and 0.004% phenol red were inoculated, except for the control tube and incubated at 30^{o}C for 48 h. Colour change from red to yellow indicated acid production (Schillinger and Lucke 1987). Different sugars such as lactose, maltose, glucose, fructose, cellobiose, mannose, rhamnose, melezitose, raffinose, ribose, xylose, sucrose, arabinose, trehalose, melibiose, salicin and mannitol were used.

Tentative Identification

Tentative identification upto genus levee of LAB isolates was performed on the basis of phenotypic and biochemical tests following the keys of Schillinger and Lucke (1987) and Holzapfel and Wood (2012).

MOLECULAR IDENTIFICATION OF LAB ISOLATES

Genomic DNA extraction

Lysozyme-mutanolysin heat lysis method was used to isolate bacterial genomic DNA (Jeyaram et al. 2010). The 5 ml of MRS culture broth incubated at 30°C for 48 h was taken and centrifuge at 8000 *g* for 5 min. Cell pellets were washed twice in 1ml of 0.5 M NaCl solution by resuspending and centrifuging. Again the, cell pellet was washed twice by re-suspending and centrifuging 1 ml of sterile Milli Q water. The washed cell pellet was resuspended at 500 µl of 1X TE buffer (pH 8) [10mM Tris-Cl, pH 8, 1mM EDTA, pH 8]. The 10 µl of 1 mg/ml Lysozyme solution or 10 µl of 1U/µl Lysozyme and 10 µl of 1U/µl Mutanolysine were added and thoroughly mixed with vortex. The mixture was incubated for 30 min at 37°C and 20 min at 95-99°C and then centrifuged

at 4°C at 10,000 *g* for 10 min. The supernatant was collected, and the quality of its DNA was measured using NanoDrop at OD260. The cell-free DNA lysate was stored at -20°C for further use.

16S rRNA sequence amplification

Polymerase chain reaction amplification targeting 16S rRNA gene sequences was performed (Romi et al. 2015). Universal primer 27F-AGAGTTTGATCCTGGCTCAG and 1492R-TACGGTTACCTTGTTACGACTT was used to amplify the sequences. PCR amplification was carried out in a final volume of 25 µl, the mixture containing Go green Taq master mix (1x) (NEB), 10µM of F primer, 10µM of R primer and nuclease-free water (NEB). PCR reaction program was set under the following PCR conditions: 94°C for 10 min; 94°C for 1 min, 65°C for 1 min and 72°C for 30 s for 35 cycles; and 72°C for 7 min. The amplified PCR amplicons were separated in 0.8% agarose gel (w/v) containing ethidium bromide (0.5µg/ml) in TAE buffer (1x) (Tris-Cl, acetic acid and EDTA) and run at 80V for 1h. The band was observed under Gel-Doc. Standard 1 kb base pair DNA ladder was used for the verification of amplicon size.

Purification of PCR products

Purification of amplified PCR product was performed by PEG (polyethylene glycol)-NaCl (sodium chloride) precipitation method with few modifications (Schmitz and Riesner 2006). Initially, 0.6 volume of 20% PEG-NaCl was added to the final volume of PCR products, vortex gently and incubated at 37°C for 20-30 min. After centrifugation at 12000 rpm for 30 min, aqueous solution was discarded by using sterile pipette. Pellet was washed twice with freshly-prepared ethanol (70%) by centrifugation at 12000 rpm for 30 min. The collected pellet was then air-dried over-night. Lastly,

20ul of nuclease-free water was added and the purified product was loaded in 1% agarose gel.

PCR amplification and sequencing of 16S rRNA gene

PCR reactions were set up for 5µl volume for single primer amplification with 1492R and 27F primers used (Lane 1991). PCR reaction was set as follows: denaturation for (96^{o}C, 10 sec), annealing (50^{o}C, 5 sec), elongation (60^{o}C, 2 min) with a stop reaction at 4^{o}C. The amplicons were then precipitated with 1µl sodium acetate (3M, pH 5.2) and 24µl of absolute alcohol, mixed briefly in Vortex and incubated at room temperature for 15 min. The tubes were then spun at 12,000 rpm for 20 min, further washed with 70% ethanol and air-dried. The air-dried samples were then suspended in 10µl formamide. Sequencing was performed in DNA Analyzer.

Phylogenetic analysis

Raw reads were checked for their quality by using Sequence Scanner software v.1.0 and good read sequences were then assembled (contiq formation) using ChromasPro v.1.34 (McCarty 1998). Chimera sequences were then checked by using Mallard v.1.0. BLAST (basic local alignment search tool) 2.0 program was performed using GenBank NCBI (National Center for Biotechnology Information) (Nucleotide BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon (https://www.ezbiocloud.net/) server for their identity. The phylogenetic tree was constructed by Neighbor-joining method (Saitou and Nei 1987) and created by using MEGA.7 software. The bootstrap consensus tree derived with 1000 replicates to Neighbor-joining method and Kimura 2-parameter. Numbers on branches depict the percent occurrence of a given branch during 1000 replicates. The assembled quality-checked sequences were then deposited

in NCBI GenBank for their accession number. Diversity indices were calculated using PAST (PAleontological STatistics) v.3.25 software (Hammer et al. 2001).

CULTURE-INDEPENDENT METHOD

Sampling

A total of 19 samples of NFM products were collected from different places of Sikkim for high-throughput sequencing studies viz. soft *chhurpi* (cow-milk) (8 samples), soft *chhurpi* (yak-milk) (4), *gheu* (yak-milk) (3), and *dahi,* (cow-milk) (3), and *dahi* (yak-milk) (1). The products were collected aseptically from the conventional production centres, placed in an ice-box and transported for examination to the laboratory and stored at -20°C for further analysis.

Metagenomic DNA extraction

The extraction of metagenomic DNA was characterized by two different methods, based on the nature of the samples. For *gheu/mar* (fat-rich) samples, DNA extraction was performed as per the method described by Keisam et al. (2016) with some modifications. Five gram of the sample was melted in low temperature and 2 ml was homogenized with 2ml of citrate buffer (2%). The 4 ml of petroleum ether:hexane (1:1) was added to the mixture and vortex and incubated for 10 min at room temperature. The 2 ml of the lower part of the mixture was transferred to a sterile 2 ml screw-cap tube containing 0.5 g of zirconia/silica beads (0.1 mm) and 4 glass beads (2 mm). The mixture was centrifuged for 10 min at 1400 × *g* at 4°C and the pellet was resuspended in 150 µl proteinase-K buffer [50 mM Tris-Cl, 10 mM EDTA (pH 8), 0.5% (w/v) SDS]. 25 µl of proteinase K (25 mg/ml) was added to the mixture and incubate

overnight at 65°C. The 150 µl of 2X breaking buffer [4% Triton X-100 (v/v), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris (pH 8), 2 mM EDTA (pH 8)] was added to the mixture. Mechanically, the cell lysis was achieved by mixing in a bead beater at maximum speed with an interval of 10 sec for 1 min. The pellet was washed twice with a mixture of chloroform:isoamyl alcohol (24:1), with centrifugation at 4°C for 10 min at 14000 x *g* per time, and eluted with absolute ethanol. This continues with twice washing with 70% ethanol and precipitate at 4°C for 15 min at 14000 x *g* after each wash by centrifugation. Lastly, the pellet was air-dried overnight and after drying the pellet was dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA).

On the other hand, casein-based samples (*dahi,* soft *chhurpi* and hard *chhurpi*), their metagenomic DNA was extracted as per the method described by Keisam et al. (2016). The 10 g of the samples were mixed in 90 ml 2% sodium citrate buffer and homogenized in a stomacher at 200 rpm for 2 min. Samples of hard *chhurpi* (hard cheese) were grinded to powder and homogenized but *chhurpi* (soft-cheese) samples were homogenized directly. In case of *dahi*, 10 mL of sample was mixed in 90 ml 2% sodium citrate buffer and homogenized in a stomacher at 200 rpm for 2 min before extraction. 1.5 mL of the homogenate was taken to a sterile centrifuge tube and centrifuge for 10 min at 18000 × *g*. TES buffer [50 mM Tris, 1 mM EDTA, 8.7% sucrose] was added to the pellet. Cell lysis was achieved by enzymatic action of lysozyme (50 KU/100 µl TES), in combination with mutanolysin (5 U/µl) and 20 U of lyticase (2 µl of 10 U/µl) in incubation time at 37°C for 1 h. Proteinase-K (25 mg/ml) was added to the mixture and incubated at 65°C for 1 h which was followed by addition of GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% sarkosyl) and cool on ice for 5 min. The pellet was precipitated by addition of 7.5M ammonium acetate which was followed by chloroform: isoamyl alcohol (24:1) and centrifugation at

15000×g for 10 min at 4ºC. Isopropanol was then added to the aqueous layer, mixed gently and stored at -20°C for 1 h which was followed by centrifugation at 4°C for 15 min at 15000 × *g*. The pellet was washed twice with 70% ethanol and DNA was precipitated by centrifuging at 4°C for 15 min at 15000 × *g*. Finally, the pellet was dissolved in TE buffer after drying. The DNA extraction kit and laboratory prepared reagents were tested for the presence of contaminant DNA by DNA extraction on blank water (sterile ultrapure) before use. After confirming the negative PCR amplification (using microbial specific primers) from the above extract, the kit and reagents were used for DNA extraction from the samples. The DNA recovery was quantified fluorometrically using Qubit dsDNA HS assay kit in Qubit 2.0 fluorometer), and the quality (A260/280) was checked spectrophotometrically as described above. DNA was stored at -20°C until required.

Barcoded Illumina MiSeq Sequencing

For in-depth bacterial community analysis, barcoded Illumina MiSeq amplicon sequencing targeting the V4-V5 region of the 16S rRNA gene was conducted as described earlier (Romi et al. 2015). The forward primer F563–577 (5'-AYTGGGYDTAAAGNG-3') and barcoded reverse primers R924–907 (5'-CCGTCAATTCMTTTRAGT-3') with an 8 bp barcode in its 5'-end was used for sample multiplexing (Romi et al. 2015). Each PCR reaction was performed in a total volume of 25µl with a template-free reaction that acts as a control. The following PCR conditions were used for amplification- initial denaturation (98 °C for 5 min); denaturation (98 °C for 15 sec), annealing (55 °C for 30 sec) and elongation (72 °C for 30 sec). The PCR reaction was run for 28 cycles with a final extension process of 72 °C for 5 min. The 430 bp sized products were separated in a 1.5% agarose gel (w/v) and

the target bands were carefully excised from the gel with a sterile scalpel blade and then purified using QIAquick gel extraction kit as per the manufacturer's instructions. The purified DNA was quantified with Qubit dsDNA BR Assay Kit in a Qubit 2.0 fluorometer and the individual were samples pooled in equimolar proportions. Amplicons were purified using AMPure XP beads to remove unused primers. Sequencing libraries were prepared using additional 8 cycles of PCR with Illumina barcoded primers with a read length of 2 x 300 bp and finally sequencing was run in an Illumina-MiSeq platform.

Bioinformatics

The raw sequence reads obtained was analysed using the default settings in MG-RAST (Meyer et al. 2008) and an open-source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME) v1.8.0 (Caporaso et al. 2010). A total of 27,01679 post-quality filtered sequences originating from 19 samples belonging to 3 food types of NFM samples were uploaded to MG-RAST server. Quality-filtered joined reads such as chimera, singleton and short sequences were denoised using deblur algorithm (q2-deblur denoise-16S) (Amir et al. 2017) against a positive filter (Greengenes 13_8) and the resulting sub-operational-taxonomic-unit (sOTUs) were then aligned with multiple alignment using fast Fourier transform (mafft) (Katoh et al. 2002) (via q2-alignment). Taxonomy assignment was performed as per the default automatic pipelines present in the MG-RAST server which generated the Operational Taxonomic Units (OTU) table used for downstream analyses (Meyer et al. 2008). OTU similarities were analyzed at four taxonomic levels- Phylum, Family, Genus and Species level against SILVA (SSU) database (Quast et al. 2012).

Predictive functionality using PICRUSt2

Quality-filtered sequences were clustered against Greengenes v13_8 databases before feeding into PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, version 2) algorithm, using via q2-vsearch-cluster-features-closed-reference (Rognes et al. 2016). PICRUSt2 deduces the predictive functionality of the marker gene by using a standard integrated genomes databases. Firstly, multiple assignment of the amplicon sequence variants (ASVs) was done using HMMER (Howard Hughes Medical Institute 2018). Placements of ASVs in the reference tree with evolutionary placement-ng (EPA-ng) algorithm (Barbera et al. 2019) and Genesis Applications for Phylogenetic Placement Analyses (GAPPA) omics (Czech and Stamatakis 2019) were applied. Prediction of gene families is run using a default castor R package (Louca et al. 2018) with the default algorthim run (maximum parsimony). Metagenome prediction was run using (metagenome_pipeline.py) (Ye and Doak 2009) and the output features were mapped into KEGG (Kyoto Encyclopaedia of Genes and Genomes) database for systematic analysis of gene functions (Kanehisa and Goto 2000). Statistical analysis was applied for hypothesis testing using Paired t-test and STAMP (statistical analysis of metagenomic profiles) software by ANOVA (analysis of variance) hypothesis testing method (Parks et al. 2014).

Statistical Analysis

Significant differences (ANOVA, Student paired two-tailed test) amongst the samples were checked at different taxonomic levels (phylum, family, genus, and species) in accordance with their respective relative abundance (Kim 2014). Comparison of the food types was evaluated for variation by multivariate PCA using Canoco software v4.52 (Jolliffe and Cadima 2016). Normalisation was performed by using log

transformation log10 (xi+1). Significant difference in the microbial community structure amongst the four food type was evaluated by Analysis of Similarities (ANOSIM) with 10,000 permutations using Bray-Curtis similarity index by PAST software (Anderson 2017). Generation of rarefaction curves of alpha diversities were calculated as per the quality-filtered OTU table at the species level for richness estimates (Chao1), diversity indices (Fisher alpha, Shannon), evenness (Shannon's equitability) and sample coverage (Good's coverage) using the multiple_rarefactions.py script in QIIME v1.8.0 bioinformatics pipeline (Caporaso et al. 2010). The significant difference between each method in the alpha diversity indices were calculated using the compare_alpha_diversity.py script in QIIME.

SCREENING OF SOME PROBIOTIC PROPERTIES

Acidification and coagulation

Acidification and coagulation of ability of LAB was analysed by method of Olasupo et al. (2001). Overnight LAB cultures were inoculated in 10% of skim milk at level (1%) and incubated at 30°C for 72 h. After incubation pH and clotting formation were measured.

Lysozyme Tolerance

Lysozyme tolerance was evaluated by following the method of Zago et al. (2011). The LAB isolates were incubated overnight in MRS broth at 37°C. Overnight grown cells were harvested by centrifugation at 7000 rpm, 4°C at 10 min. The cells were washed twice with PBS and pellets were re-suspended in Ringer solution. 100ul suspension was inoculated into sterile electrolyte solution ($CaCl_2$ 0.22g/l; NaCl 6.2g/l; KCl 2.2g/l; $NaHCO_3$; 1.2 g/l) with 100mg/l lysozyme. For control, solution without Lysozyme was taken and incubated at 37°C. After a time interval of 0 h and 2 h sample was withdrawn and dilution was made in phosphate buffer saline (PBS). 100ul of active culture was inoculated on MRS agar plates and incubated at 37°C for 48 h. Cell viability was assessed by the plate count method and the results were expressed as log cfu/ml.

Bile Tolerance

Bile salt tolerance was assessed by following the method of Ramos et al. (2013). The LAB isolates were incubated overnight in MRS broth at 37°C. Pellets of cells were centrifuged at 7000 rpm, 4°C for 10 min. The pellets were washed with PBS and

resuspended with 0.3% bile in the MRS medium. The MRS broth without bile was kept as control. After inoculation, samples were incubated at 37°C. After a time interval of 0 and 2 h samples were withdrawn and serially diluted using normal saline. Viable cell colonies were enumerated at 0 and 2 h by plating 100µl of cultures of appropriate dilutions onto MRS agar and the results were expressed as log cfu/ml.

Acid tolerance

The LAB isolates were incubated overnight in MRS broth at 37°C. Actively grown cells were harvested by centrifugation (7000 rpm, 4°C for 10 min). The MRS broth was adjusted at pH 3.0 with 1N HCl (Ramos et al. 2013). MRS broth adjusted to pH 6.5 was used as a control. Harvested cell pellets were resuspended in MRS broth with acidic pH and incubated at 37°C. After a time interval of 0 and 2 h samples were withdrawn and serially diluted in phosphate buffer saline (PBS). Samples were placed on MRS agar plates and incubated at 37°C for 48h. Cell viability was assessed by the plate count method and the results were expressed as log cfu/ml.

Bile salt hydrolase activity

The BSH activity of selected isolates were analysed by following the protocol of Nguyen et al. (2007). Active LAB cultures were streaked on MRS agar plate supplemented with 0.37g/l $CaCl_2$ and 0.5% (w/v) bile salts (sodium taurocholate, sodium cholate and sodium tauroglycocholate). Streaked plates were incubated at 37°C in an anaerobic jar for 2-3 days. BSH activity was indicated by observing precipitation zones of hydrolyzed salts around the colonies.

Hydrophobicity assay

Fresh cultures were grown in MRS broth at 30°C for 24 h and cell pellets were harvested by centrifugation at 8,000 g for 5 min. The pellets were washed with 9 ml of Ringer solution and thoroughly mixed. The absorbance of the suspension at 580 nm was also measured. 1.5 ml of suspension was mixed in duplicates and thoroughly mixed with an equivalent amount of n-hexadecane. Phases were allowed to separate at room temperature for 30 min, after which aqueous phase was carefully transferred to a new tube and the absorbance was measured at 580 nm (Shangpliang et al. 2017). The percentage hydrophobicity is expressed as follows:

Hydrophobicity % = $[A_0 - A/A] \times 100$;

Where A_0 and A, are the absorbance values of the aqueous phase before and after contact with n-hexadecane.

Antagonistic Activity of LAB

Antimicrobial activity of LAB isolates against certain pathogenic microorganisms was evaluated using a method of agar well diffusion (Ridwan et al. 2008). Tested strains were *E.coli* MCC 2413, *Salmonella enteric* subsp *enteric* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272. A 100µl of tested strains were spread by sterile cotton swab on the surface of Muller Hilton Agar (MHA) plates and wells were made using borer. A 100ul of overnight grown LAB cultures were poured into the wells and allowed to dry for 15 min. Plates were incubated at 30°C and zone of inhibition was measured after 24-48 h of incubation (Yadav et al. 2016).

Beta-galactosidase activity

For β-galactosidase activity was assessed by method of (Sharma and Sing 2014). Overnight grown cultures were streaked on MRS agar plates containing 20µl of X-Gal (5-bromo-4-chloro-3-indole-D-galactopyranoside; 20 mg/ml of DMSO). The plates were incubated at 30°C for 24-48 h. Blue colonies were observed on the plates after incubation, indicating the presence of β-galactosidase enzyme.

Detection of probiotic marker genes by PCR amplification

Molecular detection of the probiotic markers was also performed by following the protocol (Archer and Halami 2015; Turpin et al. 2011). The primer used in the present study was given in (Table B). PCR amplification was carried out in a final volume of 10 ul, the mixture containing Go green Taq master mix (1x) (NEB), 10µM of F primer, 10µM of R primer, nuclease-free water (NEB) and 50 ng of template DNA. PCR reaction program was set under the given conditions (Table B). The amplified PCR products were separated on an agarose gel (0.8%) containing ethidium bromide (0.5ug/ml) in TAE buffer (1x) (Tris-Cl, acetic acid and EDTA) and run at 80V for 1 h. The band was observed under Gel-Doc.

Table C: Primers used for probiotic gene detection

Gene	Sequences F:5'-3' R:3'-5'	Annealing temperature	Size	PCR reaction	References
Ent A	F- GGTACCACTCATAGTGGAAA R- CCCTGGAATTGCTCCACCTAA	55°C	138 bp	94°C for 5 min; 94°C for 30 sec; 55°C for 10 sec; 72°C for 40 sec; 72°C for 5 min	Ozdemir et al. (2011)
Ent B	F- CAAAATGTAAAAGAATTAAGTACG R- AGAGTATACATTTGCTAACCC	56°C	201 bp	94°C for 5 min; 94°C for 30 sec; 56°C for 10 sec; 72°C for 40 sec; 72°C for 5 min	De Vuyst et al. (2003)
Ent P	F- GCTACGCGTTCATATGGTAAT R-TCCTGCAATATTCTCTTTAGC	55°C	87 bp	94°C for 5 min; 94°C for 30 sec; 55°C for 10 sec; 72°C for 40 sec; 72°C for 5 min	Ozdemir et al. (2011)
mapA	F-TGGATTCTGCTTGAGGTAAG R-GACTAGTAATAACGCGACCG	50°C	156 bp	95°C for 5 min; 95°C for 30 sec; 50°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Ramiah et al. (2007)
mub1	F- GTAGTTACTCAGTGACGATCAATG R- TAATTGTAAAGGTATAATCGGAGG	50°C	150 bp	95°C for 5 min; 95°C for 30 sec; 50°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Ramiah et al. (2007)
apf	F- YAGCAACACGTTCTTGGTTAGCA R- GAATCTGGTGGTTCATAYWCAGC	53°C	112 bp	95°C for 5 min; 95°C for 30 sec; 53°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Goh and Klaenhammer (2010)

BGL-1	F-GTGACTATGGTAGAGTTTCC R-TCAAAACCCATTCCGTTCCCCA	50°C	1392 bp	94°C for 5 min; 94°C for 1 min; 50°C for 40 sec; 72°C for 1.2 min; 72°C for 10 min.	Spano et al. (2005) Mtshali et al. (2010)
Nisin	F-CTATGAAGTTGCGACGCATCA R-CATGCCACTGATACCCAAGT	41°C	898 bp	92°C for 5 min; 92°C for 2 min; 41°C for 40 sec; 72°C for 2 min; 72°C for 10 min	Rodriguez et al. (1995)
Lactococcin A	F-TCTGCACTCACTTCATTAGTTA R-AAGGTAATTACACCTCTTTTAT	38°C	771 bp	92°C for 5 min; 92°C for 2 min; 38°C for 40 sec; 72°C for 2 min; 72°C for 10 min	Martinez et al. (1998)
Lacticin 481	F-CAATCAGTAGAGTTATTAACATTTG R-GATTTAAAAAGACATTCGATAATTAT	51°C	366 bp	92°C for 5 min; 92°C for 2 min; 51°C for 40 sec; 72°C for 1 min; 72°C for 10 min	Martinez et al. (1998)
Enterocin AS-48	F-GAGGAGTITCATGITTAAAGA R-CATATTGTTAAATTACCAAGCAA	42°C	318 bp	92°C for 5 min; 92°C for 2 min; 42°C for 40 sec; 72°C for 2 min; 72°C for 10 min	Joosten et al. (1997)
bsh	F-GGTTGGTCGGCCAGTTCTTT R-CCAACATGCCCAAGTTCGAC	58°C	205 bp	94°C for 5 min; 94°C for 1min; 58°C for 1 sec; 72°C for 1min; 72°C for 10 min	Turpin et al. (2011)
fbp	F-AGTGCTGAAATYATGGGAAGA R-AATTGTCCACCTTGTTGCTG	60°C	835 bp	94°C for 5 min; 94°C for 1 min; 60°C for 1 sec; 72°C for 1 min; 72°C for 10 min	Archer and Halami (2015)
sor	F-CCACCTTGTACTGGTTAGTG R-GACCATTCGTGTACTTGCCG	55°C	672 bp	94°C for 5 min; 94°C for 1 min; 55°C for 1 sec; 72°C for 1 min; 72°C for 10 min	Archer and Halami (2015)

sbp	F-CAGTTCTTAGCCACAGTTTG R-GGTTCGCCGCTAATAGTAAG	55°C	805 bp	94°C for 5 min; 94°C for 1 min; 55°C for 1 sec; 72°C for 1 min; 72°C for 10 min	Archer and Halami (2015)
msa	F-GCGATTAGGGGTGTGCAAG R-GCAGTTGGTGACGTAGGCA	55°C	319 bp	94°C for 5 min; 94°C for 1 min; 55°C for 1 sec; 72°C for 1 min; 72°C for 10 min	Archer and Halami (2015)
agu A	F-GAACGACTAGCAGCTAGTTAT R-CCAATAGCCGATACTACCTTG	60°C	542 bp	95°C for 5 min; 95°C for 30 sec; 60°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Lucas et al. (2007)
clp L	F-GCTGCCTTYAAAACATCATCTGG R-AATACAATTTTGAARAACGCAGCTT	50°C	158 bp	95°C for 5 min; 95°C for 30 sec; 50°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Wall et al. (2007)
Ir1516	F-TRACCACTYTCWCCATTCAACAA R-CCACTAGCRATGACYAATACKGGTT	56.5°C	143 bp	95°C for 5 min; 95°C for 30 sec; 56.5°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Wall et al. (2007)
tdc	F-CCACTGCTGCATCTGTTTG R-CCRTARTCNGGNATAGCRAARTCNGTRTG	50°C	370 bp	95°C for 5 min; 95°C for 30 sec; 50°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Costantini et al. (2006)
LBA1446	F-GCTGGAGCCACACCGATAAC R-CAACGGGATTATGATTCCCATTAG	58°C	275 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Pfeiler et al. (2007)

	T				
groEL	F- TTCCATGGCKTCAGCRATCA R- GCTAAYCCWGTTGGCATTCG	58°C	168 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Ljungh and Wadstrom (2009)
hdc	F- AGATGGTATTGTTTCTTATG R- AGACCATACACCATAACCTT	52°C	367 bp	95°C for 5 min; 95°C for 30 sec; 52°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Costantini et al. (2006)
odc	F-TMTWCCAACHGATCGWAATGC R-CRCCCCAWGCACARTCRAA	58°C	245 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Costantini et al. (2006); Azcarate-Peril et al. (2004)
LBA1272	F-GGCTTACCAATGGCCACCTT R-GATCAAAAAGCCGGTCACGA	57.5°C	210 bp	95°C for 5 min; 95°C for 30 sec; 57.5°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Klaenhammer et al. (2005)
dlt D	F-TTCGCCTGTTCAAGCCACAT R-ACGTGCCCTTCTTTGGTTCC	58°C	283 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011)
La995	F-AACGAAGGTCCCGACAAAGG R-ACGACCTTCGGGCTGGTTAC	57.5°C	246 bp	95°C for 5 min; 95°C for 30 sec; 57.5°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Azcarate-Peril et al. (2004)
lr0085	F-RCTTTGACCGRTGGGGCTRT R-NNNATGGCCGCATGGAAA	57.5°C	150 bp	95°C for 5 min; 95°C for 30 sec; 57.5°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Whitehead et al. (2008)
lr1584	F-TAYGCCRTTCGGWTGTTTGG	55.5°C	151 bp	95°C for 5 min; 95°C for 30 sec; 55.5°C for 10 sec; 72°C for 15 sec;	Turpin et al. (2011);

	R-TCAWRATGGCRGTCCCAATG			72°C for 5 min	Whitehead et al. (2008)
LBA0552	F-GTGATTGCCCTAGCCCTGGT R-GATCCCGATCACGATGCAAG	58°C	180 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Pfeiler and Klaenhammer (2009)
LBA1429	F-AATTTCAGGATGCCCCGGTA R-CCAAGCTCCCAACAATGCAC	58°C	196 bp	95°C for 5 min; 95°C for 30 sec; 58°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Turpin et al. (2011); Pfeiler et al. (2007)
mes Y	F-ATGACGAATATGAAGTC R-TTACCAAAATCCATTTCC	45°C	182	95°C for 5 min; 95°C for 30 sec; 45°C for 10 sec; 72°C for 15 sec; 72°C for 5 min	Abriouel et al. (2008)

F: forward; R: reverse sequence

DATA AVAILABILITY

Data availability of 16S rRNA sequencing

The partial sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-National Centre for Biotechnology Information (NCBI) under the nucleotide accession number: MK574836, MK574857, MK290329, MK290330, MK290345, MK583513, MK574837, MK290369, MK290334, MK290344, MK574844, MK290357, MK290356, MK290359, MK574845, MK290343, MK290341, MK290358, MK290354, MK290355, MK290332, MK574841, MK290333, MK290360, MK290361, MK290362, MK574842, MK290331, MK574843, MK290352, MK574838, MK290347, MK574839, MK290348, MK574840, MK290349, MK290346, MK290350, MK290351, MK290353, MK574846, MK290371, MK574847, MK574848, MK574849, MK290372, MK574850, MK290370, MK574851, MK574852, MK290363, MK290364, MK574853, MK290368, MK796023, MK574854, MK290365, MK290367, MK290366, MK290338, MK290342, MK290339, MK290340, MK290337, MK290336, MK290335, MK574855 and MK574856.

Data availability of High-throughput sequencing result

The sequence data of high-throughput amplicon sequencing has uploaded at MG-RAST server with the MG-RAST ID number 4732364.3; 4732366.3; 4732367.3; 4732374.3; 4732375.3 4732376.3; 4732378.3; 4732382.3; 4732387.3; 4732388.3; 4732391.3; 4732397.3; 4732400.3; 4732401.3; 4732402.3; 4732405.3; 4732406.3; 4732410.3; 4732412.3.

RESULTS

DOCUMENTATION OF NFM PRODUCTS OF SIKKIM

A survey was conducted for documentation of traditional method of preparation of naturally fermented milk products. Questionnaire was prepared to collect an information on traditional methods of preparation, culinary, mode of consumption and socio-economy of various naturally fermented milk (NFM) products of Sikkim (Table A). The preparation methods of NFM products differ from place to place. In Sikkim, the ethnic naturally fermented milk products are prepared either from milk of cow or yak. Milk of buffalo and goat is uncommon in Sikkim. The different types of NFM products of Sikkim are shown in Table 1 and Fig. 1.

Table 1: Naturally fermented milk products of Sikkim

Milk Products	Animal's milk	Sensory property and edibles	Major consumer	Region/District
Chhurpi (soft)	Cow/yak milk	Soft, cheese-like, curry and pickle	All	All
Chhurpi (hard)	Cow/yak milk	Hard-mass; masticator	All	All
Chhu/Sheden	Cow/yak milk	Soft; strong-flavoured, dish	Bhutia, Sherpa, Lepcha	North and East Sikkim
Philu	Cow/yak milk	Cream; fried curry with butter	Bhutia, Sherpa	North and East Sikkim
Somar	Cow/yak milk	Paste, flavoured; condiment	Sherpa	West Sikkim, Rimbik of Darjeeling
Dahi	Cow/yak milk	Curd; savory	All	All
Shyow	Yak milk	Curd; savory	Bhutia, Lepcha	North and West Sikkim
Mohi	Cow milk	Butter-milk	All	All
Gheu	Cow milk	Butter	All	All
Maa	Yak milk	Butter	Bhutia	North and East Sikkim
Chur	Cow milk	Wet cheese-like; curry	Bhutia	North Sikkim

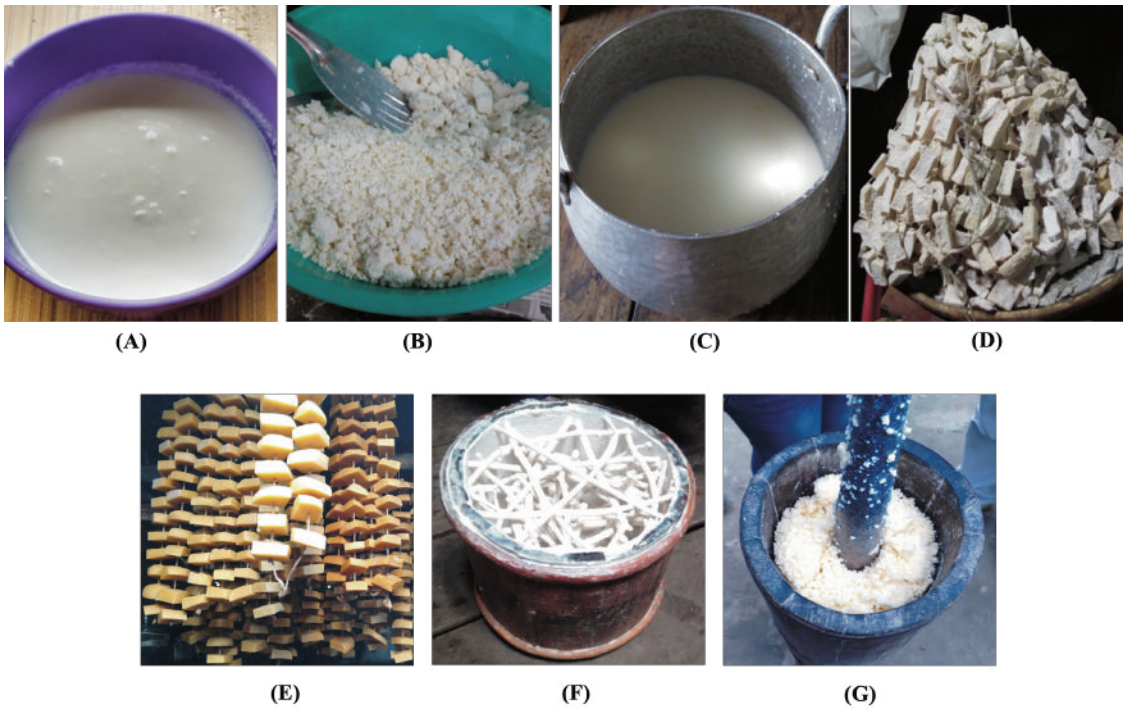


Figure 1: Different types of naturally fermented milk products of Sikkim (A) *dahi*, (B) soft *chhurpi*, (C*) mohi*, (D) *dudh chhurpi*, (E) hard *chhurpi*, (F) *philu* and (G) *gheu.*

DAHI

Dahi is a traditional fermented cow milk product in Sikkim. Varieties of fermented dairy products like *gheu, mohi*, soft *chhurpi, chhu*, etc. are obtained from *dahi*. The Nepali word is *dahi;* the Bhutia and Lepcha call it *shyow*.

Traditional method of preparation

Fresh cow milk is boiled during traditional *dahi* preparation methods, and kept at room temperature for cooling. The milk is transferred to a wooden vessel locally called *theki* and a small amount of previous *dahi* is added (back-slopping method) and left at room

temperature for natural fermentation for 1-2 days or 2-4 days depends on season (Fig. 2). *Dahi* is taken either directly or with boiled rice or *chewra* (beaten-rice).

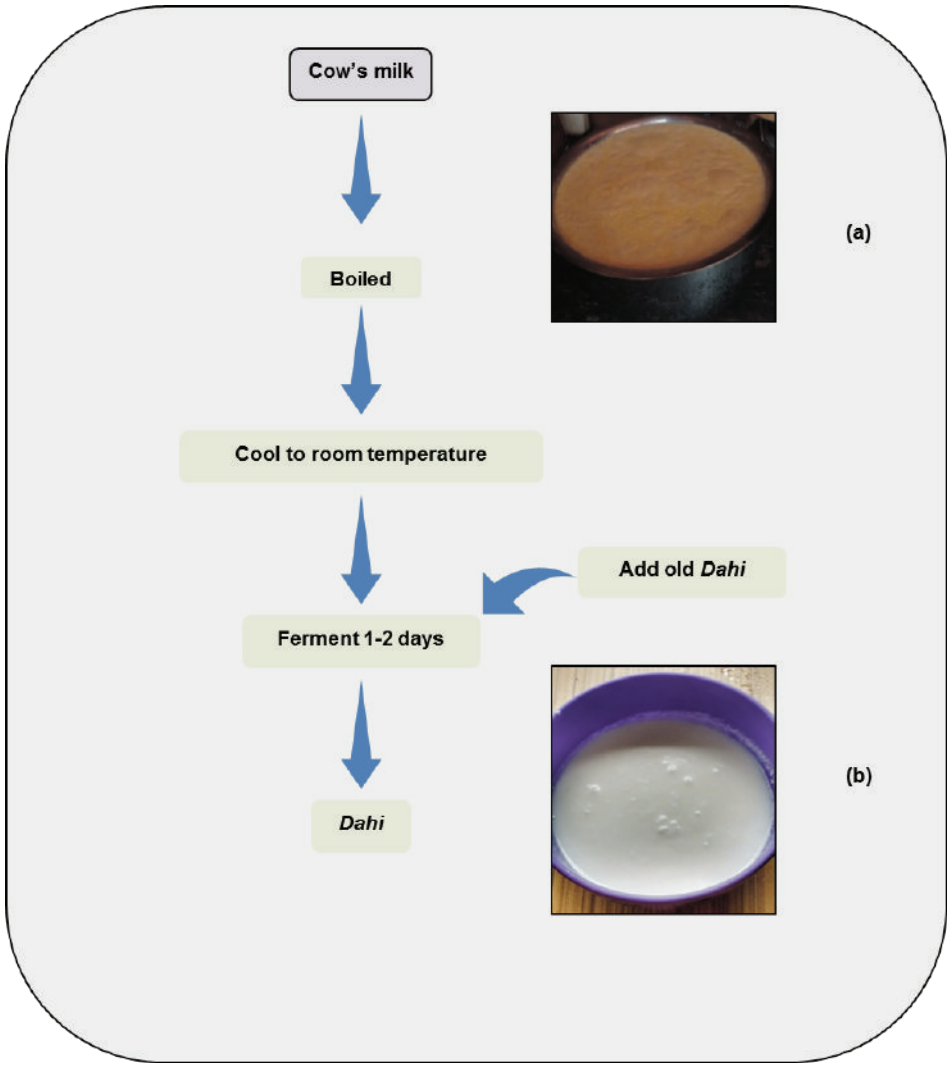


Figure 2: Traditional method of dahi preparation in the Himalayas. (a) boiled cow's milk and (b) *Dahi*

Mode of consumption

Dahi is considered a refreshing, non-alcoholic and savory beverage. People often tend to add sugar before consumption. Usually people like to mix it with rice or *chiura* (beaten rice). In addition, *dahi* is used to produce *gheu, chhurpi*, and *mohi*. *Dahi* is considered holy during a festival celebrated by the Hindu community and offered to Lord Krishna. In the Nepali culture, *dahi* is mixed with raw rice and during *dussera*, the mixture is pasted on the family members forehead by head of family. *Dahi, chiura* and banana are considered the main food in the monsoon season during the celebration of *'Ashar pandhra*' rice sowing.

GHEU

Gheu is type of artisanal butter which also known as *ghee* or *makhan* in Hindi, *maa* in Tibetan, and *mor* in Lepcha. *Gheu* is traditionally made from cow's milk. It is prepared by continuous *dahi* churning; it has a characteristic aroma and taste. It is a common dairy product in every Sikkimese household and is preferred by all ethnic communities.

Traditional method of preparation

Dahi is churned in a hollow wooden vessel called *theki*, and *madani*, consisting of the *ghurra* and a string called a *neti*. *Dahi* is kept inside the *theki* and the churning of *dahi* is done by pulling the string with either hand so that *madani* rotates in alternating clockwise and anticlockwise directions. After 15-30 min of churning a big lump of soft *gheu* is formed and carefully taken out with using the hand and transferred it to another container (Fig. 3a-c). Finally, *gheu* is separated from liquid buttermilk.

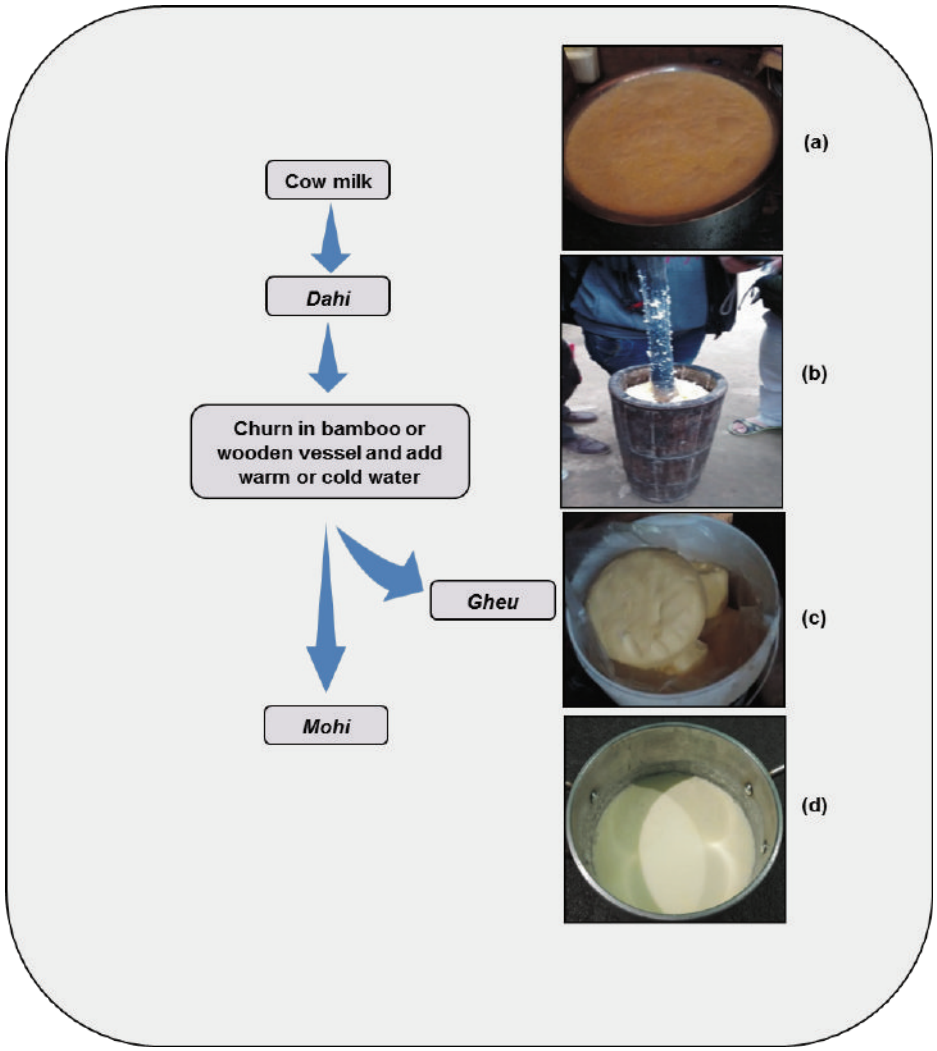


Figure 3: Indigenous method of *gheu* and *mohi* preparation in the Himalayas. (a) cow's milk, (b) churning, (c) *Gheu* and (d) *Mohi*

Mode of consumption

Fresh *gheu* is further purified by boiling until is separated by the unwanted dark-brown precipitate called *khar*. The filtered *gheu* mixes with cooked dal, curry and so on. Along with cooked rice, roti and bread it is also eaten. *Gheu* is used to prepare popular cereal based snacks and other sweet varieties. It is also used for vegetable frying and soup preparation.

MOHI

Mohi is a liquid by-product which is prepared by *dahi* churning. It is usually called buttermilk. It is primarily taken by the local people as a refreshing beverage. *Mohi* is similar to Western Himalayan *lassi*.

Traditional method of preparation

Preparation method of *moh*i is same as *gheu*, as both products are prepared simultaneously by *dahi* churning (Fig. 3a-d). During process, *dahi* is churned to produce *gheu* (butter), and the remaining liquid portion is known as *mohi*, which is a Nepali word. It is a *dahi* by-product, formed during *gheu* (curd) preparation. *Kachhu* is the term used in the Bhutia and Lepcha language for buttermilk.

Mode of consumption

Mohi is usually mixed with cooked rice or maize of *dheroh* by Gorkha. It is drunk as a cooling beverage and is also processed further to produce other fermented milk products like soft *chhurpi, chhu, dudh chhurpi*, etc.

SOFT *CHHURPI*

Chhurpi (soft variety) is a traditional cheese-like product, prepared by raw or boiled milk of cow and is widely consumed by ethnic people of Sikkim.

Traditional method of preparation

During *chhurpi* preparation, milk is kept for fermentation at 15-25°C overnight and then butter is separated by churning of *dahi*. The remaining buttermilk is boiled in a

heat till a soft, white mass is formed and filtered it in a clean cotton cloth. The leftover water is squeeze out by pressing gently and kept it in container for further use (Fig. 4).

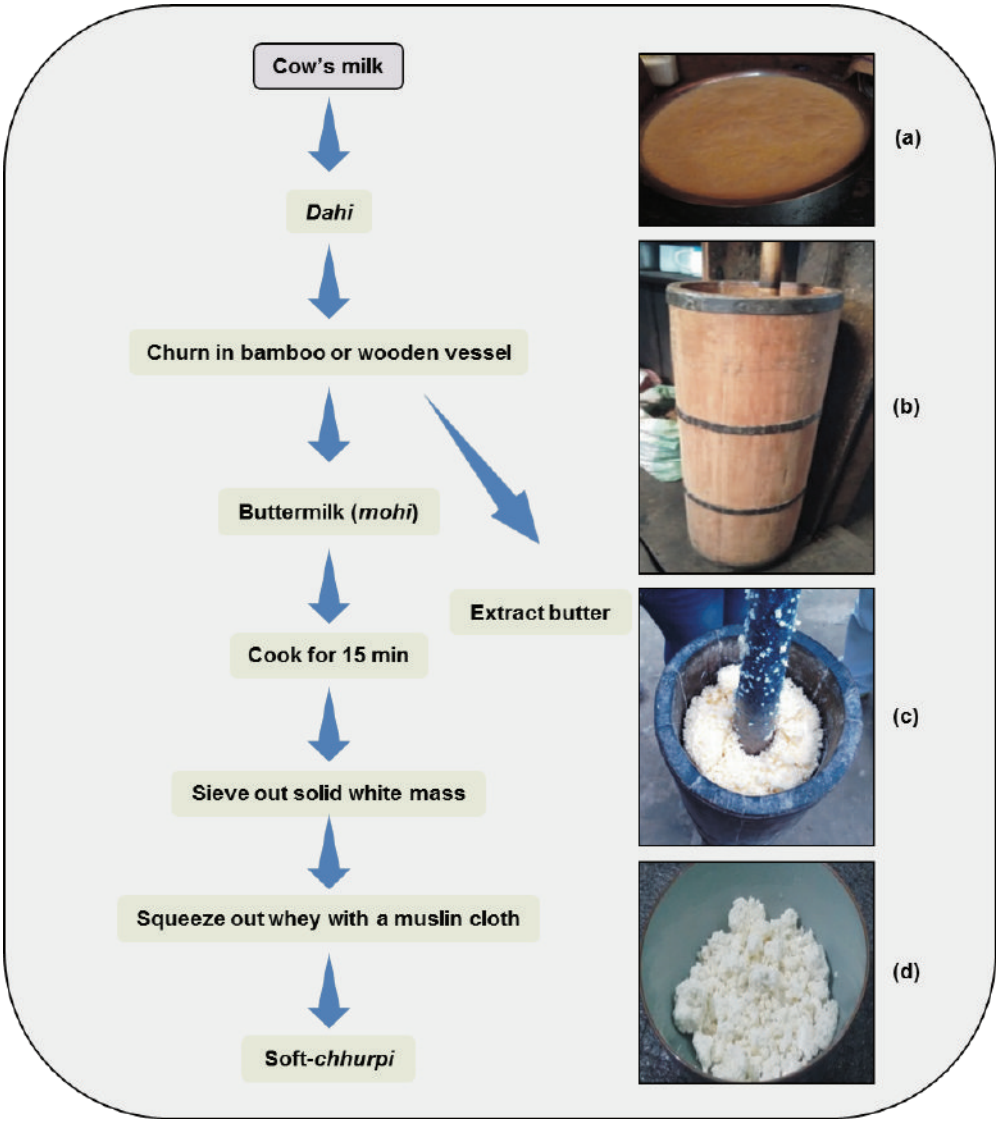


Figure 4: Indigenous method of soft *chhurpi* preparation in Sikkim. (a) *dahi*, (b) wooden vessel, (c) *gheu* (butter), and (d) soft-*chhurpi*

Mode of consumption

Soft *chhurpi* is consumed with rice as a side dish curry in main meal, and also as soup. Curry is also prepared with wild edible ferns locally called *ningro* (*Diplaziume sculentum*).

HARD *CHHURPI*

Hard-variety *chhurpi* is mostly prepared from yak milk in North Sikkim and West Sikkim (2100 to 4500 m) and is used as masticator due its characteristic gumminess and chewiness. Two types of hard-*chhurpi* are prepared one is common *chhurpi* (hard) and other is *dudh chhurpi*. The Bhutia and Lepcha call it *khamu.*

Traditional method of preparation

During hard *chhurpi* preparation, fresh yak milk is boiled until a thick white layer is formed, and previous whey is added into the milk. The white mass is collected by sieving out using a clean cotton cloth and pressed it to drain out the remaining liquid. Further the wrapped white mass is pressed by heavy stones for 2-3 h and then kept in traditional oven for 2-3 days. After that the hard white mass is cut into long pieces and sieve in a coconut thread and kept for drying for almost 15 days (Fig. 5).

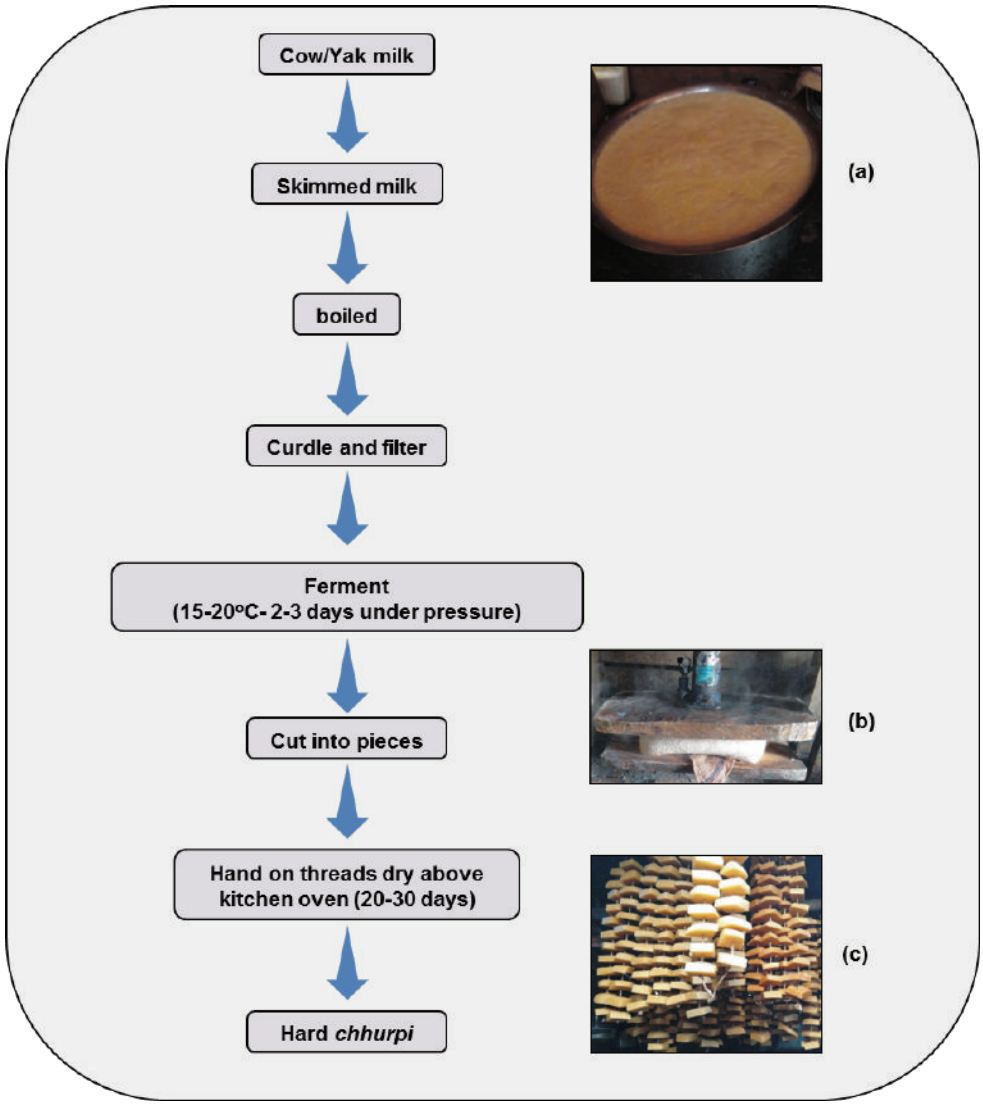


Figure 5: Indigenous method of hard-variety *chhurpi* preparation in Sikkim. (a) cow/yak milk, (b) squeezed under pressure (c) Hard-*chhurpi*

Mode of consumption

Hard *chhurpi* is taken as masticator as chewing gum; it provides extra energy at high altitudes. It is available in small cubes and sold in local market costing 200 per kg.

PHILU

Philu is a typical indigenous fermented butter-like milk product obtained from cow or yak milk, with an inconsistent semi-solid texture. It is commonly eaten by the Bhutia and also by Sherpa who calls it *philuk* of these regions.

Traditional method of preparation

During *philu* preparation, the fresh milk is poured into the wooden vessel for two or three times a day, where a thick mesh of dried creeper or bamboo sticks are kept inside that holds the milk. After pouring fresh milk into the vessel, rotate it gently so that milk cream adhere to the wall of the vessels and transfer the remaining milk into another container. Repeat the process until a thick creamy mass is formed and kept it for 6-7 days and some would even keep it for up to 15 days for fermentation. Scrape off the white mass from the branches and wall of the vessels and kept for further use (Fig. 6).

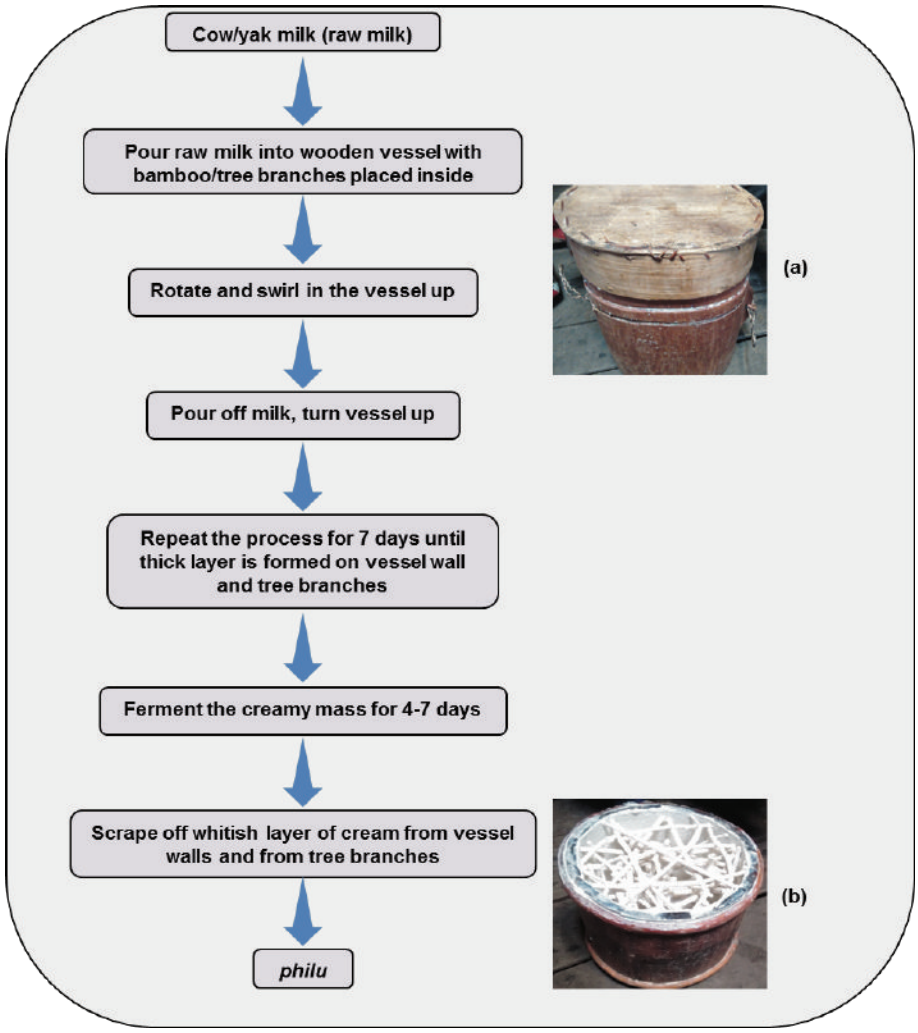


Figure 6: Indigenous method of *philu* preparation in Sikkim. (a) Wooden vessel and (b) *philu*

Mode of consumption

Philu is consumed as a curry along with steamed rice. It is a high-fat milk product cooked with butter and salt to make gravy, occasionally mixed with meat and green *vegetables. Philu* is most expensive ethnic milk product prepared in Himalayas and sold in local markets costing Rs. 600/- per kilogram.

DUDH CHHURPI

Dudh chhurpi is a type of hard cheese made from both cow and yak milk. It is hard type *chhurpi* covered with sweet and dried skim milk power.

Traditional method of preparation

During *dudh chhurpi* preparation, buttermilk is boiled in a container and little amount of previous whey is added and cooked until a white solid mass is formed. The white mass is taken out and placed on a sack and pressed with a heavy stone for 2-3 h to squeeze out the left over water. The solid mass is taken out and cut into small cubes. Further, milk is boiled make a thick creamy layer; mixed with the cubes and then threaded and hung above a traditional kitchen oven for drying for 12-15 days (Fig. 7).

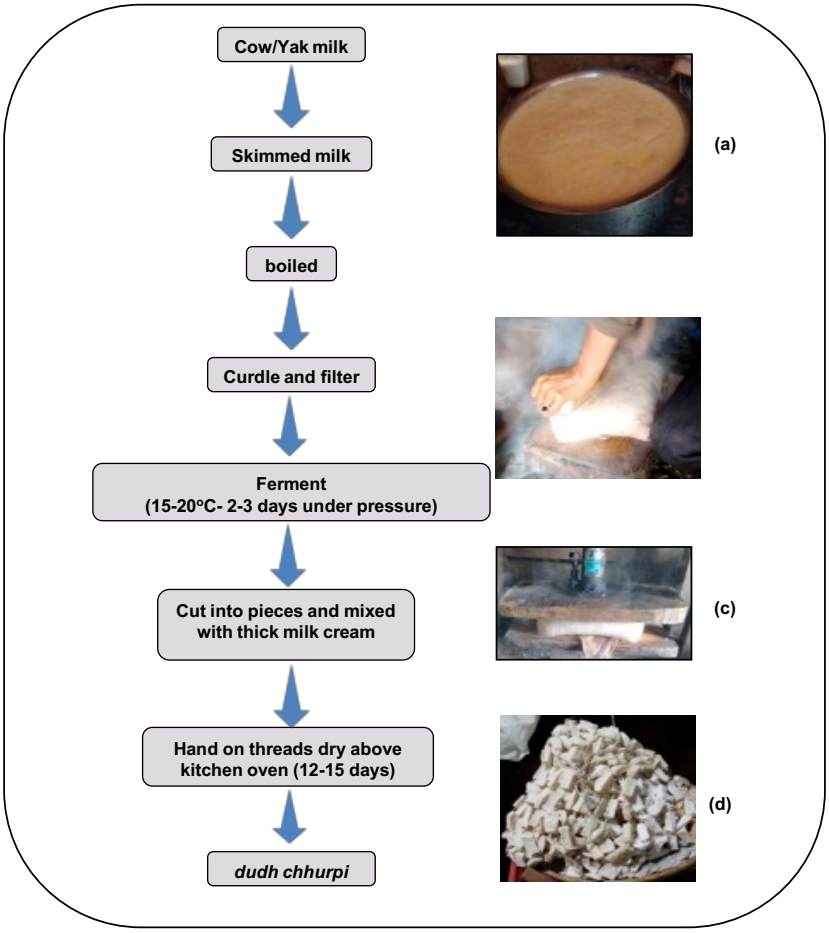


Figure 7: Indigenous method of *dudh-chhurpi* preparation in Sikkim. (a) cow/yak milk, (b) drain out water, (c) squeezed under pressure (d) *dudh-chhurpi*.

Mode of consumption

Dudh chhurpi is also eaten as masticator, same as hard *chhurpi;* it provides extra energy at high altitudes. It is available in small cubes and sold mostly by women in local market costing Rs. 200/- per kg.

MICROBIOLOGICAL ANALYSIS AND pH

Information on sample collections sites and locations of 22 different varieties of naturally fermented milk products viz. *dahi* (cow-milk), *dahi* (yak-milk), soft *chhurpi* (cow-milk), soft *chhurpi* (yak-milk), *mohi* (cow-milk), hard *chhurpi* (yak-milk), and *philu* (yak-milk) and their geography coordinates was sought (Table 2). The pH of samples was determined, and found the average pH of 4.3 in *dahi* (cow-milk), pH 4.5 in *dahi* (yak-milk), pH 5.1 in soft *chhurpi* (cow milk), pH 5.2 in soft *chhurpi* (yak-milk), pH 5.0 in *mohi* (cow milk), pH 6.5 in hard *chhurpi* (yak milk) and pH 5.1 in *philu* (yak milk), respectively (Table 2). The bacterial load of different types of NFM products were calculated which showed the range from 4.54 to 8.77 log cfu/g or ml (Table 2). The microbial load of *dahi* (cow-milk) ranged from 5.69-7.61 log cfu/ml, *dahi* (yak-milk) 7.56-8.77 log cfu/ml, soft *chhurpi* (cow-milk) 7.20-8.34 log cfu/g, soft *chhurpi* (yak-milk) 6.23-7.23 log cfu/g, *mohi* (cow-milk) 7.47-8.41 log cfu/ml, hard *chhurpi* (yak-milk) 4.54-7.49 log cfu/g and *philu* (yak-milk) 7.39-8.77 log cfu/g, respectively (Table 2).

Table 2: Information on sample collections sites and geography coordinates in Sikkim

Sample	District	Sample Collection Site	Altitude (Metre)	Latitude	Longitude	pH	Microbial load (log_{10} cfu/ml* or g**)
*Dahi** (cow-milk) (n=4)	East Sikkim	Aritar	1380	27°16′ 43.428″N	88° 41' 7.8" E	4.3 (4.1-4.4)	6.97 (5.69-7.61)
		Entel, 6th mile	1189	27°19′ 49.764″N	88° 36' 48.78" E		
	South Sikkim	Tarku	1401	27°16′ 43.428″N	88° 41' 7.8" E		
	West Sikkim	Thingling	1183	27°12′ 59.988″N	88° 15' 0" E		
*Dahi** (yak-milk) (n=3)	North Sikkim	Yumesamdong	5055	27°40′ 56.3232″N	88° 44' 19.0752" E	4.5 (4.2-4.6)	8.04 (7.56-8.77)
	West Sikkim	Chongri	2671	27°21′ 49.536″N	88° 11' 15.324" E		
Soft *chhurpi* ** (cow-milk) (n=3)	South Sikkim	Yangang	1983	27°16′ 43.428″N	88° 41' 7.8" E	5.1 (5.0-5.2)	7.90 (7.20-8.34)
		Pakyong	1120	27°14′ 11.436″N	88° 35' 32.748" E		

Soft *chhurpi* ** (yak-milk) (n=3)	North Sikkim	Yumesamdong	5055	27°40′ 56.3232″N	° 44' 19.0752" E	5.2 (5.1-5.3)	7.27 (6.23-7.23)
*Mohi** (cow-milk) (n=3)	West Sikkim	Martam	1665	27°21′ 49.536″N	88° 11' 15.324" E	5.0 (4.9-5.2)	8.01 (7.47-8.41)
	East Sikkim	Battis mile	681	27°13′ 54.48″N	88° 29' 54.96" E		
Hard *chhurpi* ** (yak-milk) (n=3)	North Sikkim	Yumesamdong	5055	27°40′ 56.3232″N	88° 44' 19.0752" E	6.5 (6.4-6.5)	6.42 (4.54-7.49)
	West Sikkim	Chongri	2671	27°21′ 49.536″N	88° 11' 15.324" E		
*Philu*** (yak-milk) (n=3)	North Sikkim	Yumesamdong	5055	27°40′ 56.3232″N	88° 44' 19.0752" E	5.1 (4.9-5.3)	7.9 (7.4-8.77)

n, number of samples in parenthesis; cfu, colony forming units.

PHENOTYPIC CHARACTERIZATION

A total of 272 bacterial isolates were isolated from 22 samples of different NFM product of Sikkim such as *dahi* (n=7), soft *chhurpi* (n=6), *mohi* (n=3), hard *chhurpi* (n=3), and *philu* (n=3). We have characterized 272 isolates by phenotypic characterizations including colony and cell morphology, different temperatures, pH, and different concentration of NaCl, Gram stain, catalase test, biochemical and sugar fermentation. All LAB isolates from NFM products were Gram-positive and catalase negative. Of the 272 isolates, 259 were cocci, and 13 were coccobacilli (Table 3-9).

Table 3: Colony and cell morphology of LAB isolated from *dahi* samples prepared from cow's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DA1	Circular, creamy white with halo zone	Cocci in chain	-	+
DA2	Circular, creamy white with halo zone	Cocci in pair	-	+
DA3	Circular, creamy white with halo zone	Cocci in chain	-	+
DA4	Circular, creamy white with halo zone	Cocci in pair	-	+
DA5	Circular, creamy white with halo zone	Cocci in chain	-	+
DA6	Circular, creamy white with halo zone	Cocci in chain	-	+
DA7	Circular, creamy white with halo zone	Cocci in pair	-	+
DA8	Circular, creamy white with halo zone	Cocci in pair	-	+
DA9	Circular, creamy white with halo zone	Cocci in chain	-	+
DA10	Circular, creamy white with halo zone	Cocci in chain	-	+
DA11	Circular, creamy white with halo zone	Cocci in chain	-	+
DA12	Circular, creamy white with halo zone	Cocci in pair	-	+
DA13	Circular, creamy white with halo zone	Cocci in chain	-	+
DA14	Circular, creamy white with halo zone	Cocci in pair	-	+
DA15	Circular, creamy white with halo zone	Cocci in chain	-	+
DA16	Circular, creamy white with halo zone	Cocci in chain	-	+
DA17	Circular, creamy white with halo zone	Cocci in pair	-	+
DA18	Circular, creamy white with halo zone	Cocci in pair	-	+

(Cont.)

solate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DA19	Circular, creamy white with halo zone	Cocci in chain	-	+
DA20	Circular, creamy white with halo zone	Cocci in chain	-	+
DA21	Circular, creamy white with halo zone	Cocci in chain	-	+
DA22	Circular, creamy white with halo zone	Cocci in pair	-	+
DA23	Circular, creamy white with halo zone	Cocci in chain	-	+
DA24	Circular, creamy white with halo zone	Cocci in pair	-	+
DA25	Circular, creamy white with halo zone	Cocci in chain	-	+
DA26	Circular, creamy white with halo zone	Cocci in chain	-	+
DA27	Circular, creamy white with halo zone	Cocci in pair	-	+
DA28	Circular, creamy white with halo zone	Cocci in pair	-	+
DA29	Circular, creamy white with halo zone	Cocci in chain	-	+
DA30	Circular, creamy white with halo zone	Cocci in chain	-	+
DA31	Circular, creamy white with halo zone	Cocci in chain	-	+
DA32	Circular, creamy white with halo zone	Cocci in pair	-	+
DA33	Circular, creamy white with halo zone	Cocci in chain	-	+
DA34	Circular, creamy white with halo zone	Cocci in pair	-	+
DA35	Circular, creamy white with halo zone	Cocci in chain	-	+
DA36	Circular, creamy white with halo zone	Cocci in chain	-	+

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DA37	Circular, creamy white with halo zone	Cocci in pair	-	+
DA38	Circular, creamy white with halo zone	Cocci in pair	-	+
DA39	Circular, creamy white with halo zone	Cocci in chain	-	+
DA40	Circular, creamy white with halo zone	Cocci in chain	-	+
DA41	Circular, creamy white with halo zone	Cocci in chain	-	+
DA42	Circular, creamy white with halo zone	Cocci in pair	-	+
DA43	Circular, creamy white with halo zone	Cocci in chain	-	+
DA44	Circular, creamy white with halo zone	Cocci in pair	-	+
DA45	Circular, creamy white with halo zone	Cocci in chain	-	+
DA46	Circular, creamy white with halo zone	Cocci in chain	-	+
DA47	Circular, creamy white with halo zone	Cocci in pair	-	+
DA48	Circular, creamy white with halo zone	Cocci in pair	-	+
DA49	Circular, creamy white with halo zone	Cocci in chain	-	+
DA50	Circular, creamy white with halo zone	Cocci in chain	-	+
DA51	Circular, creamy white with halo zone	Cocci in chain	-	+
DA52	Circular, creamy white with halo zone	Cocci in pair	-	+
DA53	Circular, creamy white with halo zone	Cocci in chain	-	+
DA54	Circular, creamy white with halo zone	Cocci in pair	-	+

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DA55	Circular, creamy white with halo zone	Cocci in chain	-	+
DA56	Circular, creamy white with halo zone	Cocci in chain	-	+
DA57	Circular, creamy white with halo zone	Cocci in pair	-	+
DA58	Circular, creamy white with halo zone	Cocci in pair	-	+
DA59	Circular, creamy white with halo zone	Cocci in chain	-	+
DA60	Circular, creamy white with halo zone	Cocci in chain	-	+
DA61	Circular, creamy white with halo zone	Cocci in chain	-	+
DA62	Circular, creamy white with halo zone	Cocci in pair	-	+
DA63	Circular, creamy white with halo zone	Cocci in chain	-	+
DA64	Circular, creamy white with halo zone	Cocci in pair	-	+
DA65	Circular, creamy white with halo zone	Cocci in chain	-	+
DA66	Circular, creamy white with halo zone	Cocci in chain	-	+
DA67	Circular, creamy white with halo zone	Cocci in pair	-	+
DA68	Circular, creamy white with halo zone	Cocci in pair	-	+
DA69	Circular, creamy white with halo zone	Cocci in chain	-	+

(+) positive; (-) negative.

Table 4: Colony and cell morphology of LAB isolated from *dahi* sample prepared from yak's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DY1	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY2	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY3	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY4	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY5	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY6	Circular, creamy white with halo zone	Coccobacilli in pair	-	Gram positive
DY7	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
DY8	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY9	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY10	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY11	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY12	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY13	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY14	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY15	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY16	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY17	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY18	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DY19	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY20	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY21	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY22	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY23	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY24	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY25	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY26	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY27	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY28	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY29	Circular, creamy white with halo zone	Coccobacilli in chains	-	Gram positive
DY30	Circular, creamy white with halo zone	Coccobacilli in chains	-	Gram positive
DY31	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY32	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY33	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY34	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY35	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY36	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
DY37	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY38	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY39	Circular, creamy white with halo zone	Coccobacilli in chains	-	Gram positive
DY40	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY41	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
DY42	Circular, creamy white with halo zone	Coccobacilli in chains	-	Gram positive

(+) positive; (-) negative.

Table 5: Colony and cell morphology of LAB isolated from soft *chhurpi* sample prepared from cow's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
SC1	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC2	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC3	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC4	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC5	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC6	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC7	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC8	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC9	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC10	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC11	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC12	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC13	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC14	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC15	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC16	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC17	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC18	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
SC19	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC20	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC21	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC22	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC23	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC24	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC25	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC26	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC27	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC28	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC29	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC30	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC31	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC32	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
SC33	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC34	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
SC35	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive

(+) positive; (-) negative.

Table 6: Colony and cell morphology of LAB isolated from soft *chhurpi* prepared from yak milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS3:01	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:02	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:03	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:04	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS3:05	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:06	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:07	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:08	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS3:09	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS3:10	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS4:01	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS4:02	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS4:03	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS4:04	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS4:05	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS4:06	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS4:07	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS4:08	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS4:09	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS4:10	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS5:01	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS5:02	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS5:03	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS5:04	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS5:05	Circular, creamy white with halo zone	Cocci in pair	-	Gram positive
YS5:06	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS5:07	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS5:08	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS5:09	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS5:10	Circular, creamy white with halo zone	Cocci in pair	-	Gram positive

(+) positive; (-) negative.

Table 7: Colony and cell morphology of LAB isolated from *mohi* sample prepared from cow's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
MH1	Circular, creamy white with halo zone	Cocci in chain	-	+
MH2	Circular, creamy white with halo zone	Cocci in pair	-	+
MH3	Circular, creamy white with halo zone	Cocci in chain	-	+
MH4	Circular, creamy white with halo zone	Cocci in pair	-	+
MH5	Circular, creamy white with halo zone	Cocci in chain	-	+
MH6	Circular, creamy white with halo zone	Cocci in chain	-	+
MH7	Circular, creamy white with halo zone	Cocci in pair	-	+
MH8	Circular, creamy white with halo zone	Cocci in pair	-	+
MH9	Circular, creamy white with halo zone	Cocci in chain	-	+
MH10	Circular, creamy white with halo zone	Cocci in chain	-	+
MH11	Circular, creamy white with halo zone	Cocci in chain	-	+
MH12	Circular, creamy white with halo zone	Cocci in chain	-	+
MH13	Circular, creamy white with halo zone	Cocci in chain	-	+
MH14	Circular, creamy white with halo zone	Cocci in chain	-	+
MH15	Circular, creamy white with halo zone	Cocci in chain	-	+
MH16	Circular, creamy white with halo zone	Cocci in chain	-	+
MH17	Circular, creamy white with halo zone	Cocci in pair	-	+
MH18	Circular, creamy white with halo zone	Coccobacilli in chain	-	+

.

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
MH19	Circular, creamy white with halo zone	Cocci in chain	-	+
MH20	Circular, creamy white with halo zone	Cocci in pair	-	+
MH21	Circular, creamy white with halo zone	Cocci in chain	-	+
MH22	Circular, creamy white with halo zone	Cocci in pair	-	+
MH23	Circular, creamy white with halo zone	Cocci in pair	-	+
MH24	Circular, creamy white with halo zone	Cocci in pair	-	+
MH25	Circular, creamy white with halo zone	Coccobacilli in chain	-	+
MH26	Circular, creamy white with halo zone	Cocci in chain	-	+
MH27	Circular, creamy white with halo zone	Cocci in chain	-	+
MH28	Circular, creamy white with halo zone	Cocci in chain	-	+
MH29	Circular, creamy white with halo zone	Cocci in pair	-	+
MH30	Circular, creamy white with halo zone	Cocci in chain	-	+
MH31	Circular, creamy white with halo zone	Cocci in chain	-	+
MH32	Circular, creamy white with halo zone	Cocci in pair	-	+
MH33	Circular, creamy white with halo zone	Cocci in pair	-	+
MH34	Circular, creamy white with halo zone	Cocci in pair	-	+
MH35	Circular, creamy white with halo zone	Coccobacilli in chain	-	+

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
MH36	Circular, creamy white with halo zone	Cocci in chain	-	+
MH37	Circular, creamy white with halo zone	Cocci in chain	-	+
MH38	Circular, creamy white with halo zone	Cocci in chain	-	+
MH39	Circular, creamy white with halo zone	Cocci in pair	-	+
MH40	Circular, creamy white with halo zone	Cocci in chain	-	+

(+) positive; (-) negative.

Table 8: Colony and cell morphology of LAB isolated from hard *chhurpi* prepared from yak's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS6:01	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS6:02	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS6:03	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS6:04	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
YS6:05	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS6:06	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS6:07	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
YS6:08	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS6:09	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS6:10	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:1	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS7:2	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:3	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:4	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS7:5	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:6	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:7	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
YS7:8	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS7:9	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS7:10	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
YS7:11	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS7:12	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:13	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:14	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:15	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:16	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:17	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:18	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:19	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS7:20	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive

(+) positive; (-) negative.

Table 9: Colony and cell morphology of LAB isolated from *philu* prepared from yak's milk

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS8:01	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS8:02	Circular, creamy white with halo zone	Coccobacilli in pairs	-	Gram positive
YS8:03	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS8:04	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:05	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS8:06	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:07	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:08	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS8:09	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:10	Circular, creamy white with halo zone	Cocci in pairs	-	Gram positive
YS8:11	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:12	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:13	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:14	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:15	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:16	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:17	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:18	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive

(Cont.)

Isolate Code	Colony morphology	Cell morphology	Catalase test	Gram reaction
YS8:19	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:20	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:21	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:22	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:23	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:24	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:25	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive
YS8:26	Circular, creamy white with halo zone	Cocci in chains	-	Gram positive

(+) positive; (-) negative.

The sample-wise phenotypic characterization of LAB is given in Table 10-16. LAB morphology on MRS agar was circular in colony, 1-2 mm in size and creamy white in color (Fig. 8). Out of 272 isolates, 122 (44%) isolates produced CO_2; 65% of the isolates grew well at 15°C while 44.4 % of the LAB isolates grew at 45°C. Similarly, 25 % of LAB grew at pH 3.9, 22 % at pH 9.6 and only 8 % of LAB isolates grew at 6.5 % of NaCl concentration. It was found that most of the sugars except raffinose, arabinose, galactose, and sorbitol were fermented by LAB isolates.

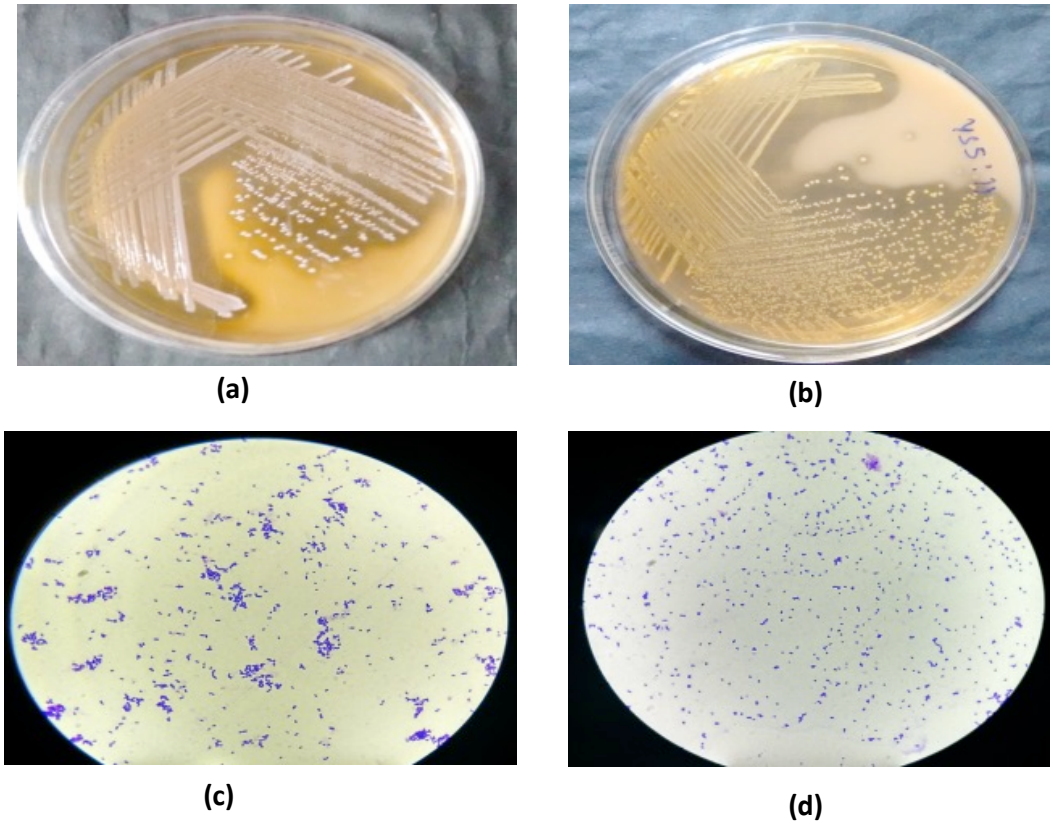


Figure 8: Colony morphology of LAB on MRS agar plate (a) and (b), cell morphology (cocci shaped) (c) and (d).

Table 10: Phenotypic characteristics of LAB from *dahi* (cow-milk)

Strain ID	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	6.5% NaCl	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
DA1	Cocci (chains)	+	+	+	-	W +	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-
DA2	Cocci (chains)	-	+	-	-	+	+	-	-	+	-	W +	-	W +	-	-	-	-	-	-	-	-	-	-	-	-
DA3	Cocci (chains)	-	+	+	-	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	+	+
DA4	Cocci (chains)	-	+	+	+	W +	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	+	-	+	+	+
DA5	Cocci (chains)	+	+	+	-	+	+	-	-	+	+	W +	+	+	-	-	-	W +	-	+	-	-	-	-	-	-
DA6	Cocci (chains)	+	+	+	-	-	W +	+	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	W +	+

DA12	DA11	DA10	DA9	DA8	DA7	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	+	+	+	-	+	**CO_2 pdtn**
+	+	+	-	+	+	**10˚C**
+	+	-	-	+	+	**15°C**
+ W	-	+	-	+	-	**45°C**
+ W	-	-	+	-	+ W	**pH 3.9**
+	+	-	+	+	+	**pH 9.6**
+	+	-	-	-	+	**6.5% NaCl**
+	+	-	-	-	+	**Lactose**
+	+	+	+	+	+	**Maltose**
+	+	+	+	+	+	**Glucose**
+	+	+	+	+	+	**Fructose**
-	-	-	-	-	-	**Cellobiose**
+	+	+	+ W	+	+	**Mannose**
-	-	-		-	-	**Rhamnose**
-	-	-	-	-	-	**Melezitose**
-	-	-	-	-	-	**Raffinose**
+	-	-	+ W	+	-	**Ribose**
-	-	-	-	-	-	**Xylose**
-	+	+	+	-	+	**Sucrose**
-	-	-	-	-	-	**Arabinose**
+	+	+	+	+	+	**Trehalose**
-	-	-	-	-	-	**Melibiose**
-	-	-	-	-	-	**Salicin**
+	-	-	-	+	-	**Mannitol**
+	+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

DA17	DA16	DA15	DA14	DA13	**Strain ID**
Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	+	+	-	-	**CO_2 pdtn**
+	+	-	+	+	**10°C**
+ w	-	-	-	+	**15°C**
-	-	-	-	-	**45°C**
-	-	+	+ w	+	**pH 3.9**
+	+	+	+	+	**pH 9.6**
+	-	-	-	+	**6.5% NaCl**
+	-	-	-	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	-	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	+	-	-	-	**Cellobiose**
+	-	-	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	+	+	**Ribose**
-	-	-	-	-	**Xylose**
+	+	-	-	-	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+ w	-	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	+	-	**Salicin**
-	-	-	+	+	**Mannitol**
+	+	-	+	+	**Arginine hydrolysis**

(Cont.)

DA22	DA21	DA20	DA19	DA18	**Strain ID**
Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	**Cell morphology**
-	-	+	+	+	**CO_2 pdtn**
	-	+	+	+	**10°C**
-	-	+	-	+	**15°C**
+	-	-	-	-	**45°C**
+	+	+	+	-	**pH 3.9**
+	+ W	+	+ W	+	**pH 9.6**
	+	+	-	+	**6.5% NaCl**
-	+	+	-	+	**Lactose**
+	+	+	+	+	**Maltose**
-	+	+	+ W	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
-	+	+	-	-	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
+	-	-	-	-	**Raffinose**
-	-	-	-	+	**Ribose**
-	-	-	-	-	**Xylose**
-	+	+	+	-	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+	-	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	+ W	**Salicin**
-	-	-	-	+	**Mannitol**
	-	+	-	+	**Arginine hydrolysis**

(Cont.)

DA27	DA26	DA25	DA24	DA23	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morpholog**
+	-	-	-	+	**CO_2 pdtn**
+	-	-	-	+	**10°C**
+	-	+ W	-	-	**15°C**
-	-	-	-	-	**45°C**
+	+	+	+	+	**pH 3.9**
+	+	+	+	+ W	**pH 9.6**
+	-	-	-	-	**6.5% NaCl**
-	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	-	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	+ W	-	**Cellobiose**
+	+	+	+	-	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	+ W	+ W	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	-	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
+ W	-	-	+	-	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

DA32	DA31	DA30	DA29	DA28	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	-	+	+	**CO_2 pdtn**
+	-	+	+	+	**10°C**
+	-	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+	+	+	+	+	**pH 3.9**
+ W	+	+	+ W	+ W	**pH 9.6**
+	-	+	+	+	**6.5% NaCl**
-	+ W	-	+	+ W	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
+ W	+	+ W	+ W	+ W	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

Strain ID	DA33	DA34	DA35	DA36	DA37
Cell morphology	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)
CO_2 pdtn	-	+	-	-	-
10°C	+	+	+	+	-
15°C	-	+	-	+	-
45°C	+	-	-	+	-
pH 3.9	+	+	W +	+	+
pH 9.6	+	-	+	+	+
6.5% NaCl	-	+	-	-	-
Lactose	W +	-	+	+	+
Maltose	+	+	+	+	+
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Cellobiose	-	-	-	-	-
Mannose	+	+	+	+	+
Rhamnose	-	-	-	-	-
Melezitose	-	-	-	-	-
Raffinose	W +	-	W +	-	W +
Ribose	-	-	-	+	-
Xylose	-	-	-	-	-
Sucrose	+	+	+	-	+
Arabinose	-	-	-	-	-
Trehalose	+	+	+	+	+
Melibiose	-	-	-	-	-
Salicin	-	-	-	+	-
Mannitol	+	W +	-	+	-
Arginine hydrolysis	-	+	+	+	+

(Cont.)

DA42	DA41	DA40	DA39	DA38	**Strain ID**
Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
+	+	+	+	-	**10°C**
-	-	-	-	-	**15°C**
-	-	-	-	-	**45°C**
+	+	+	+	+	**pH 3.9**
+	+	+	+	+	**pH 9.6**
+	+	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	+ W	+ W	**Raffinose**
+	+ W	+	-	-	**Ribose**
+	+	+	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
+	+	+	-	-	**Salicin**
+	+	+	-	-	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

DA47	DA46	DA45	DA44	DA43	**Strain ID**
Cocci (chains)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
-	-	-	-	-	**15°C**
+	-	-	-	-	**45°C**
+ W	+	+	+	+	**pH 3.9**
+	+	+	+	+	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
-	+	+	+	+	**Fructose**
+	+	+	+	+	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
+ W	+ W	+ W	+	+ W	**Ribose**
+	+	+	+	+	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
+	+	-	+	+	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

DA52	DA51	DA50	DA49	DA48	**Strain ID**
Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
+	+	-	-	+	**10°C**
+	-	-	-	-	**15°C**
-	-	+	+	-	**45°C**
+	+	+	+	+/-	**pH 3.9**
+	+	+	+	+/-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	+	-	**Cellobiose**
+	+	+	+	-	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	+	**Raffinose**
+/-	+/-	+/-	+/-	-	**Ribose**
+	+	+	+	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	-	**Trehalose**
-	-	-	-	-	**Melibiose**
+	+	+	-	-	**Salicin**
+	+	+	+	-	**Mannitol**
+	+	-	+	+	**Arginine hydrolysis**

(Cont.)

DA57	DA56	DA55	DA54	DA53	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
+	+	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+ W	-	**45°C**
+	+	+	+	+	**pH 3.9**
+	+	+	+	+	**pH 9.6**
-	+	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	+	+	+	**Cellobiose**
+	-	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	+ W	+ W	**Ribose**
-	-	-	+	+	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	+	+	**Salicin**
-	-	-	+	+	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

DA62	DA61	DA60	DA59	DA58	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	+	+	+	+	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	-	+	+	**15°C**
-	-	+	+	+	**45°C**
+	+	+	+	+	**pH 3.9**
+	+	+	+	+	**pH 9.6**
+	-	-	+	+	**6.5% NaCl**
W +		+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	W +	-	+	+	**Glucose**
+	W +	W +	+	+	**Fructose**
-	-	-	+	-	**Cellobiose**
+	-	-	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	-	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
W +	-	-	-	-	**Salicin**
W +	-	-	-	-	**Mannitol**
-		+	+	+	**Arginine hydrolysis**

(Cont.)

Strain ID	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	6.5% NaCl	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
DA63	Cocci (chains)	+	+	+	-	+	+	-	+	+	W +	W +	-	W +	-	-	-	W +	-	+	-	-	-	-		+
DA64	Cocci (chains)	-	+	-	-	+	+	-	-	+	+	+	-	-	-	-	-	W +	-	+	-	+	-	+	-	-
DA65	Cocci (chains	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+
DA66	Cocci (chains	-	+	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+
DA67	Cocci (chains	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+
DA68	Cocci (chains)	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+
DA69	Cocci (chains)	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+

(-) Negative; (+) Positive; (w+) Weakly positive.

Table 11: Phenotypic characterization of lab isolated from *dahi* (yak-milk)

DY5	DY4	DY3	DY2	DY1	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	+	-	-	+	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	+	**15°C**
-	-	-	-	-	**45°C**
-	+ w	+	+ w	+ w	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	+	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
-	-	-	-	+	**Glucose**
-	-	-	-	-	**Fructose**
-	+	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	-	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
-	-	-	-	-	**Arginine hydrolysis**

(Cont.)

DY12	DY10	DY9	DY8	DY7	DY6	Strain ID
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Coccobacillin (pairs)	Coccobacilli (pairs)	Cell morphology
+	-	+	-	+	+	CO_2 pdtn
+	+	+	+	+	+	10°C
+	+	+	+	+	+	15°C
-	-	-	-	-	-	45°C
-	+	+	+	+	+	pH 3.9
-	-	-	-	-	-	pH 9.6
-	-	-	-	-	-	6.5% NaCl
+	+	+	+	-	+	Lactose
+	+	+	+	+	+	Maltose
-	-	-	-	-	-	Glucose
-	+	-	-	-	-	Fructose
-	-	-	-	-	-	Cellobiose
+	+	+	+	+	+	Mannose
-	-	-	-	-	-	Rhamnose
-	-	-	-	-	-	Melezitose
-	-	-	-	-	-	Raffinose
-	-	-	-	-	-	Ribose
-	-	-	-	-	-	Xylose
+	+	+	+	+	+	Sucrose
-	-	-	-	-	-	Arabinose
+	+	+	+	+	+	Trehalose
-	-	-	-	-	-	Melibiose
-	-	-	-	-	-	Salicin
-	-	-	-	-	-	Mannitol
-	-	-	+	+	+	Arginine hydrolysis

(Cont.)

DY17	DY16	DY15	DY14	DY13	Strain ID
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cell morphology
-	-	+	-	+	CO_2 pdtn
+	+	+	+	+	10°C
+	+	+	+	+	15°C
-	-	-	-	-	45°C
+	+	+	-	+	pH 3.9
-	-	-	-	-	pH 9.6
-	-	-	-	-	6.5% NaCl
+	+	-	+	+	Lactose
+	+	+	+	+	Maltose
+	-	-	-	+	Glucose
-	-	-	-	-	Fructose
-	-	-	-	-	Cellobiose
+	+	+	+	+	Mannose
-	-	-	-	-	Rhamnose
-	-	-	-	-	Melezitose
-	-	-	-	-	Raffinose
-	-	-	-	-	Ribose
-	-	-	-	-	Xylose
+	+	+	+	+	Sucrose
-	-	-	-	-	Arabinose
+	+	+	+	+	Trehalose
-	-	-	-	-	Melibiose
-	-	-	-	-	Salicin
-	-	-	-	-	Mannitol
-	+	-	-	-	Arginine hydrolysis

(Cont.)

DY22	DY21	DY20	DY19	DY18	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	+	+	+	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	+	**15°C**
-	-	-	-	-	**45°C**
+	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
-	+	+	+	-	**Glucose**
-	+	+	+	-	**Fructose**
-	-	-	-	-	**Cellobiose**
-	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

DY27	DY26	DY25	DY24	DY23	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	+	+	-	+	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	+	**15°C**
-	-	-	-	-	**45°C**
-	+	-	+	+	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
-	-	+	-	-	**Glucose**
-	-	-	-	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	-	-	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	+	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

DY31	DY30	DY29	DY28	**Strain ID**
Cocci (chains)	Coccobacil-li (chains)	Coccobacil-li (chains)	Cocci (chains)	**Cell morphology**
-	+	+	-	**CO_2 pdtn**
+	+	+	+	**10°C**
+	+	+	+	**15°C**
-	-	-	-	**45°C**
+	+ W	+ W	+ W	**pH 3.9**
-	-	-	-	**pH 9.6**
-	-	-	-	**6.5% NaCl**
+	+	+	-	**Lactose**
+	+	+	+	**Maltose**
-		+	-	**Glucose**
+	+	+	-	**Fructose**
-	-	-	-	**Cellobiose**
+	+	+	+	**Mannose**
-	-	-	-	**Rhamnose**
-	-	-	-	**Melezitose**
-	-	-	-	**Raffinose**
-	-	-	-	**Ribose**
-	-	-	-	**Xylose**
+	+	+	+	**Sucrose**
-	-	-	-	**Arabinose**
+	+	-	+	**Trehalose**
-	-	-	-	**Melibiose**
-	-	-	-	**Salicin**
-	-	-	-	**Mannitol**
-	+	+	-	**Arginine hydrolysis**

(Cont.)

DY36	DY35	DY34	DY33	DY32	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	+	-	+	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	+	**15°C**
-	-	-	-	-	**45°C**
+ W	+	+	-	+ W	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
+	+	+	-	-	**Lactose**
+	+	+	+	+	**Maltose**
-	-	-	+	+	**Glucose**
+	+	+	-	-	**Fructose**
+	-	-	-	-	**Cellobiose**
-	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
-	-	-	-	-	**Arginine hydrolysis**

DY42	DY41	DY40	DY339	DY38	DY37	**Strain ID**
Coccobacilli (chains)	Cocci (chains)	Cocci (chains)	Coccobacilli (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	-	+	+	-	**CO_2 pdtn**
+	+	+	+	+	+	**10°C**
+	+	+	+	+	+	**15°C**
-	-	-	-	-	-	**45°C**
+	+	+	-	+W	+W	**pH 3.9**
-	-	-	-	-	-	**pH 9.6**
-	-	-	-	-	-	**6.5% NaCl**
+	+	+	+	-	+	**Lactose**
+	+	+	+	-	+	**Maltose**
-	-	-	-	-	+	**Glucose**
-	-	+	+	+	+	**Fructose**
-	-	-	-	+	-	**Cellobiose**
+	+	+	+	+	+	**Mannose**
-	-	-	-	-	-	**Rhamnose**
-	-	-	-	-	-	**Melezitose**
-	-	-	-	-	-	**Raffinose**
-	-	-	-	-	-	**Ribose**
-	-	-	-	+	-	**Xylose**
+	+	+	+	+	+	**Sucrose**
-	-	-	-	-	-	**Arabinose**
+	+	-	+	+	+	**Trehalose**
-	-	-	-	-	-	**Melibiose**
-	-	-	-	-	-	**Salicin**
-	-	-	-	-	-	**Mannitol**
+	+	-	+	-	+	**Arginine hydrolysis**

(-) Negative; (+) Positive; (w+) Weakly positive.

Table 12: Phenotypic characteristics of LAB from soft *chhurpi* (cow-milk)

Strain ID	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	6.5% NaCl	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
SC1	Cocci (chains)	+	+	+	+	w +	w +	+	-	w +	+	+	w +	+	w +	w +	w +	+	-	+	-	+	-	+	+	+
SC2	Cocci (chains)	+	-	+	+	w +	w +	-	+	+	+	+	w +	+	-	w +	-	w +	-	+	-	+	-	+	+	+
SC3	Cocci (chains)	+	-	+	-	w +	-	-	w +	-	+	w +	w +	w +	w +	w +	-	+	-	+	-	+	-	w +	-	+
SC4	Cocci (chains)	-	-	+	+	w +	w +	-	+	+	+	+	w +	+	w +	w +	w +	w +	-	+	-	+	-	+	+	+
SC5	Cocci (chains)	+	-	+	+	w +	w +	-	+	w +	+	+	-	+	w +	w +	w +	+	-	+	-	+	-	w +	+	+

(Cont.)

SC 10	SC9	SC8	SC7	SC6	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	+	+	+	+	**CO_2 pdtn**
+	-	+	-	-	**10°C**
+	+	+	+	+	**15°C**
+ W	+	+	+	+	**45°C**
+ W	-	+ W	+ W	+ W	**pH 3.9**
+	-	-	+ W	+ W	**pH 9.6**
-	-	+	-	-	**6.5% NaCl**
-	+	+	+	-	**Lactose**
-	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+ W	+ W	+ W	+	+	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	+ W	+ W	+ W	**Rhamnose**
+ W	+ W	+ W	+ W	+ W	**Melezitose**
-	-	+	+ W	-	**Raffinose**
+	+	+ W	+	+	**Ribose**
-	-	-	-	-	**Xylose**
-	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	+	+ W	+	+ W	**Salicin**
-	+	+	+	+	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

SC 15	SC 14	SC 13	SC 12	SC 11	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	+	+	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
-	-	-	-	+ V	**15°C**
+	-	-	+ V	-	**45°C**
+ V	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
-	-	-	-	-	**Lactose**
-	-	-	-	-	**Maltose**
+	-	-	-	-	**Glucose**
-	-	-	-	-	**Fructose**
+	+ V	+	+ V	+ V	**Cellobiose**
-	-	-	-	-	**Mannose**
+ V	+ V	+ V	+ V	+ V	**Rhamnose**
+ V	-	+ V	+ V	+ V	**Melezitose**
+	+	+	+	+ V	**Raffinose**
+	-	+	+	+ V	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+ V	+	+	**Trehalose**
-	+	+	+	+	**Melibiose**
-	-	+ V	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
+	+	+	+	-	**Arginine hydrolysis**

(Cont.)

SC 20	SC 19	SC 18	SC 17	SC 16	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	+	-	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+ W	-	+	-	**15°C**
-	+ W	-	+	-	**45°C**
+ W	+ W	+ W	+ W	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
-	-	-	+	-	**Lactose**
-	-	-	-	-	**Maltose**
+	+	-	+	-	**Glucose**
-	+ W	-	+	-	**Fructose**
+	+ W	+	+	+ W	**Cellobiose**
-	+ W	-	+	-	**Mannose**
+ W	+ W	+	+ W	+ W	**Rhamnose**
+ W	+ W	+ W	+ W	-	**Melezitose**
-	-	+	+ W	+	**Raffinose**
-	+	+	+	-	**Ribose**
-	-	-	-	-	**Xylose**
-	+	+	-	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+	+	-	**Trehalose**
-	-	+	-	+	**Melibiose**
+	+	+	+ W	+ W	**Salicin**
-	+	-	+	-	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

SC 25	SC 24	SC 23	SC 22	SC 21	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	+	+	-	**CO_2 pdtn**
-	-	-	-	+	**10°C**
-	-	-	-	+	**15°C**
+ V	-	+	+	+	**45°C**
+ V	+ V	-	-	+	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	+	**6.5% NaCl**
-	+ V	-	-	+ V	**Lactose**
-	-	-	-	+	**Maltose**
-	+	+	-	+	**Glucose**
-	+ V	-	-	+	**Fructose**
+	+	+ V	+	+	**Cellobiose**
-	+ V	-	-	+	**Mannose**
+ V	+ V	+ V	+	-	**Rhamnose**
+ V	+ V	+ V	+ V	+	**Melezitose**
+	+ V	+	+	+ V	**Raffinose**
+ V	+	-	+	+	**Ribose**
-	-	-	-	+ V	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	-	+	**Trehalose**
+	-	-	-	-	**Melibiose**
+	+ V	-	+	-	**Salicin**
+	+	-	-	+	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

SC 30	SC 29	SC 28	SC27	SC 26	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	+	+	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+ V	+	-	-	-	**45°C**
+ V	+ V	+ V	-	+ V	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**6.5% NaCl**
-	-	-	-	-	**Lactose**
-	-	-	-	-	**Maltose**
-	+ V	+	-	+	**Glucose**
+	-	+ V	+ V	+ V	**Fructose**
+	+	+	+ V	+	**Cellobiose**
-	-	-	-	-	**Mannose**
+ V	+ V	+ V	+ V	+ V	**Rhamnose**
+ V	+ V	+ V	+ V	+ V	**Melezitose**
-	+	+	+ V	+	**Raffinose**
+ V	-	+	+ V	+	**Ribose**
+	+	-	+	+	**Xylose**
+	+	+	-	-	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	-	+	**Trehalose**
-	-	+ V	-	-	**Melibiose**
-	-	-	-	-	**Salicin**
-	-	-	-	-	**Mannitol**
+	-	+	+	+	**Arginine hydrolysis**

(Cont.)

Strain ID	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	6.5% NaCl	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
SC 31	Cocci (chains)	+	-	+	w +	-	-	-	-	-	-	-	-	-	-	-	w +	w +	-	-	-	+	-	-	-	+
SC 32	Cocci (chains)	+	-	+	+	w +	-	-	-	-	+	+	+	-	w +	-	+	w +	+	+	-	+	+	-	-	+
SC33	Cocci (chains)	+	-	+	+	w +	-	-	-	-	-	-	+	-	w +	w +	+	w +	-	+	-	+	-	-	-	+
SC 34	Cocci (chains)	+	-	+	-	w +	-	-	+	-	-	-	+	-	-	w +	+	-	+	-	-	+	-	-	+	+
SC 35	Cocci (chains)	+	-	+	-	w +	-	-	-	-	+	-	+	-	w +	w +	+	w +	+	+	-	+	-	-	-	+

(-) Negative; (+) Positive; (w+) Weakly positive

Table 13: Phenotypic characterization of lab isolated from soft *chhurpi* (Yak-milk)

Strain ID	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	6.5% NaCl	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
YS3:1	Cocci (pairs)	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	w +	+	-	+	-	-	-	-
YS3:2	Cocci (pairs)	+	-	+	+	w +	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	+	w +	-	-
YS3:3	Cocci (pairs)	+	-	+	+	w +	+	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	+	w +	-	-	-
YS3:4	Cocci (chains)	-	-	-	+	w +	+	w +	+	+	+	+	-	+	-	-	-	-	+	+	-	-	w +	-	+	+
YS3:5	Cocci (pairs)	+	-	+	+	w +	w +	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	-	w +	+	+	+

(Cont.)

YS3:10	YS3:9	YS3:8	YS3:7	YS3:6	**Strain ID**
Cocci (chains)	Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	**Cell morphology**
-	-	+	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
-	-	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+	+ V	+	+	+ V	**pH 3.9**
+ V	+	+	+	+	**pH 9.6**
+	+	+	+	+	**6.5% NaCl**
-	+	-	+	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	+ V	+ V	+ V	**Raffinose**
-	-	+ V	+ V	+ V	**Ribose**
+ V	+	+	+ V	+	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	-	-	+	-	**Trehalose**
+ V	+ V	+ V	+ V	+ V	**Melibiose**
-	-	+ V	+ V	+	**Salicin**
-	-	-	+	+	**Mannitol**
-	-	+	-	-	**Arginine hydrolysis**

(Cont.)

YS4:5	YS4:4	YS4:3	YS4:2	YS4:1	**Isolate code**
Cocci (pairs)	Cocci (chains)	Cocci (chains)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
+	+	-	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ ≥	+ ≥	+ ≥	+ ≥	+	**pH 3.9**
+ ≥	+	+ ≥	+ ≥	+	**pH 9.6**
+ ≥	+ ≥	+ ≥	+ ≥	+ ≥	**NaCl 6.5%**
-	+	+	-	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+ ≥	+ ≥	-	-	+	**Mannose**
-	+	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	+ ≥	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	-	-	**Trehalose**
+	+	-	+	-	**Melibiose**
-	-	-	-	-	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

YS4:10	YS4:9	YS4:8	YS4:7	YS4:6	**Isolate code**
Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Cocci (pairs)	**Cell morphology**
+	+	-	-	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ ≥	+ ≥	-	+ ≥	+ ≥	**pH 3.9**
+ ≥	+ ≥	+ ≥	+ ≥	+ ≥	**pH 9.6**
+ ≥	+ ≥	+ ≥	+ ≥	+ ≥	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	+	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	+	-	-	-	**Raffinose**
-	-	+ ≥	-	-	**Ribose**
+	+ ≥	-	-	+ ≥	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	-	+	-	-	**Trehalose**
+	+	+ ≥	+	+	**Melibiose**
-	-	+ ≥	+ ≥	+ ≥	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	+	**Arginine hydrolysis**

(Cont.)

YS5:5	YS5:4	YS5:3	YS5:2	YS5:1	**Isolate code**
Cocci (pairs)	Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	**Cell morphology**
+	+	+	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ w	+ w	+ w	+ w	+	**pH 3.9**
+ w	+ w	+ w	+ w	+	**pH 9.6**
+ w	+ w	+ w	+ w	+ w	**NaCl 6.5%**
-	+	+	-	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+ w	+ w	-	-	+	**Mannose**
-	+	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	+ w	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	-	-	**Trehalose**
+	+	-	+	-	**Melibiose**
-	-	-	-	-	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

YS5:10	YS5:9	YS5:8	YS5:7	YS5:6	**Isolate code**
Cocci (pairs)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (pairs)	**Cell morphology**
+	+	+	+	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**pH 3.9**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**pH 9.6**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	+	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	+	-	-	-	**Raffinose**
-	-	+ ≤	-	-	**Ribose**
+	+ ≤	-	-	+ ≤	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	-	+	-	-	**Trehalose**
+	+	+ ≤	+	+	**Melibiose**
-	-	+ ≤	+ ≤	+ ≤	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	+	**Arginine hydrolysis**

Table 14: Phenotypic characterization of lab isolated from *mohi* (cow-milk)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
MH1	Cocci (chains)	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+
MH2	Cocci (pairs)	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	+	-	+
MH3	Cocci (chains)	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+
MH4	Cocci (pairs)	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	+	-	+
MH5	Cocci (chains)	+	+	+	-	-	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	-	+	-	-

(Cont.)

MH10	MH9	MH8	MH7	MH6	**Isolate code**
Cocci (chains)	Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
-	-	+	-	+	**15°C**
-	-	-	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
-	-	-	-	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	+	+	**Cellobiose**
+	-	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
-	-	-	-	-	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	+	+	+	+	**Salicin**
-	-	+	-	-	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

MH15	MH14	MH13	MH12	MH11	**Isolate code**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
+	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
-	-	+	-	-	**15°C**
+	-	-	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
-	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	+	+	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
+	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
-	-	-	-	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+ w	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
+	+	+	+	+	**Salicin**
-	-	-	-	-	**Mannitol**
-	+	+	+	+	**Arginine hydrolysis**

(Cont.)

MH20	MH19	MH18	MH17	MH16	**Isolate code**
Cocci (pairs)	Cocci (chains)	Coccobacilli (chains)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
-	-	+	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	+	+	+	-	**15°C**
+	+	-	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
-	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
-	+	-	-	+	**Glucose**
+	+	+	+	+	**Fructose**
+ w	+	+	+	+	**Cellobiose**
+	-	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	-	-	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+ w	+	-	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
-	+	+	+	+	**Salicin**
-	-	-	-	-	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

MH25	MH24	MH23	MH22	MH21	**Isolate code**
Coccobacil -li (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
+	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	-	-	-	-	**15°C**
-	-	-	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
+	+	+	-	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	-	+	**Cellobiose**
+	+	+ ⩾	+	+ ⩾	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	+	-	-	-	**Xylose**
-	-	-	+	-	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+ ⩾	+ ⩾	-	**Trehalose**
-	-	-	-	-	**Melibiose**
-	+	-	-	-	**Salicin**
-	+	-	-	-	**Mannitol**
-	+	-	-	-	**Arginine hydrolysis**

(Cont.)

MH30	MH29	MH28	MH27	MH26	**Isolate code**
Cocci (chains)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
+	-	-	-	-	**15°C**
-	+	+	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	+	+	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	+ W	**Ribose**
-	-	-	-	+	**Xylose**
-	-	-	+	-	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	+	+	+	**Trehalose**
-	-	-	-	-	**Melibiose**
+	+	+	+	+	**Salicin**
-	-	-	-	+	**Mannitol**
-	+	-	-	+	**Arginine hydrolysis**

(Cont.)

MH35	MH34	MH33	MH32	MH31	**Isolate code**
Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Cocci (pairs)	**Cell morphology**
+	-	-	-	-	**CO_2 pdtn**
+	+	+	+	+	**10°C**
-	-	-	+	+	**15°C**
-	-	-	-	-	**45°C**
-	-	-	-	-	**pH 3.9**
-	-	-	-	-	**pH 9.6**
-	-	-	-	-	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
+	+	+	+	+	**Cellobiose**
+	w+	+	+	+	**Mannose**
-	-	-	-	+	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	w+	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	-	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	+	+	**Trehalose**
w+	-	-	-	-	**Melibiose**
-	w+	-	+	+	**Salicin**
-	-	-	-	-	**Mannitol**
-	-	-	-	-	**Arginine hydrolysis**

(Cont.)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
MH36	Cocci (chains)	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-
MH37	Cocci (chains)	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	+	-	-
MH38	Cocci (chains)	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	w+	-	+	-	+	-	+	-	+
MH39	Cocci (chains)	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	-	-	-
MH40	Cocci (pairs)	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-

- Negative; + Positive; w+ Weakly positive.

Table 15: Phenotypic characterization of lab isolated from hard *chhurpi* (yak-milk)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
YS6:01	Cocci (pairs)	+	-	+	+	w +	w +	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	w +	-	-
YS6:02	Cocci (pairs)	+	-	+	+	w +	w +	+	+	+	+	+	-	w +	+	-	-	-	-	+	-	+	+	w +	-	-
YS6:03	Cocci (chains)	-	-	+	+	w +	w +	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	w +	-	-	-
YS6:04	Cocci (pairs)	-	-	-	+	w +	+	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	-	w +	-	+	+
YS6:05	Cocci (pairs)	+	-	+	+	+	w +	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	-	w +	-	+	+

(Cont.)

YS6:10	YS6:09	YS6:08	YS6:07	YS6:06	**Isolate code**
Coccobacilli (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (pairs)	**Cell morphology**
+	-	-	-	+	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	-	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ ≤	+ ≤	+ ≤	+ ≤	-	**pH 3.9**
+ ≤	+	+ ≤	+ ≤	+ ≤	**pH 9.6**
+	+	+	+	+	**NaCl 6.5%**
+	+	-	+	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	-	-	+	-	**Trehalose**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**Melibiose**
+ ≤	-	-	-	+ ≤	**Salicin**
-	-	-	+	+	**Mannitol**
-	-	+	-	-	**Arginine hydrolysis**

(Cont.)

YS7:5	YS7:4	YS7:3	YS7:2	YS7:1	**Isolate code**
Cocci (pairs)	Coccobacilli (pairs)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
+	+	-	+	-	**CO_2 pdtn**
-	-	-	-	-	**10°C**
-	+	+	-	+	**15°C**
+	+	+	+	+	**45°C**
-	+ V	+ V	+ V	+ V	**pH 3.9**
+	+ V	+ V	+ V	+ V	**pH 9.6**
+ V	+ V	+ V	+	+	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+ V	+	**Mannose**
+	+	+	+	+	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	+ V	+ V	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	+	+	+	+	**Trehalose**
+	-	+ V	+	+ V	**Melibiose**
+ V	+ V	+ V	+ V	-	**Salicin**
+	-	-	+	+	**Mannitol**
+	+	+	+	+	**Arginine hydrolysis**

(Cont.)

YS7:10	YS7:9	YS7:8	YS7:7	YS7:6	**Isolate code**
Cocci (pairs)	Cocci (pairs)	Cocci (chains)	Coccobacilli (pairs)	Cocci (chains)	**Cell morphology**
-	-	+	+	-	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ V	+ V	+ V	-	+ V	**pH 3.9**
+	+ V	+	+	+	**pH 9.6**
+	+	+	+	+	**NaCl 6.5%**
-	-	-	-	+	**Lactose**
+	-	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+ V	-	-	+	+	**Mannose**
+	+	+	+	+	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	+ V	-	-	+ V	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	-	-	-	-	**Trehalose**
+	+	+	+	-	**Melibiose**
+ V	-	-	+ V	+ V	**Salicin**
-	+	+	+	+	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

YS7:15	YS7:14	YS7:13	YS7:12	YS7:11	**Isolate code**
Cocci (pairs)	Cocci (chains)	Cocci (pairs)	Cocci (pairs)	Cocci (chains)	**Cell morphology**
+	+	+	+	-	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	+	+	**15°C**
+	+	+	+	+	**45°C**
+ V	+ V	+ V	+ V	+ V	**pH 3.9**
+	+	+ V	+	+	**pH 9.6**
+ V	+ V	-	+ V	+ V	**NaCl 6.5%**
-	-	+	-	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+ V	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	+ V	-	+ V	-	**Xylose**
-	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	-	-	-	-	**Trehalose**
+	+	+	+ V	+	**Melibiose**
-	-	-	-	+ V	**Salicin**
+	+	+	-	-	**Mannitol**
+	+	+	-	-	**Arginine hydrolysis**

(Cont.)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
YS7:16	Cocci (pairs)	-	-	+	+	w+	+	w+	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-
YS7:17	Coccobacilli (pairs)	+	-	+	+	w+	+	w+	+	+	+	+	-	+	-	-	-	-	w+	+	-	+	+	-	+	-
YS7:18	Cocci (pairs)	+	-	+	+	w+	+	w+	-	+	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-
YS7:19	Cocci (chains)	-	-	+	+	w+	+	w+	-	+	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	+
YS7:20	Coccobacilli (pairs)	-	-	+	+	w+	w+	w+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	+

(-) Negative; (+) Positive; (w+) weakly positive.

Table 16: Phenotypic characterization of lab isolated from *philu* (yak-milk)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
YS8:01	Cocci (pairs)	-	-	+	+	w +	w +	-	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	w +	-	-
YS8:02	Coccobacilli (pairs)	+	-	+	+	w +	+	w +	+	+	+	+	-	w +	+	-	-	-	-	+	-	+	+	w +	-	-
YS8:03	Cocci (pairs)	-	-	-	-	w +	+	w +	+	+	+	+	-	+	+	-	-	-	-	+	-	+	w +	-	-	-
YS8:04	Cocci (chains)	-	-	-	+	w +	+	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	-	w +	-	+	+
YS8:05	Cocci (pairs)	+	-	+	+	w +	+	w +	+	+	+	+	-	+	-	-	-	-	-	+	-	-	w +	-	+	+

(Cont.)

YS8:10	YS8:09	YS8:08	YS8:07	YS8:06	**Isolate code**
Cocci (pairs)	Cocci (chains)	Cocci (pairs)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	+	-	-	**CO_2 pdtn**
-	-	-	-	-	**10°C**
-	-	+	-	+	**15°C**
+	+	+	-	+	**45°C**
+ ≤	+ ≤	+	+ ≤	+ ≤	**pH 3.9**
+ ≤	+ ≤	+	+	+	**pH 9.6**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**NaCl 6.5%**
+	+	-	+	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	-	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	-	-	+	-	**Trehalose**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**Melibiose**
+ ≤	-	-	-	+ ≤	**Salicin**
-	-	-	+	+	**Mannitol**
-	-	+	-	-	**Arginine hydrolysis**

YS8:15	YS8:14	YS8:13	YS8:12	YS8:11	**Isolate code**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	+	+	**CO_2 pdtn**
-	-	-	+	+	**10°C**
+	+	-	+	+	**15°C**
+	+	-	+	+	**45°C**
+ ≤	+ ≤	+ ≤	+ ≤	+	**pH 3.9**
+ ≤	+ ≤	+ ≤	+ ≤	+	**pH 9.6**
+ ≤	+ ≤	+ ≤	+ ≤	+ ≤	**NaCl 6.5%**
-	+	+	-	-	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+ ≤	+ ≤	-	-	+	**Mannose**
-	+	-	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	-	-	-	-	**Raffinose**
-	-	-	-	-	**Ribose**
-	-	+ ≤	-	-	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
+	+	-	-	-	**Trehalose**
+	+	-	+	-	**Melibiose**
-	-	-	-	-	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

YS8:20	YS8:19	YS8:18	YS8:17	YS8:16	**Isolate code**
Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	Cocci (chains)	**Cell morphology**
-	-	-	-	-	**CO_2 pdtn**
-	-	-	-	-	**10°C**
+	+	+	-	-	**15°C**
+	+	+	-	-	**45°C**
w+	w+	w+	w+	w+	**pH 3.9**
w+	w+	w+	w+	w+	**pH 9.6**
w+	w+	w+	w+	w+	**NaCl 6.5%**
+	+	+	+	+	**Lactose**
+	+	+	+	+	**Maltose**
+	+	+	+	+	**Glucose**
+	+	+	+	+	**Fructose**
-	-	-	-	-	**Cellobiose**
+	+	+	+	+	**Mannose**
-	-	+	-	-	**Rhamnose**
-	-	-	-	-	**Melezitose**
-	+	-	-	-	**Raffinose**
-	-	w+	-	-	**Ribose**
+	w+	-	-	w+	**Xylose**
+	+	+	+	+	**Sucrose**
-	-	-	-	-	**Arabinose**
-	-	+	-	-	**Trehalose**
+	+	w+	+	+	**Melibiose**
-	-	w+	w+	w+	**Salicin**
+	+	+	+	+	**Mannitol**
+	+	-	-	-	**Arginine hydrolysis**

(Cont.)

Isolate code	Cell morphology	CO_2 pdtn	10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melezitose	Raffinose	Ribose	Xylose	Sucrose	Arabinose	Trehalose	Melibiose	Salicin	Mannitol	Arginine hydrolysis
YS8:21	Cocci (chains)	-	-	+	+	+	+	w+	-	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	+	-
YS8:22	Cocci (chains)	-	-	+	+	w+	w+	w+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-
YS8:23	Cocci (chains)	-	-	-	-	w+	w+	w+	+	+	+	+	-	-	-	-	-	-	w+	+	-	-	-	-	+	-
YS8:24	Cocci (chains)	-	-	+	+	w+	w+	w+	+	+	+	+	-	w+	+	-	-	-	-	+	-	+	+	-	+	+
YS8:25	Cocci (chains)	-	-	+	+	w+	w+	w+	-	+	+	+	-	w+	-	-	-	-	-	+	-	+	+	-	+	+
YS8:26	Cocci (chains)	-	-	+	+	w+	w+	w+	+	+	+	+	-	+	-	-	-	-	w+	+	-	-	+	w+	+	+

(-) Negative; (+) Positive; (w+) Weakly positive.

On the basis of phenotypically characters including physiological, biochemical and sugar fermentation tests, 272 bacterial isolates were tentatively identified as *Leuconostoc* (44.8%), *Lactococcus* (38.2%), *Enterococcus* (11.3%), and *Streptococcus* (2.5%) including unidentified group (2.9%) (Table 17, Fig. 9). *Leuconostoc* spp. dominated the overall distribution of LAB identified by phenotypic characterization from NFM of Sikkim.

Table 17: Tentative identification of lactic acid bacteria osolated NFM products of Sikkim based on phenotypic characterization

Arginine hydrolysis	CO_2 roduction	Temperature			pH		Salt	Sugar fermentation															Tentative identity (No. of isolates)
		10°C	15°C	45°C	pH 3.9	pH 9.6	NaCl 6.5%	Lactose	Maltose	Glucose	Fructose	Cellobiose	Mannose	Rhamnose	Melizitose	Raffinose	Ribose	Xylose	Sucrose	Trehalose	Melibiose	Salicin	Mannitol	
+(43) v(0) -(79)	+ (122)	- (122)	+(99) v(2) -(21)	+(65) v(16) -(41)	+(29) v(61) -(32)	+(42) v(33) -(47)	+(12) v(32) -(78)	+(64) v(3) -(55)	+(99) v(2) -(21)	+(88) v(3) -(31)	+(88) v(4) -(30)	+(31) v(11) -(80)	+(77) v(11) -(34)	+(13) v(20) -(89)	+(0) v(25) -(97)	+(20) v(9) -(93)	+(28) v(9) -(85)	+(12) v(11) -(99)	+(106) v(0) -(16)	+(77) v(3) -(42)	+(34) v(13) -(75)	+(26) v(16) -(80)	+(36) v(2) -(84)	*Leuconostoc* (122)
+(41) v(0) -(63)	- (104)	- (104)	+(68) v(1) -(35)	+(31) v(2) -(71)	+(24) v(34) -(46)	+(10) v(21) -(73)	+(7) v(20) -(77)	+(91) v(1) -(12)	+(101) v(0) -(3)	+(82) v(1) -(21)	+(93) v(1) -(10)	+(40) v(4) -(60)	+(89) v(8) -(7)	+(12) v(3) -(89)	+(1) v(4) -(99)	+(0) v(10) -(99)	+(11) v(12) -(81)	+(5) v(9) -(90)	+(79) v(0) -(25)	+(79) v(3) -(22)	+(13) v(12) -(79)	+(30) v(13) -(61)	+(35) v(1) -(68)	*Lactococcus* (104)
+(10) v(0) -(21)	- (31)	- (31)	+(11) v(0) -(20)	+(20) v(1) -(10)	+(12) v(7) -(12)	+(3) v(5) -(15)	+(3) v(5) -(23)	+(24) v(1) -(6)	+(30) v(0) -(1)	+(24) v(0) -(7)	+(25) v(1) -(5)	+(7) v(0) -(24)	+(28) v(2) -(1)	+(0) v(1) -(30)	+(0) v(0) -(31)	+(0) v(2) -(29)	+(6) v(4) -(21)	+(1) v(1) -(29)	+(26) v(0) -(5)	+(26) v(0) -(5)	+(0) v(8) -(23)	+(1) v(1) -(29)	+(2) v(0) -(29)	*Enterococcus* (31)
+(1) v(0) -(6)	- (7)	- (7)	- (122)	+(5) v(0) -(2)	+(5) v(0) -(2)	+(5) v(0) -(2)	-	+(5) v(0) -(2)	+(7) v(0) -(0)	+(6) v(0) -(1)	+(7) v(0) -(0)	+(4) v(0) -(3)	+(6) v(0) -(1)	+(0) v(0) -(7)	+(0) v(0) -(7)	+(0) v(1) -(6)	+(2) v(0) -(5)	+(0) v(0) -(7)	+(3) v(0) -(4)	+(4) v(0) -(3)	+(0) v(0) -(7)	+(6) v(0) -(2)	+(3) v(4) -(0)	*Streptococcus* (7)
- (8)	- (8)	- (8)	- (8)	- (8)	- (8)	- (8)	- (8)	+ (8)	+ (8)	+ (8)	- (8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	+(0) v(0) -(8)	- (122)	- (122)	+(3) v(0) -(5)	+(3) v(0) -(5)	Unidentified isolates (8)

+, Positive; -, Negative; v, Variable

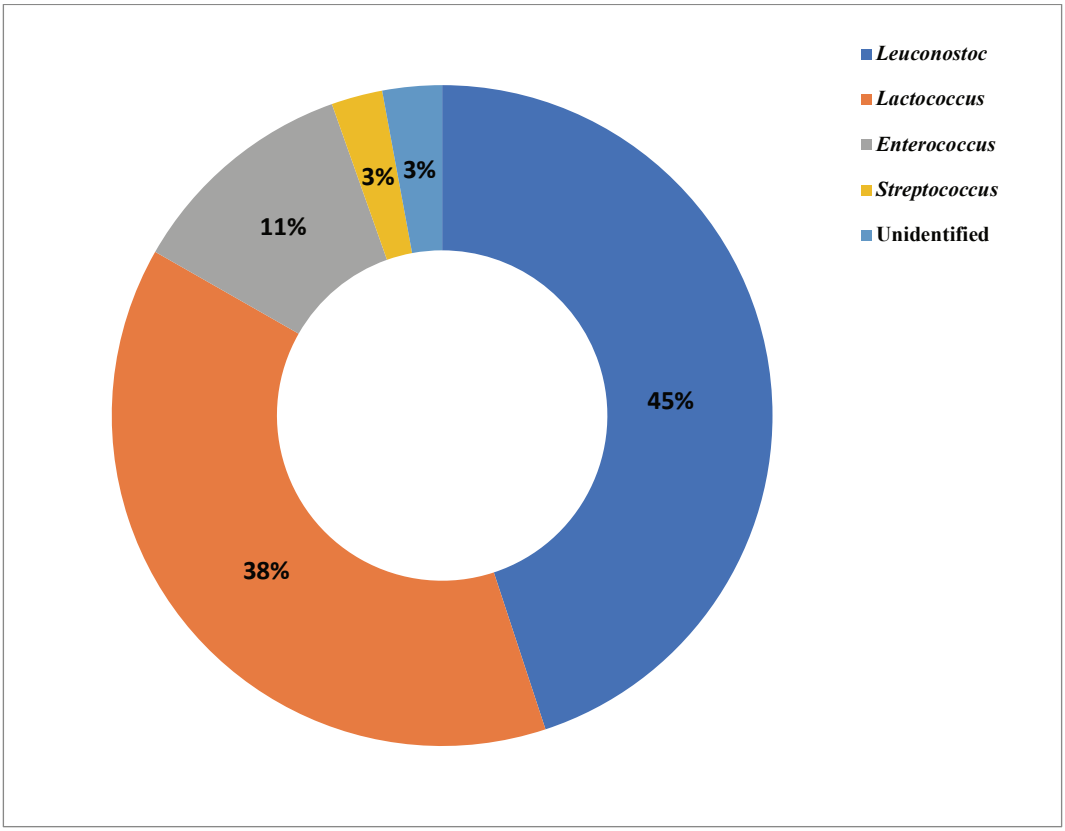


Figure 9: Tentative identification and distribution of LAB from NFM products of Sikkim based on phenotypic parameters.

On the basis of phenotypic characterizations and biochemical tests, 272 bacterial isolates were randomly grouped into 68 representative strains. These representative strains were: cow-milk *dahi* (10 representative strains)- DA1, DA3, DA4, DA8, DA10, DA11, DA14, DA35, DA41, DA66; yak-milk *dahi* (10)- DY2, DY3, DY14, DY16, DY18, DY19, DY29, DY30, DY36, DY42; cow-milk soft *chhurpi* (10)- SC3, SC4, SC5, SC7, SC11, SC17, SC19, SC22, SC26, SC30; yak-milk soft *chhurpi* (10)- YS4-1, YS4-3, YS4-4, YS4-7, YS4-8, YS4-9, YS4-10, YS4-11, YS4-14, YS4-15; cow-milk *mohi* (9)- MH3, MH4, MH9, MH15, MH18, MH20, MH22, MH39, MH40; hard yak-milk *chhurpi* (10)- YS7-1, YS7-2, YS7-3, YS7-4, YS7-5, YS7-7, YS7-8, YS7-10, YS7-12, YS7-13; and yak-milk *philu* (9)- YS8-1, YS8-3, YS8-4, YS8-5, YS8-7, YS8-8, YS8-10, YS8-11, YS8-13, respectively.

MOLECULAR IDENTIFICATION BY 16S rRNA GENE SEQUENCING

Bacterial Diversity

The genomic DNA of each isolate of 68 representative bacterial strains were extracted and PCR of each isolate was performed targeting 16S rRNA gene (Fig. 10). Amplified products were purified (Fig. 11) and genomic identification was done by Sanger sequencing method. For bacterial identification, sequences were assigned by comparing them with those available in the GenBank NCBI database using a BLAST 2.0. Phylogenetic tree based upon the Neighbor-Joining of 16S rDNA sequences derived by PCR with *Escherichia coli* ATCC 11775^{T} as outgroup was constructed using the Neighbor-joining method with 1000 bootstrap value replicates (Fig. 12). Several genera with species/sub-species isolated from naturally fermented milk products of Sikkim were identified by 16S rRNA gene sequence based on Basic Local Alignment Search Tool

(BLAST) (Table 18-19). Overall bacterial diversity in NFM products of Sikkim showed *Leuconostoc mesenteriodes* (43%) as the most dominant genus, followed by *Lactococcus lactis* subsp. *cremoris* (19%), *Lactococcus lactis* (16%), *Leuconostoc mesenteriodes* subsp. *jonggajibkimchii* (9%), *Enterococcus faecalis* (7%), *Lactococcus lactis* subsp. *hordniae* (2%), *Lactococcus lactis* subsp. *tructae* (2%), *Enterococcus italicus* (1%), and *Enterococcus pseudoavium* (1%) (Fig. 13)

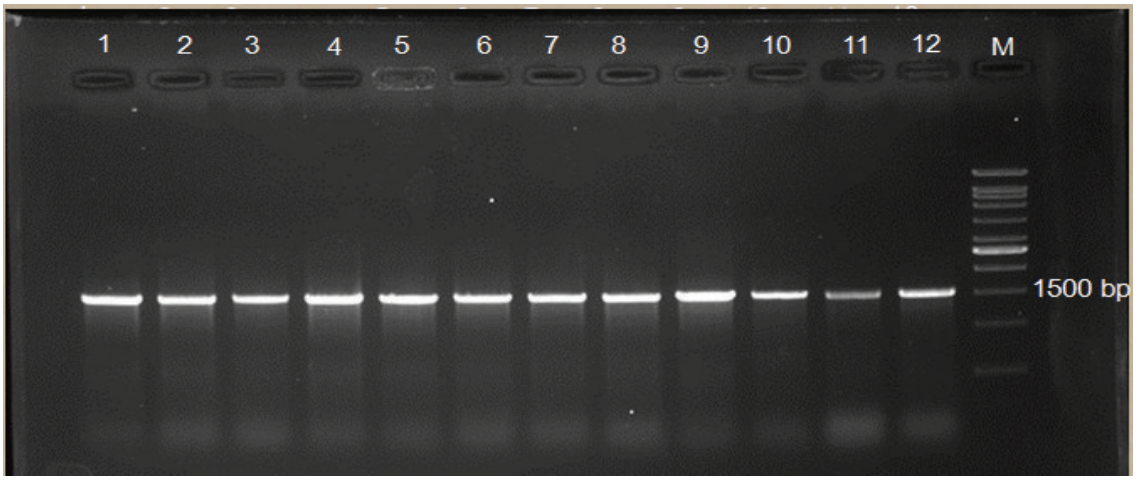


Figure 10: Agarose gel electrophoresis showing PCR amplification products of bacterial DNA targeting 16S rRNA region. Isolate code: 1-12, M: 1 kb DNA ladder. Isolate code 1-12; M 1kb ladder.

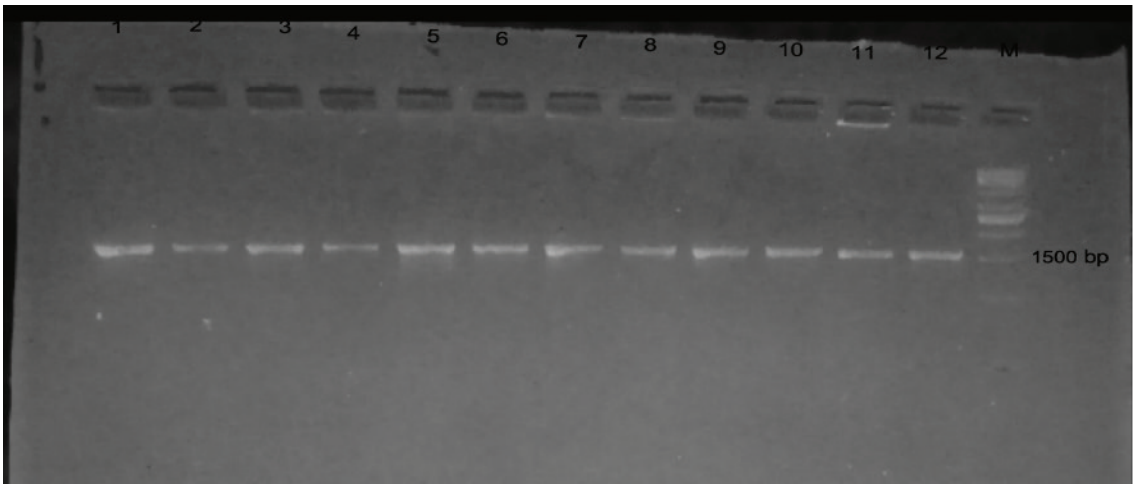


Figure 11: Agarose gel electrophoresis showing purified PCR amplification products of bacterial DNA targeting 16S rRNA region. Isolate code: 1-12, M: 1 kb DNA ladder.

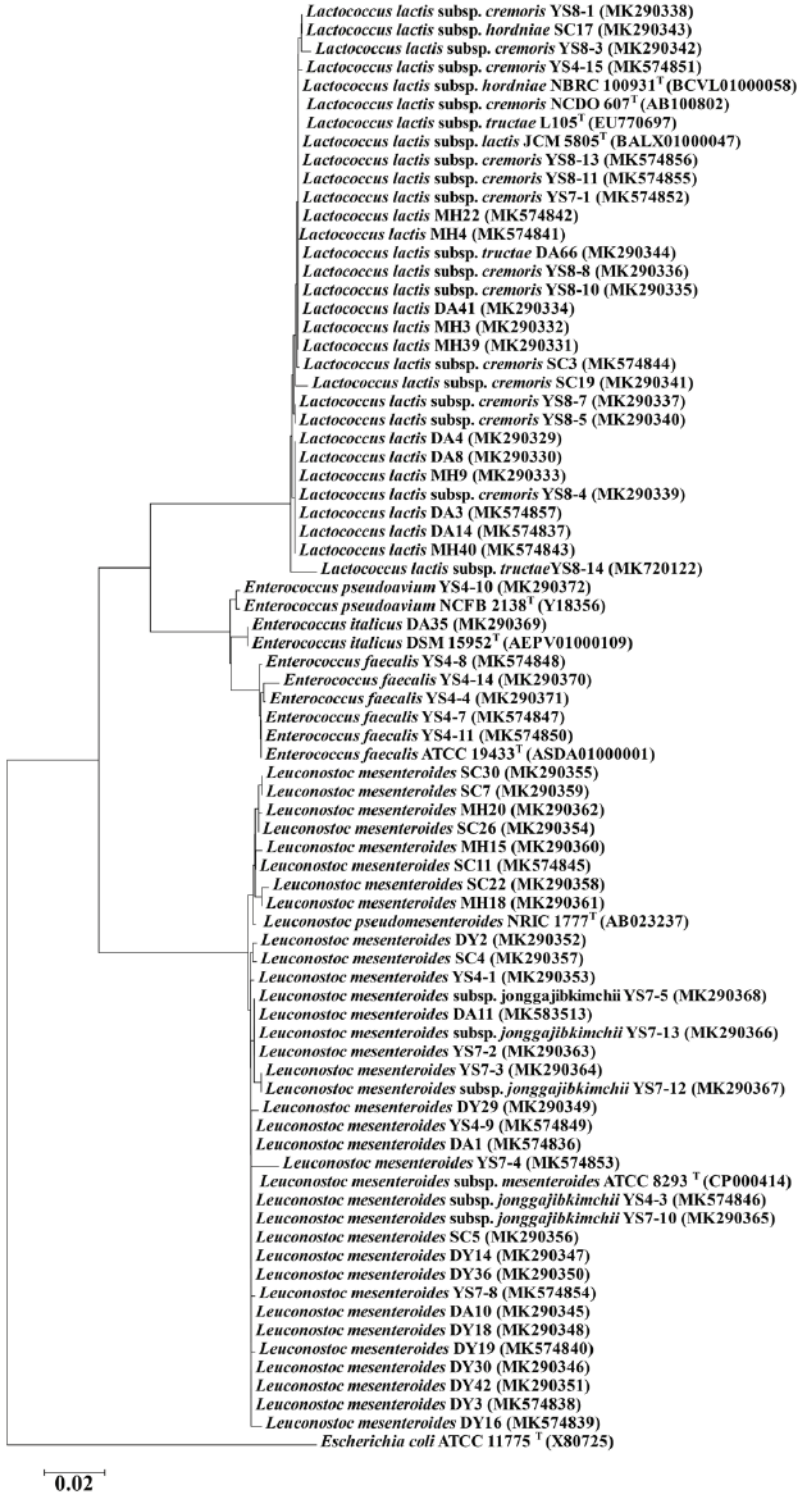


Figure 12: Molecular phylogenetic analysis of 68 bacterial isolates from naturally fermented milk products based on 16S rRNA region sequencing constructed by Neighbour-joining method using MEGA 7 with *Escherichia coli* ATCC 11775 as the out group.

Table 18: Molecular identification of bacterial strains isolated from naturally fermented cow-milk products of Sikkim by 16S rRNA gene sequence based on Basic Local Alignment Search Tool (BLAST)

Product	Isolate code	Identity	Type Species (% Similarity)	GenBank Accession Number	Size (base pair)
Dahi	DA1	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK574836	1439
	DA3	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (99%)	MK574857	1435
	DA4	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (98%)	MK290329	1412
	DA8	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (98%)	MK290330	1402
	DA10	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK290345	1400
	DA11	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK583513	1224
	DA14	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (99%)	MK574837	1391
	DA35	*Enterococcus italicus*	*Enterococcus italicus* strain LMG 21727 (99%)	MK290369	1432
	DA41	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (99%)	MK290334	1411
	DA66	*Lactococcus lactis* subsp. *tructae*	*Lactococcus lactis* subsp. *tructae* strain L105 (99%)	MK290344	1420
Soft *Chhurpi*	SC3	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK574844	1280
	SC4	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (97%)	MK290357	1431
	SC5	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii*	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii* strain DRC1506 (99%)	MK290356	1424
	SC7	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290359	1428
	SC11	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK574845	1406
	SC17	*Lactococcus lactis* subsp. *hordniae*	*Lactococcus lactis* subsp. *hordniae* strain NBRC 100931 (99%)	MK290343	1426

	SC19	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK290341	1265
	SC22	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain NRIC 1777 (99%)	MK290358	1433
	SC26	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290354	1431
	SC30	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290355	1435
Mohi	MH3	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (97%)	MK290332	1431
	MH4	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (100%)	MK574841	1300
	MH9	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (99%)	MK290333	1436
	MH15	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain NRIC 1777 (99%)	MK290360	1434
	MH18	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290361	1424
	MH20	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290362	1424
	MH22	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (100%)	MK574842	1332
	MH39	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (98%)	MK290331	1425
	MH40	*Lactococcus lactis*	*Lactococcus lactis* strain NBRC 100933 (99%)	MK574843	1368

Table 19: Molecular identification of bacterial strains isolated from naturally fermented yak-milk products of Sikkim by 16S rRNA gene sequence based on Basic Local Alignment Search Tool (BLAST)

Product	Isolate code	Identity	Type Species (% Similarity)	GenBank Accession Number	Size (base pair)
Dahi	DY2	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290352	1426
	DY3	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK574838	1420
	DY14	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK290347	1400
	DY16	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK574839	1387
	DY18	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK290348	1421
	DY19	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK574840	1401
	DY29	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290349	1428
	DY30	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK290346	1419
	DY36	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290350	1415
	DY42	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK290351	1413
Soft *Chhurpi*	YS4-1	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290353	1424
	YS4-3	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii*	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii* strain DRC1506 (99%)	MK574846	1348
	YS4-4	*Enterococcus faecalis*	*Enterococcus faecalis* strain ATCC 19433 (99%)	MK290371	1415
	YS4-7	*Enterococcus faecalis*	*Enterococcus faecalis* strain ATCC 19433 (100%)	MK574847	1358
	YS4-8	*Enterococcus faecalis*	*Enterococcus faecalis* strain ATCC 19433 (99%)	MK574848	1407
	YS4-9	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK574849	1326
	YS4-10	*Enterococcus pseudoavium*	*Enterococcus pseudoavium* strain NBRC 100491 (99%)	MK290372	1441

	YS4-11	*Enterococcus faecalis*	*Enterococcus faecalis* strain NBRC 100480 (99%)	MK574850	1356
	YS4-14	*Enterococcus faecalis*	*Enterococcus faecalis* strain ATCC 19433 (99%)	MK290370	1429
	YS4-15	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK574851	1334
Hard *Chhurpi*	YS7-1	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK574852	1385
	YS7-2	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290363	1450
	YS7-3	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (99%)	MK290364	1417
	YS7-4	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK574853	1291
	YS7-5	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii*	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii* strain DRC1506 (97%)	MK290368	1386
	YS7-7	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii*	*Leuconostoc mesenteroides subsp. jonggajibkimchii* strain DRC1506 (98%)	MK796023	1429
	YS7-8	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* strain ATCC 8293 (98%)	MK574854	1299
	YS7-10	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	*Leuconostoc mesenteroides subsp. jonggajibkimchii* strain DRC1506 (97%)	MK290365	1474
	YS7-12	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	*Leuconostoc mesenteroides subsp. jonggajibkimchii* strain DRC1506 (99%)	MK290367	1434
	YS7-13	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	*Leuconostoc mesenteroides subsp. jonggajibkimchii* strain DRC1506 (99%)	MK290366	1431

Philu	YS8-1	*Lactococcus lactis subsp. cremoris*	*Lactococcus lactis subsp. cremoris strain* NBRC 100676 (99%)	MK290338	1421
	YS8-3	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (97%)	MK290342	1344
	YS8-4	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (98%)	MK290339	1468
	YS8-5	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (97%)	MK290340	1338
	YS8-7	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis subsp. cremoris* strain NBRC 100676 (97%)	MK290337	1462
	YS8-8	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis subsp. cremoris* strain NBRC 100676 (98%)	MK290336	1434
	YS8-10	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis subsp. cremoris* strain NBRC 100676 (98%)	MK290335	1419
	YS8-11	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK574855	1348
	YS8-13	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris* strain NBRC 100676 (99%)	MK574856	1338

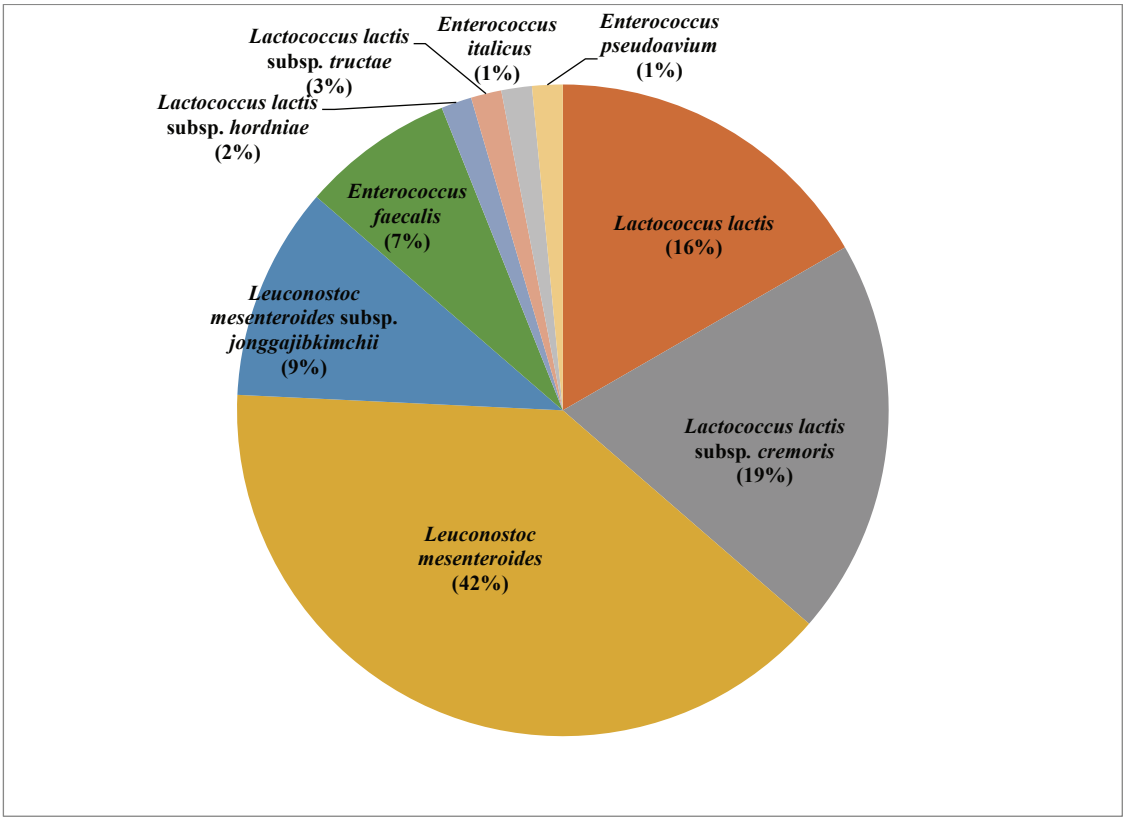


Figure 13: Percentile distribution of lactic acid bacteria present in naturally fermented milk products of Sikkim identified based on 16s rRNA sequencing method.

In sample-wise distribution of lactic acid bacteria in NFM prepared from cow's milk, it was found that *dahi* sample was dominated by *Lactococcus lactis* (50%) followed by *Leuconostoc mesenteriodes* (30%), *Lactococcus lactis* subsp. *tructae* (10%), and *Enterococcus italicus* (10%) (Fig. 14). Similarly, bacterial diversity in NFM products prepared from yak milk was also observed. *Leuconostoc mesenteriodes* (100%) was found predominate in the *dahi* sample, with no other LAB genera detected (Fig. 15).

In soft *chhurpi* (cow-milk), the genus *Leuconostoc mesenteroides* (70%) was found dominant genus followed by *Lactococcus lactis* subsp. *cremoris* (20%), and *Lactococcus lactis* subsp. *hordniae* (10%) (Fig. 16). In soft *chhurpi* (yak-milk), *Enterococcus faecalis* (50%) was most dominant followed by *Leuconostoc mesenteroides* (20%), *Lactococcus lactis* subsp. *cremoris* (10%), *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* (10%), and *Enterococcus pseudoavium* (10%) (Fig. 17). In *mohi* sample (cow-milk), it was found *Lactococcus lactis* (67%) was predominant genus followed by *Leuconostoc mesenteriodes* (33%) (Fig. 18).

In hard *chhurpi* (yak-milk), the dominant genus was *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* (50%) followed by *Leuconostoc mesenteroides* (40%), and *Lactococcus lactis* subsp. *cremoris* (10%) (Fig. 19). *Lactococcus lactis* subsp. *cremoris* (100%) was the only genus detected in *philu* sample prepared from yak-milk (Fig. 20).

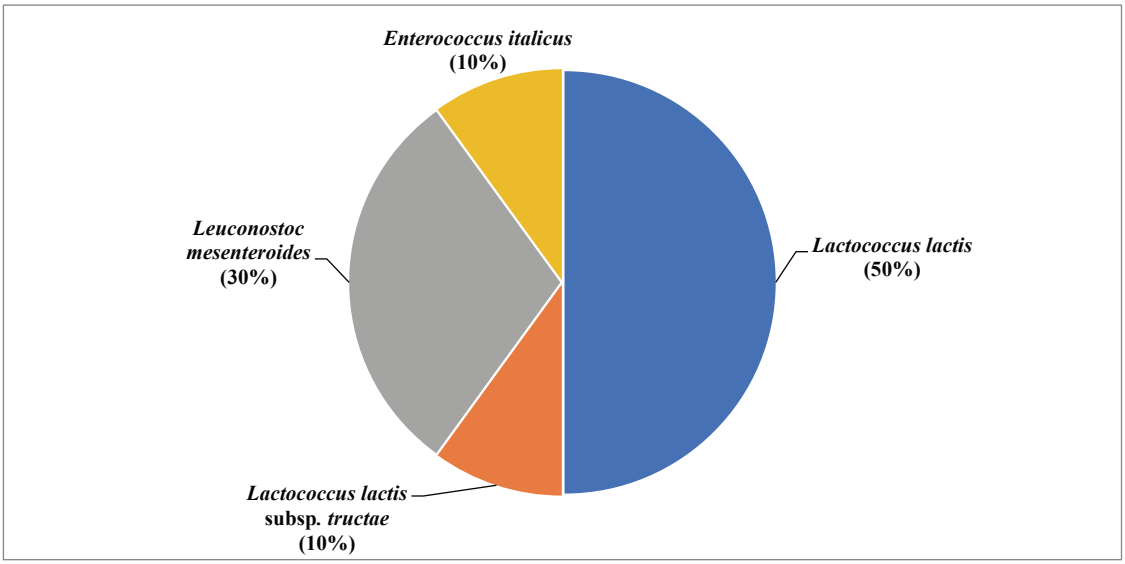


Figure 14: Percentile distribution of different species of lactic acid bacteria present in *dahi* sample prepared from cow's milk.

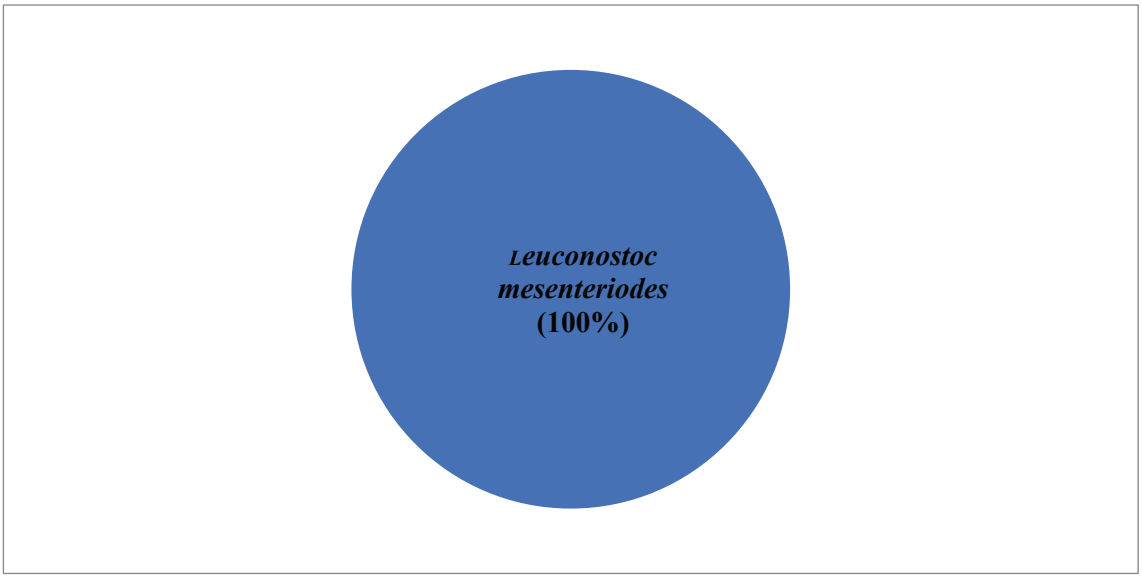


Figure 15: Percentile distribution of different species of lactic acid bacteria present *dahi* sample prepared from yak's milk.

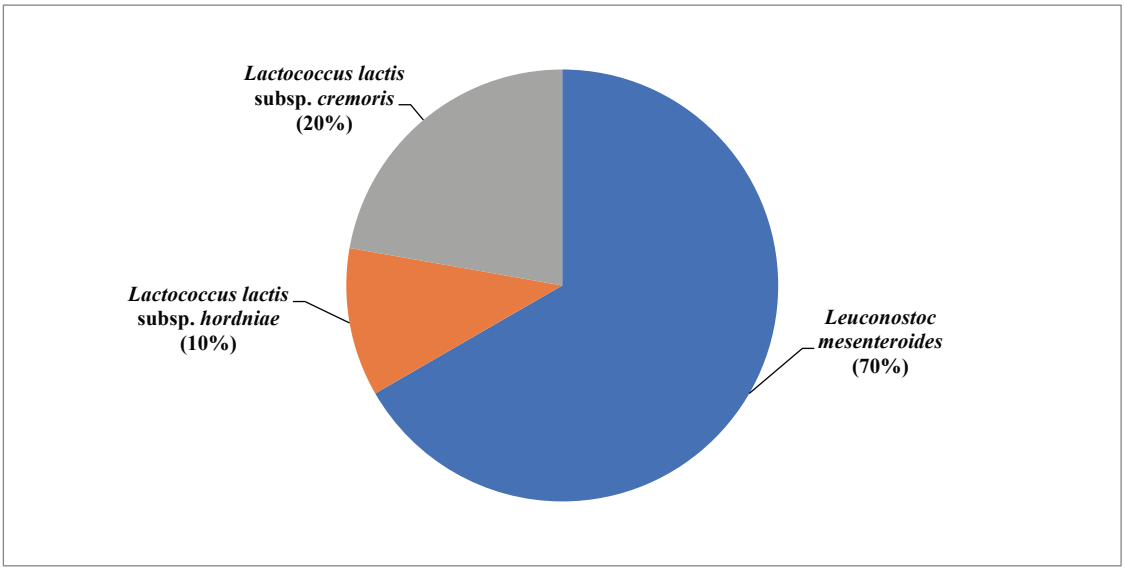


Figure 16: Percentile distribution of different species of lactic acid bacteria present in soft *chhurpi* sample prepared from cow's milk.

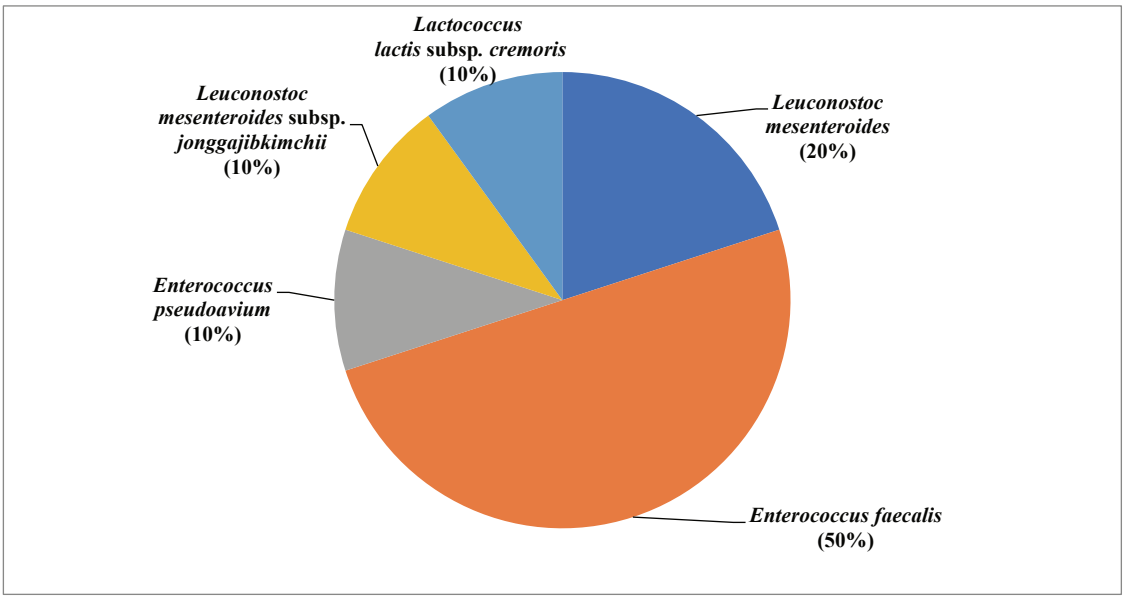


Figure 17: Percentile distribution of different species of lactic acid bacteria present in soft *chhurpi* sample prepared from yak's milk.

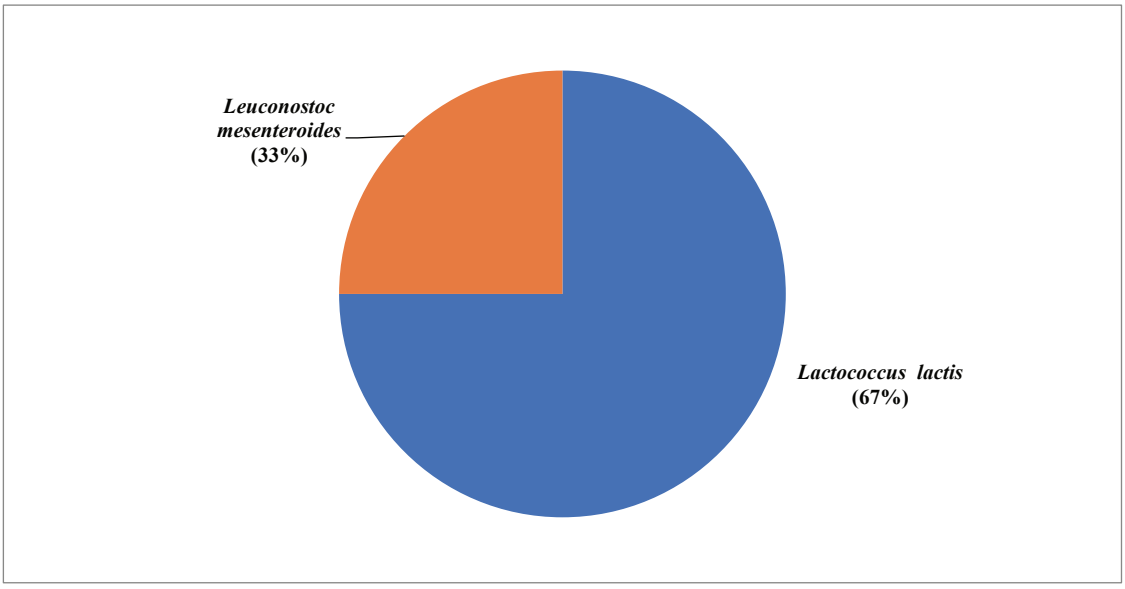


Figure 18: Percentile distribution of different species of lactic acid bacteria present in *mohi* sample prepared from cow's milk.

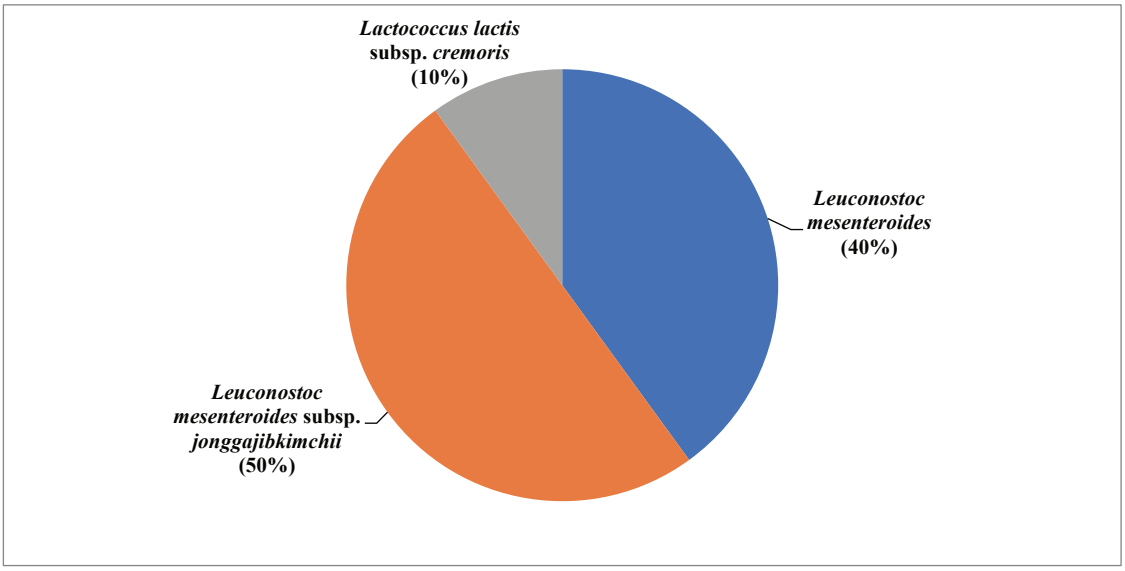


Figure 19: Percentile distribution of different species of lactic acid bacteria present in hard *chhurpi* sample prepared from yak's milk.

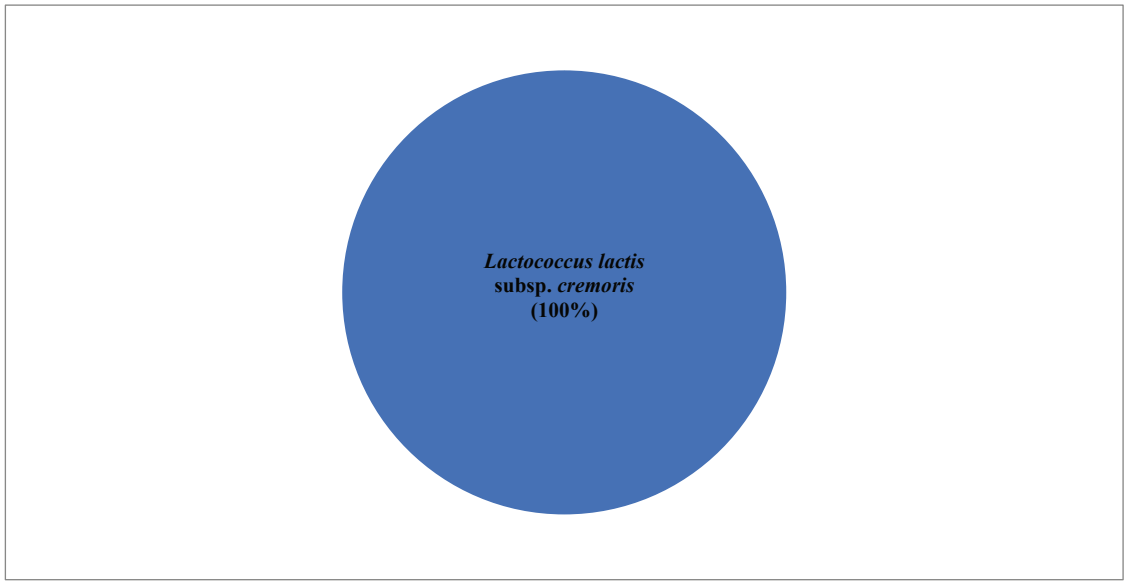


Figure 20: Percentile distribution of different species of lactic acid bacteria present in *philu* sample prepared from yak's milk.

In summary, distribution of LAB in NFM products of Sikkim is as follows:

Dahi **(cow-milk)**	*Leuconostoc mesenteroides, Lactococcus lactis, Enterococcus italicus, Lactococcus lactis* subsp. *tructae.*
Soft *chhurpi* (cow-milk)	*Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii, Lactococcus lactis* subsp. *hordniae, Lactococcus lactis* subsp. *cremoris.*
Mohi **(cow-milk)**	*Lactococcus lactis, Leuconostoc mesenteroides*.
Dahi **(yak-milk)**	*Leuconostoc mesenteroides.*
Soft *chhurpi* (yak-milk)	*Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii, Enterococcus faecalis, Enterococcus pseudoavium, Lactococcus lactis* subsp. *cremoris.*
Philu **(yak-milk)**	*Lactococcus lactis* subsp. *cremoris.*
Hard *chhurpi* (yak-milk)	*Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii*.

Diversity Indices

Diversity indices of species of LAB from different NFM products were calculated by Simpson's index (1-*D*), Shannon's index (*H*), Dominance (*D*) and Chao-1 (Table 20).

The Simpson's diversity index (1-*D*) values ranged between 0.4444 in *mohi* (cow-milk) to 0.64 in *dahi* (cow-milk). Shannon diversity index *H* for assessing bacterial diversity was recorded the highest from *dahi* (cow-milk) (*H*: 1.168), and lowest from *mohi* (cow-milk) (*H*: 0.6365). The dominance D values were 0.36 in *dahi* (cow-milk), 1 in *dahi* (yak-milk), 0.54 in soft *chhurpi* (cow-milk), 0.32 in soft *chhurpi* (yak-milk), 0.5556 in *mohi* (cow-milk), 0.42 in hard *chhurpi* (yak-milk) and 1 in *philu* (yak-milk) respectively. Chao 1 index showed the evaluation of species richness based on abundance. The highest species richness was recorded 6.5 in soft *chhurpi* (yak-milk) and lowest 1 in *dahi* (yak-milk) and *philu* (yak-milk), respectively (Table 20).

Table 20: Diversity indices of different NFM products of Sikkim				
Product	**Diversity indices**			
	Simpson's index (1-*D*)	**Shannon's index (*H*)**	**Dominance (*D*)**	**Chao-1**
Dahi (cow-milk)	0.64	1.168	0.36	5
Dahi (yak-milk)	0	0	1	1
Soft *chhurpi* (cow-milk)	0.46	0.8018	0.54	3
Soft *chhurpi* (yak-milk)	0.68	1.359	0.32	6.5
Mohi (cow-milk)	0.4444	0.6365	0.5556	2
Hard *chhurpi* (yak-milk)	0.58	0.9433	0.42	3
Philu (yak-milk)	0	0	1	1

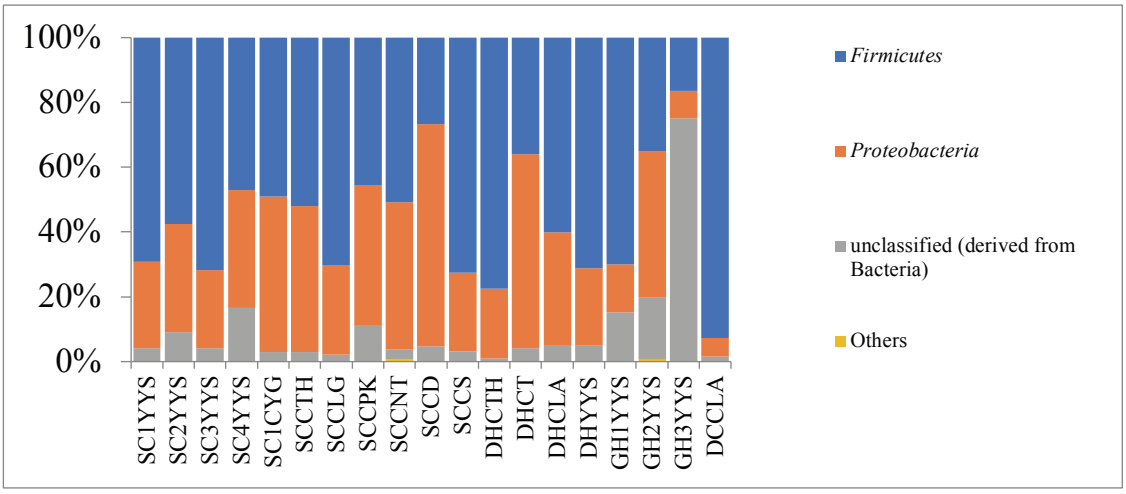
CULTURE-INDEPENDENT METHOD BY HIGH-THROUGHPUT SEQUENCING METHOD

Culture-independent analysis of the microbial structure of NFM of Sikkim was performed using the next-generation sequencing (NGS) technique by high-throughput sequencing (HTS) method for profiling the bacterial communities present in naturally fermented milk products of Sikkim. We selected 19 samples of NFM products of Sikkim for HTS studies viz. soft *chhurpi* (cow-milk) (8 samples), soft *chhurpi* (yak-milk) (4), *gheu* (yak-milk) (3), and *dahi,* (cow-milk) (3), and *dahi* (yak-milk) (1).

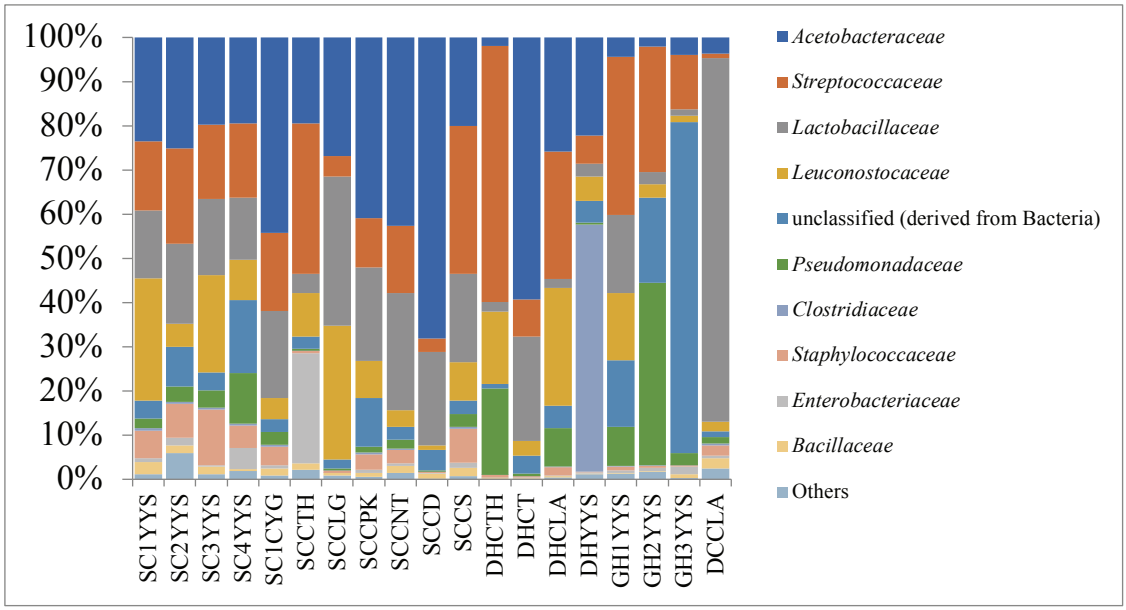
Overall bacterial communities in NFM products of Sikkim

Firmicutes (56.38%) was most dominant phylum at >1% abundance in NFM products viz. soft *chhurpi* (cow/yak), *gheu* and *dahi* (yak/cow) of Sikkim, followed by phylum *Proteobacteria* (33.58%), unclassified phyla (9.91%) and others phyla (0.13%) detected at <1% abundance (Fig. 21a). Under phylum *Firmicutes*, family *Streptococcaceae* (19.4%) was dominant followed by *Lactobacillaceae* (18.2%), *Leuconostocaceae* (10.7%), *Staphylococcaceae* (3%), *Bacillaceae* (1%) and *Clostridiaceae* (3.1%). Similarly, under phylum *Proteobacteria*, family *Acetobacteraceae* (24.8%) was dominant followed by *Pseudomonadaceae* (6%), *Enterobacteriaceae* (2.1%) and uncultured bacteria (9.9%) (Fig. 21b). In both cases, the relative abundance of those members with less than 1% was characterized as others (1.2%). At species level, the overall diversity of these NFM products was dominated by lactic acid bacteria- *Lactococcus lactis* (13.0%), *Lactobacillus helveticus* (10.7%), *Pseudomonas fluorescens* (5.1%), *Leuconostoc mesenteroides* (4.2%), *Leuconostoc pseudomesenteroides* (3.2%), *Lactococcus piscium* (2.9%), *Lactococcus raffinolactis* (2.8%), *Lactobacillus delbrueckii*

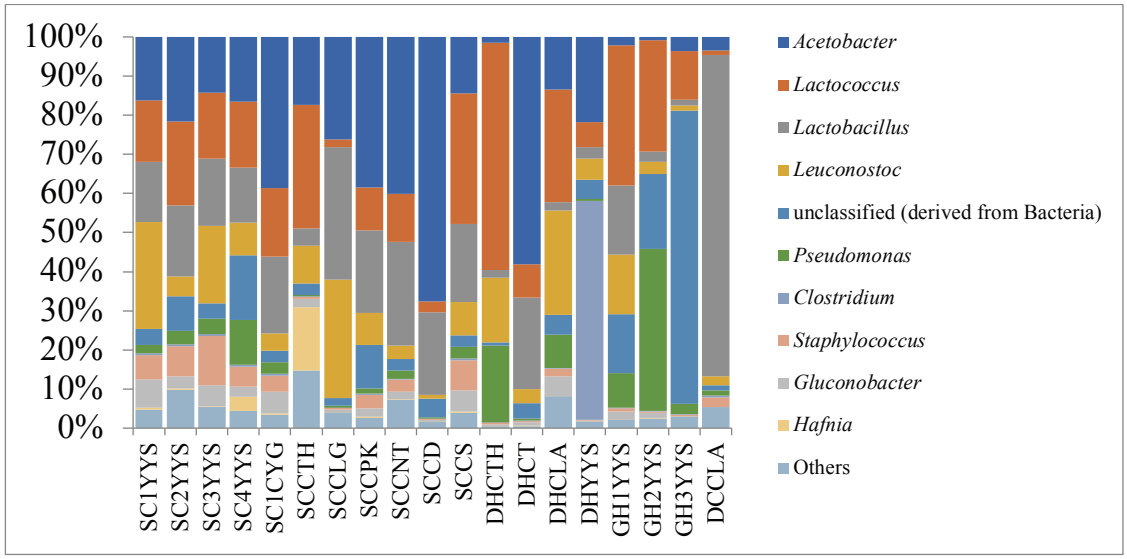
(2.7%), *Leuconostoc lactis* (2.1%) and *Lactobacillus gasseri* (1.4%) (Fig. 21d). Additionally, species belonging to the acetic acid bacteria were also detected which included *Acetobacter lovaniensis* (5.18%), *Acetobacter pasteurianus* (8.5%), *Gluconobacter oxydans* (1.6%), *Acetobacter syzygii* (6.8%), *Hafnia alvei* (1.2%) and others (12.8%). *Staphylococcus cohnii* (2.2%) and *Clostridium tyrobutyricum* (3.1%) were also detected from the samples. Approximately 9.8% of uncultured bacteria were also detected in NFM products of Sikkim.



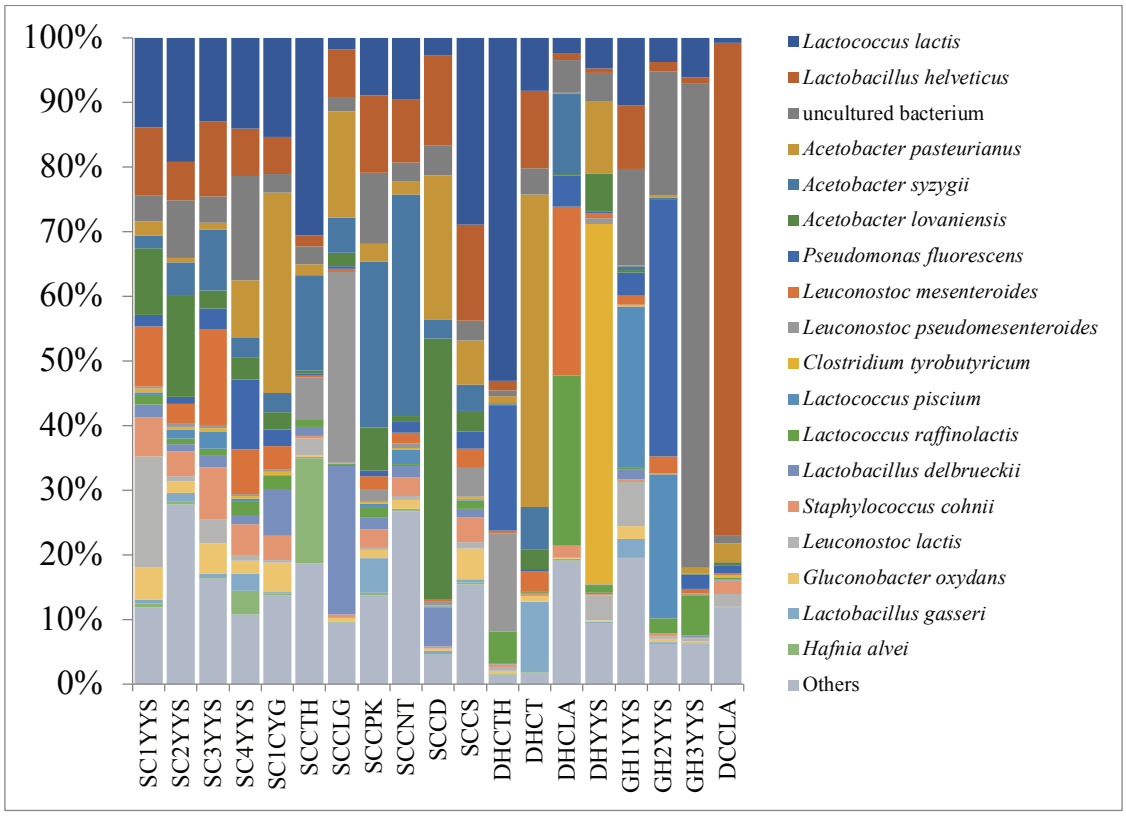
(a)



(b)



(c)

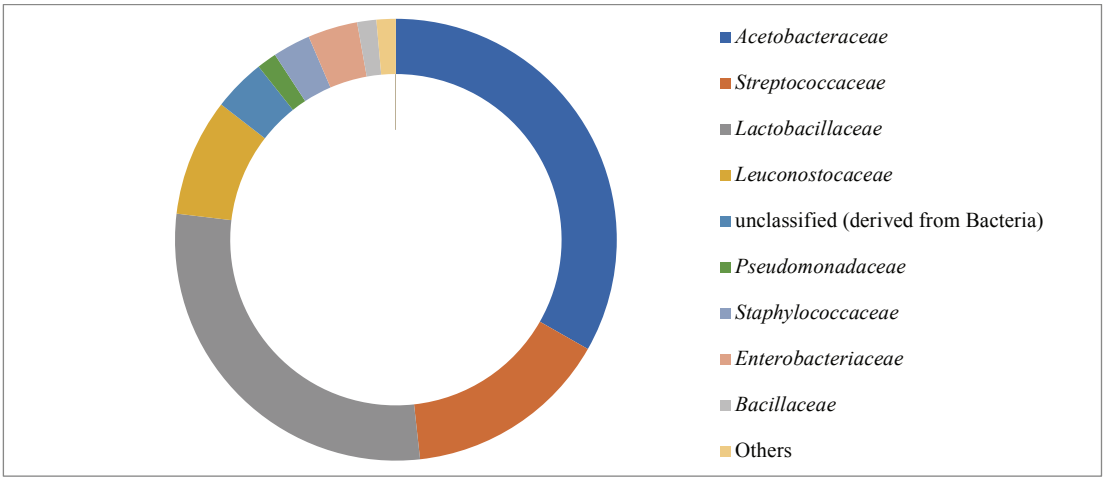


(d)

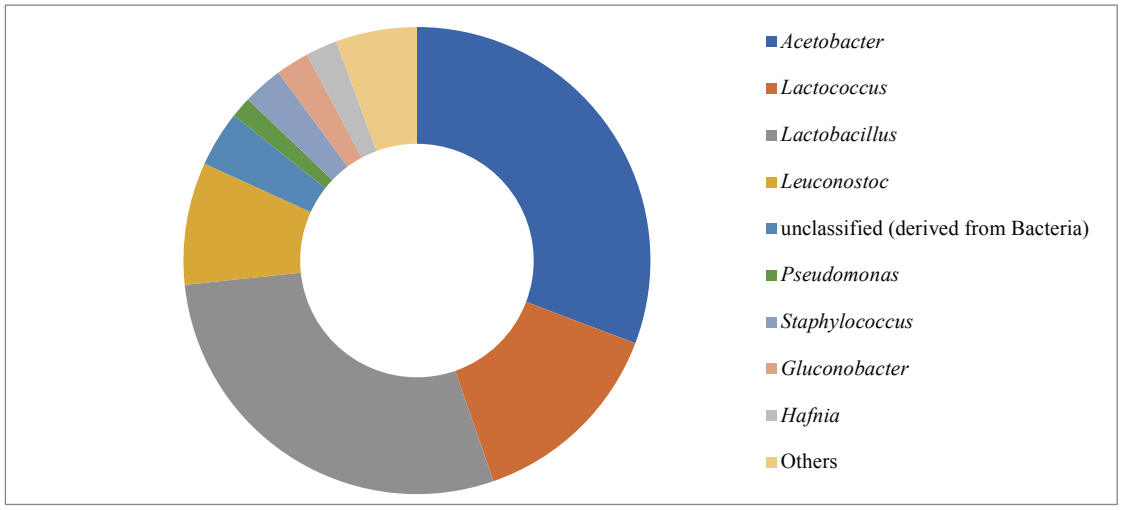
Figure 21: The overall bacterial composition of the four NFMs *chhurpi*, *dahi, gheu and* dudh *chhurpi* at different taxonomic level [a] Phylum; [b] Family; [c] Genus and [d] species.

Microbial diversity in soft *chhurpi* (cow-milk)

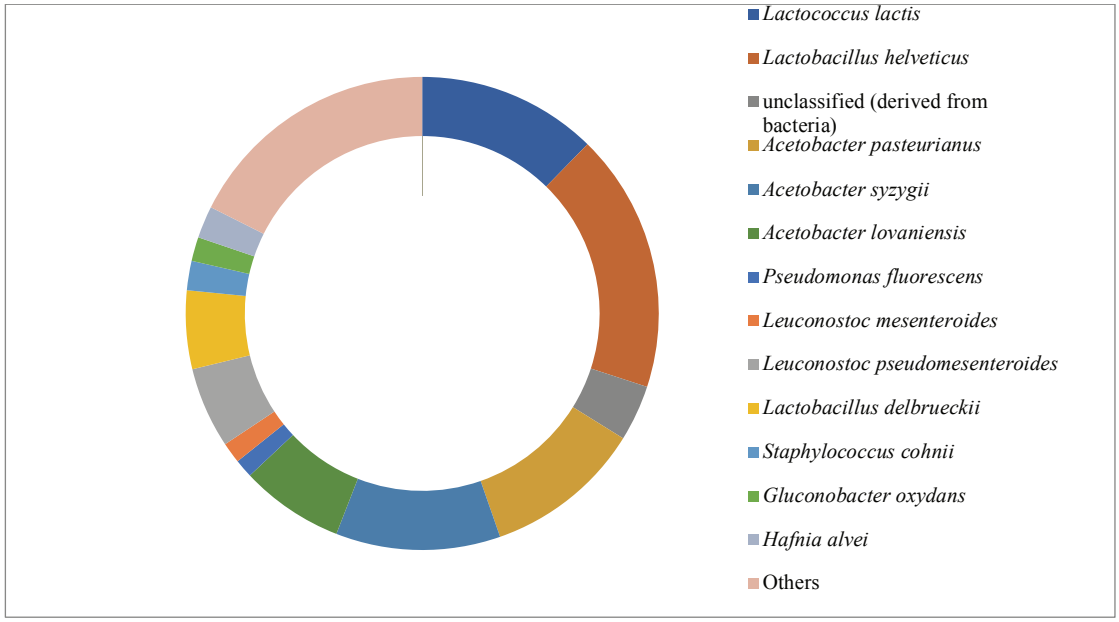
In samples of soft *chhurpi* (cow-milk) *Acetobacteraceae* (33.2%) was the most abundant family detected at >1% abundance followed by families *Lactobacillaceae* (28.6%), *Streptococcaceae* (15%), *Leuconostocaceae* (8.5%), *Enterobacteriaceae* (3.6%), *Staphylococcaceae* (2.7%), *Pseudomonaceae* (1.4%), *Bacillaceae* (1.4%) and others (1.4%). An average of 3.8% of uncultured bacteria was also recorded (Fig. 22a). In genus level, the samples were predominated by *Acetobacter* (30.7%), followed by *Lactobacillus* (28.6%), *Lactococcus* (13.9%), *Leuconostoc* (8.4%), *Staphylococcus* (2.7%), *Gluconobacter* (2.3%), *Hafnia* (2.1%) and *Pseudomonas* (1.4%). The relative abundance of those members with less than 0.1% (others) was (5.6%) (Fig. 22b). Similarly, in species level the following bacterial species were detected viz. *Lactobacillus helveticus* (17.6%), *Lactococcus lactis* (12.2%), *Acetobacter syzygii* (11.2%), *Acetobacter pasteurianus* (10.7%), *Acetobacter lovaniensis* (7%), *Leuconostoc pseudomesenteriodes* (5.5%), *Lactobacillus delbrueckii* (5.3%), *Staphylococcus cohnii* (2.0%), *Hafnia alveli* (2.1%), *Gluconobacter oxydans* (1.6%), *Leuconostoc mesenteriodes* (1.4%) and *Pseudomonas fluorescens* (1.2%), and others (17.2%) (Fig. 22c). An average of 3.8% of uncultured bacteria was recorded.



(a)



(b)



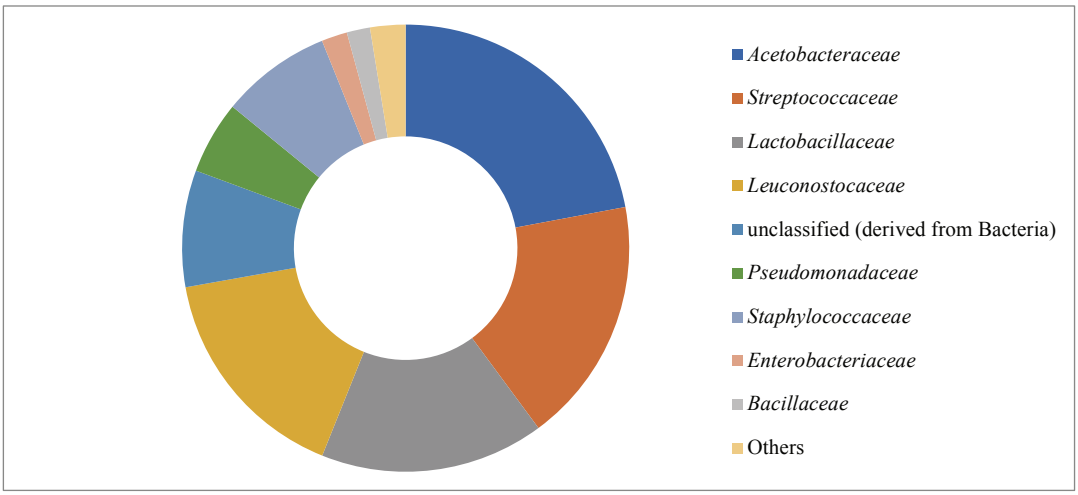
(c)

Figure 22: The bacterial composition of soft *chhurpi* (cow-milk) at different taxonomic level [a] Family [b] Genus and [c] species.

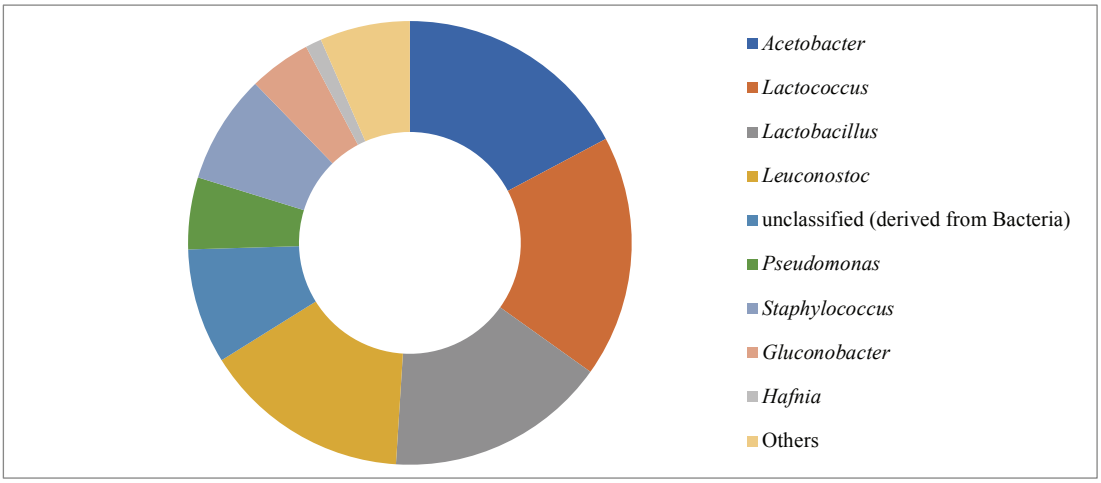
Microbial diversity in soft *chhurpi* (yak-milk)

Likewise, bacterial diversity in family level of soft *chhurpi* (yak-milk) was predominated by *Acetobacteraceae* (21.9%), followed by *Streptococcaceae* (17.7%), *Lactobacillaceae* (16.1%), *Leuconostocaceae* (16%), *Staphylococcaceae* (7.9%), *Pseudomonadaceae* (5.2%), *Enterobacteriaceae* (1.8%), *Bacillaceae* (1.6%) and others (2.5%) (Fig. 23a). Additionally, 8.3% of uncultured bacteria were also found.

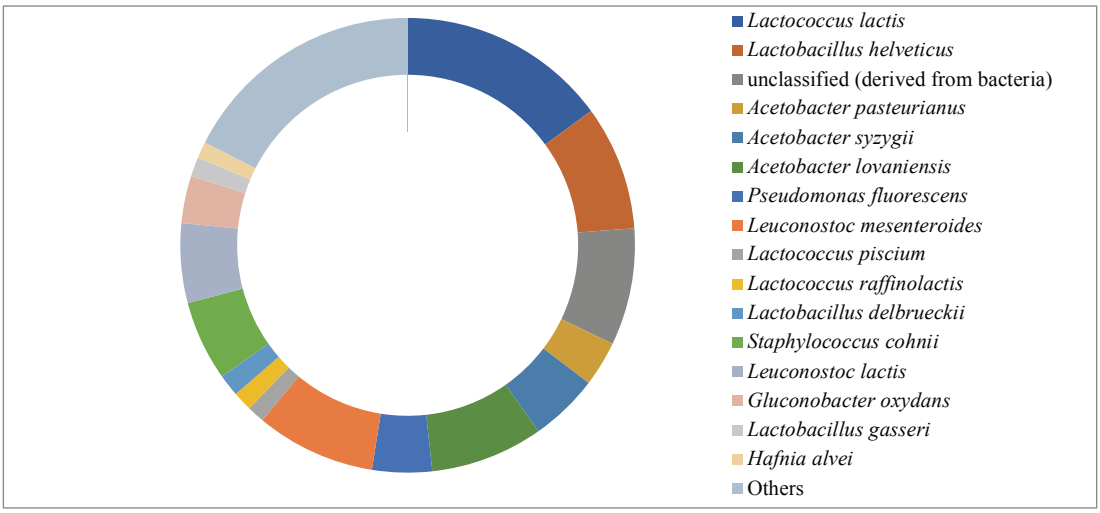
In genus level, the samples were predominated by *Lactococcus* (17.6%), *Acetobacter* (17.2%), *Lactobacillus* (16.1%), *Leuconostoc* (15.1%), *Staphylococcus* (7.9%), *Pseudomonas* (5.2%), *Gluconobacter* (4.5%), *Hafnia* (1.1%), and others (6%) (Fig. 23b). In species level distribution *Lactococcus lactis* (14.9%) was the most dominant followed by *Lactobacillus helveticus* (8.8%), *Leuconostoc mesenteroides* (8.4%), *Acetobacter lovaniensis* (8.0%), *Staphylococcus cohnii* (5.6%), *Leuconostoc lactis* (5.6%), *Acetobacter syzygii* (4.8%), *Pseudomonas fluorescens* (4.2%), *Gluconobacter oxydans* (3.3%), *Acetobacter pasteurianus* (3.2%), *Lactobacillus delbrueckii* (1.5%), *Lactococcus raffinolactis* (1.3%), *Lactobacillus gasseri* (1.3%), *Lactococcus piscium* (1.2%), *Hafnia alvei* (1.1%), and others (17.4%) (Fig. 23c).



(a)



(b)

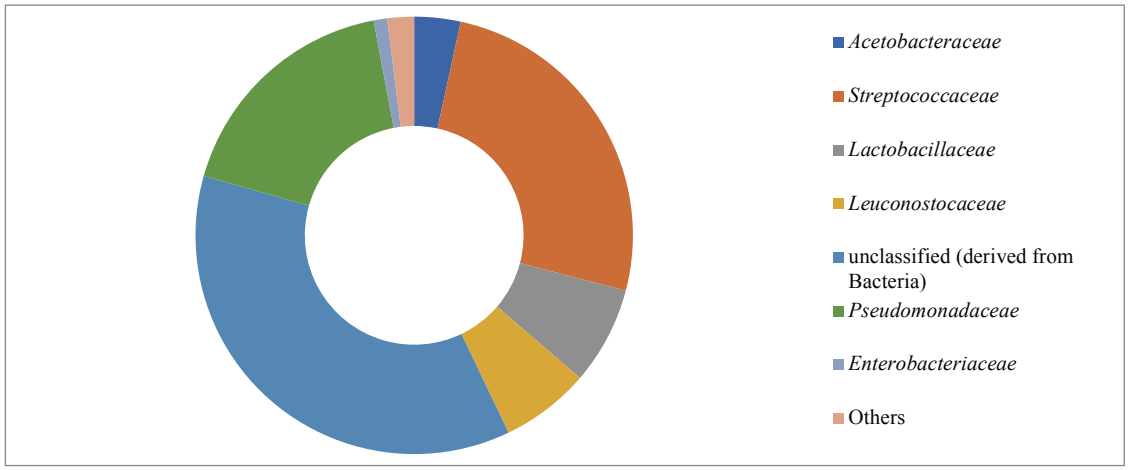


(c)

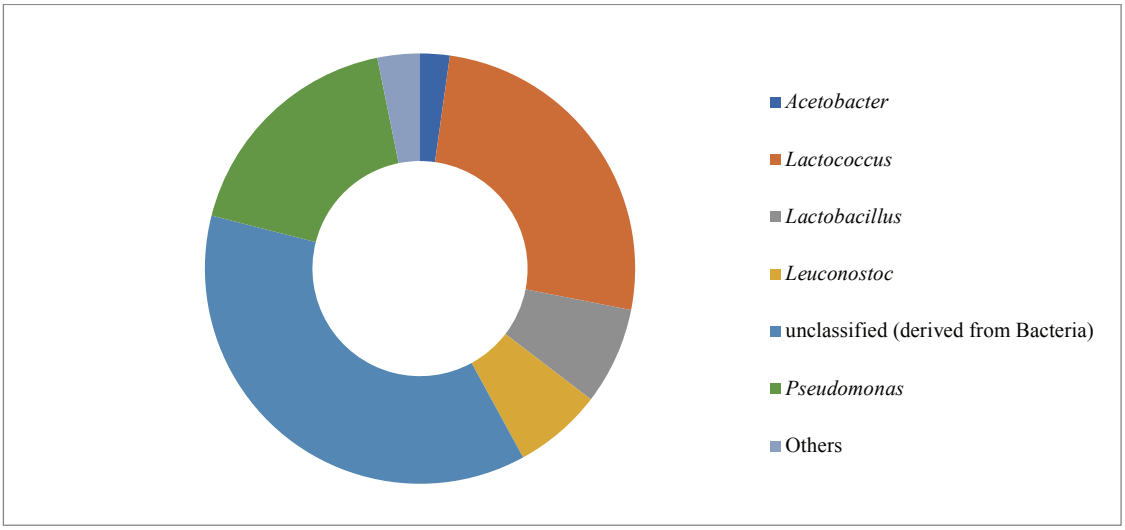
Figure 23: The bacterial composition of soft *chhurpi* (yak-milk) at different taxonomic level [a] Family [b] Genus and [c] species.

Microbial diversity in *Gheu* (yak-milk)

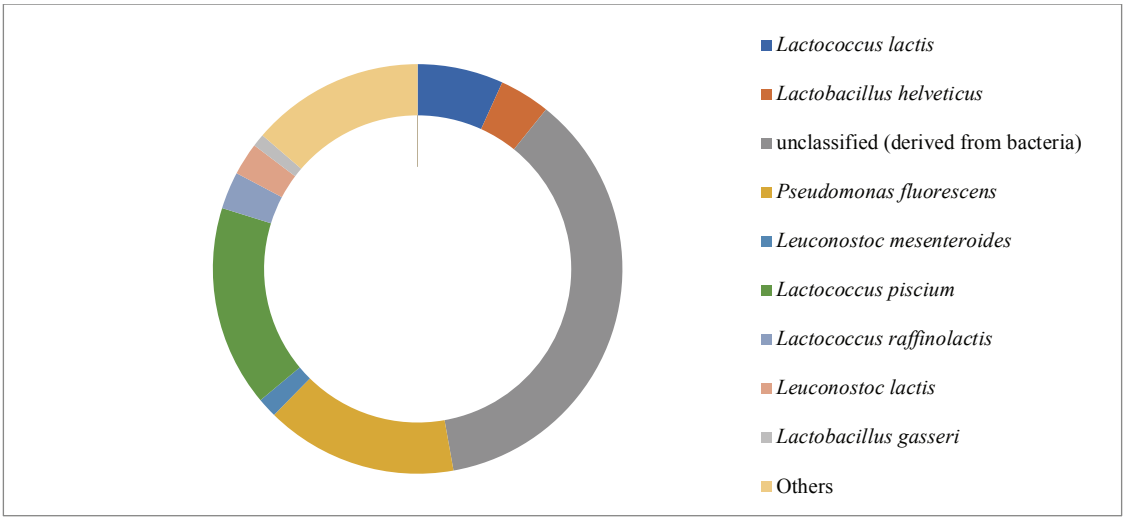
Bacterial diversity in *gheu* samples was predominated by family *Streptococcaceae* (25.6%), followed by *Pseudomonadaceae* (17.6%), *Lactobacillaceae* (7.2%), *Leuconostocaceae* (6.5%), *Acetobacteraceae* (3.4%), *Enterobacteriaceae* (1%) and others (1.9%) (Fig 24a). Uncultured bacteria (36.4%) were also reported. In genus level- *Lactococcus* (25.5%) was dominant genus followed by *Pseudomonas* (17.6%), *Lactobacillus* (7.2%), *Leuconostoc* (6.5%), *Acetobacter* (2.1%), *Gluconobacter* (1.2%) and others (3.1%) (Fig. 24b). In species level, *Lactococcus piscium* (15.8%), *Pseudomonas fluorescens* (15.1%), *Lactococcus lactis* (6.7%), *Lactobacillus helveticus* (4%), *Lactococcus raffinolactis* (2.9%), *Leuconostoc lactis* (2.5%), *Leuconostoc mesenteroides* (1.5%), *Lactobacillus gasseri* (1%), uncultured bacteria (36.4%) and others (13.6%) detected at <1% abundance (Fig. 24c).



(a)



(b)

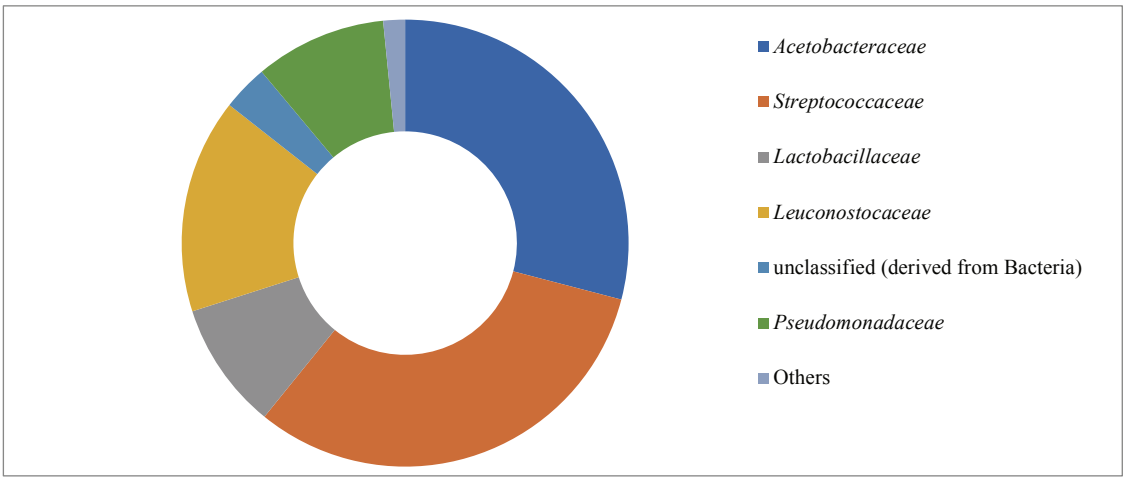


(c)

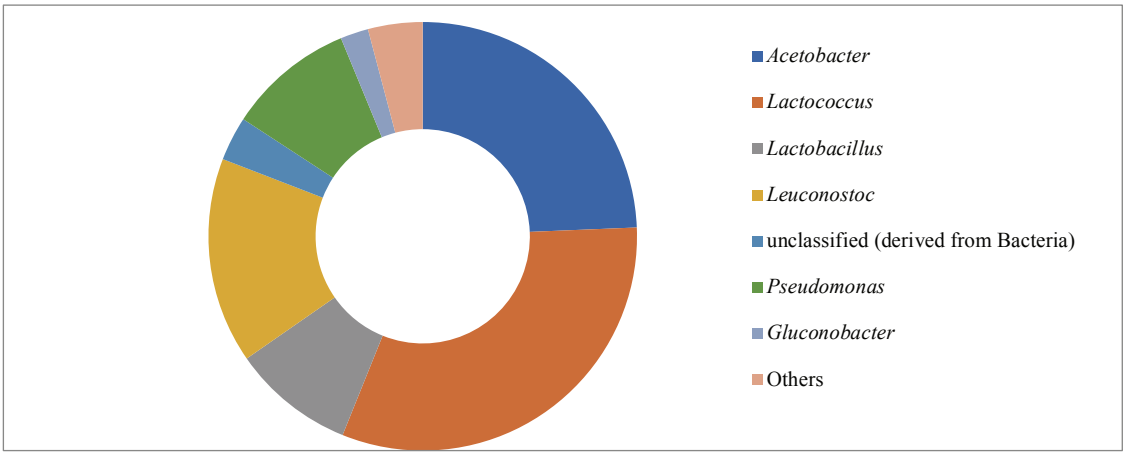
Figure 24: The bacterial composition of *Gheu* (yak-milk) at different taxonomic level [a] Family [b] Genus and [c] species.

Microbial diversity in *Dahi* (cow-milk)

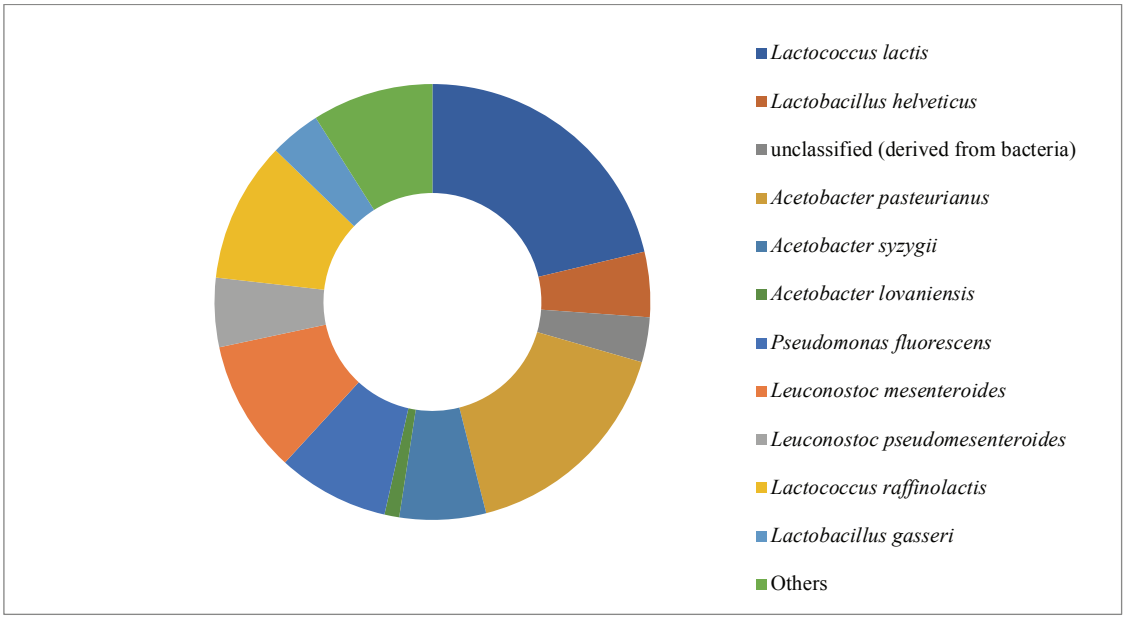
Family *Streptococcaceae* (31.7%) was found dominant in *dahi* (cow-milk) samples followed by *Acetobacteraceae* (29%), *Leuconostocaceae* (15.5%), *Pseudomonadaceae* (9.5%), *Lactobacillaceae* (9.2%) and others (1.5%) (Fig. 25a). Uncultured bacteria (3.3%) were also reported. In genus level dominant genus was *Lactococcus* (31.7%), followed by *Acetobacter* (24.3%), *Leuconostoc* (15.5%), *Pseudomonas* (9.5%), *Lactobacillus* (9.2%), *Gluconobacter* (2.1%) and others (4%) (Fig. 25b). In species level, *Lactococcus lactis* (21.2%), was dominant species followed by *Acetobacter pasteurianus* (16.5%), *Lactococcus raffinolactis* (10.4%), *Leuconostoc mesenteroides* (9.8%), *Pseudomonas fluorescens* (8.2%), *Acetobacter syzygii* (6.3%), *Leuconostoc pseudomesenteroides* (5.1%), *Lactobacillus helveticus* (4.8%), *Lactobacillus gasseri* (3.7%), *Acetobacter lovaniensis* (1%), and others (8.9%) (Fig. 25c).



(a)



(b)

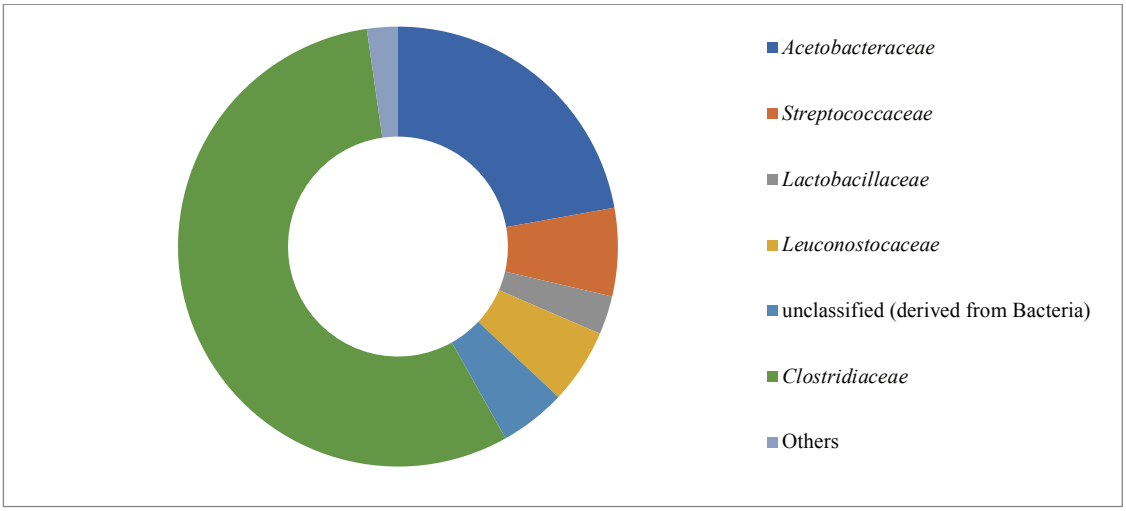


(c)

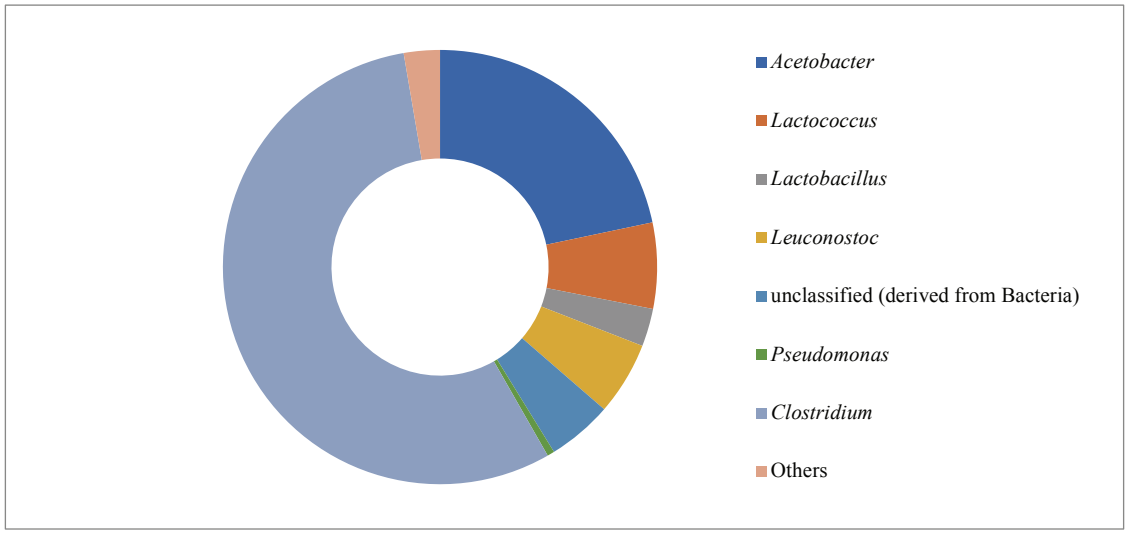
Figure 25: The bacterial composition of *dahi* (cow-milk) at different taxonomic level [a] Family [b] Genus and [c] species.

Microbial diversity in *dahi* (yak-milk)

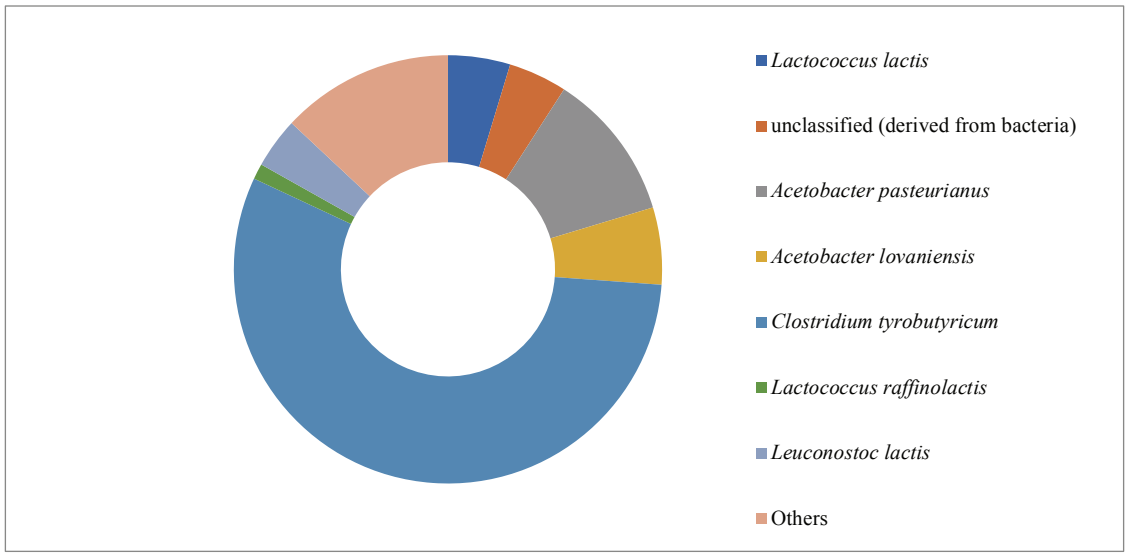
Similarly, the microbial diversity in *dahi* (yak-milk) samples was predominated by family *Clostridiaceae* (55.8%), followed by *Acetobacteraceae* (22%), *Streptococcaceae* (6.4%), *Leuconostocaceae* (5.5%), *Lactobacillaceae* (2.8%) and others (2.7%) (Fig. 26a). The relative abundance of 4.8% of uncultured bacteria was also reported. In genus level it was recorded that *Clostridium* (55.8%) was dominant genus followed by *Acetobacter* (21.8%), *Lactococcus* (6.4%), *Leuconostoc* (5.4%), *Lactobacillus* (2.8%) and others (2.5%) (Fig. 26b). Distribution of bacterial diversity in species level- *Clostridium tyrobutyricum* (55.8%), *Acetobacter pasteurianus* (11.2%), *Acetobacter lovaniensis* (5.8%), *Lactococcus lactis* (4.6%), *Leuconostoc lactis* (3.8%), *Lactococcus raffinolactis* (1.1%) and others (12.3%) (Fig. 26c).



(a)



(b)



(c)

Figure 26: The bacterial composition of *dahi* (yak-milk) at different taxonomic level (a) Family (b) Genus and (c) species.

Additionally, uncultured bacteria were detected in *gheu* samples with 36.4% of abundance followed by 8.3% in soft *chhurpi* (yak-milk); 4.8% in *dahi* (yak-milk); 3.8% in soft *chhurpi* (cow-milk); and 3.3% in *dahi* (cow-milk), respectively.

Profile of bacterial diversity in NFM products of Sikkim based on Culture-dependent and Culture-independent Methods

We detected 4 genera by phenotypic characterization without any species in NFM products of Sikkim. Similarly, we detected 3 genera and 9 species/subspecies in same products by 16S rRNA gene sequencing method. Finally, by application of HTS (culture-independent method), we detected 9 genera and 17 species having relative abundance above 1% in NFM products of Sikkim (Table 21).

Table 21: Profile of bacterial speceis in NFM products of Sikkim revealed by Culture-dependent and Culture-independent Methods

NFM Product	Culture-dependenet		Culture-independent
	Phenotypic	**Genotypic (16S rRNA gene sequeneng)**	**High-thorughput Sequeineung**
	Bacterials species		
Dahi (cow-milk)	*Leuconostoc, Lactococcus, Enterococcus* and *Streptococcus*	*Leuconostoc mesenteroides, Lactococcus lactis, Enterococcus italicus, Lactococcus lactis* subsp. *tructae*	*Lactococcus lactis, Lactobacillus helveticus, Acetobacter pasteurianus, Acetobacter syzygii, Acetobacter lovaniensis, Pseudomonas fluorescens, Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides, Lactococcus raffinolactis, Lactobacillus gasseri*
Dahi (yak-milk)	*Leuconostoc, Lactococcus, Enterococcus*	*Leuconostoc mesenteroides*	*Lactococcus lactis, Acetobacter pasteurianus, Acetobacter lovaniensis, Clostridium tyrobutyricum, Lactococcus raffinolactis, Leuconostoc lactis*
Soft *chhurpi*	*Leuconostoc, Lactococcus,*	*Lactococcus lactis* subsp. *cremoris,*	*Lactococcus lactis, Lactobacillus helveticus, Acetobacter*

(cow-milk)	*Enterococcus*	*Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii, Lactococcus lactis* subsp. *hordniae, Lactococcus lactis* subsp. *cremoris*	*pasteurianus, Acetobacter syzygii, Acetobacter lovaniensis, Pseudomonas fluorescens, Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides, Lactobacillus delbrueckii, Staphylococcus cohnii, Gluconobacter oxydans, Hafnia alvei*
Soft *chhurpi* (yak-milk)	*Leuconostoc, Lactococcus, Enterococcus*	*Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii, Enterococcus faecalis, Enterococcus pseudoavium, Lactococcus lactis* subsp. *cremoris.*	*Lactococcus lactis, Lactobacillus helveticus, Acetobacter pasteurianus, Acetobacter syzygii, Acetobacter lovaniensis, Pseudomonas fluorescens, Leuconostoc mesenteroides, Lactococcus piscium, Lactococcus raffinolactis, Lactobacillus delbrueckii, Staphylococcus cohnii, Leuconostoc lactis, Gluconobacter oxydans, Lactobacillus gasseri, Hafnia alvei*
Mohi (cow-milk)	*Leuconostoc, Lactococcus, Streptococcus*	*Lactococcus lactis, Leuconostoc mesenteroides*	ND
Hard *chhurpi* (yak-milk)	*Leuconostoc, Lactococcus, Enterococcus*	*Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *jonggajibkimchii*	ND
Gheu (yak-milk)	ND	ND	*Lactococcus lactis, Lactobacillus helveticus, Pseudomonas fluorescens, Leuconostoc mesenteroides, Lactococcus piscium, Lactococcus raffinolactis, Leuconostoc lactis, Lactobacillus gasseri*
Philu (yak-milk)	*Leuconostoc, Lactococcus, Enterococcus*	*Lactococcus lactis subsp. cremoris*	ND

ND: not determined

SCREENING OF PROBIOTIC PROPERTIES

Furthermore, selected representative strains were screened for probiotic attributes. The strains were selected from five different NFM products prepared from cow or yak milk. Sample-wise strains were selected from cow-milk *dahi* (10 strains), yak-milk *dahi* (10), cow-milk *mohi* (9), cow-milk soft *chhurpi* (10), yak-milk soft *chhurpi* (10), yak-milk hard *chhurpi* (10) and yak-milk *philu* (9), respectively for screening of probiotic attributes.

Acidification and Coagulation

Skim milk broth was inoculated with LAB isolate, and broth pH was recorded after incubation. LAB strains isolated from NFM products showed remarkable acidification properties with a lowest pH of 4.53, strains such as *Lactococcus lactis* DA41 (isolated from cow-milk *dahi*) and *Leuconostoc mesenteroides subsp. jonggajibkimchii* YS7-10 isolated from hard *chhurpi* (yak-milk) (Table 22). The pH value ranged from 4.4-5.9 in *dahi* (cow-milk), 4.5-5.6 in *dahi* (yak-milk), 4.8-5.8 in soft *chhurpi* (cow-milk), 4.5-6.0 in soft *chhurpi* (yak-milk), 4.5-5.5 in *mohi* (cow-milk), 4.9-5.9 in hard *chhurpi* (yak-milk) and 4.7-5.5 in *philu* (yak-milk), respectively. Nearly 93% of LAB strains isolated from NFM products could coagulate the skim milk under laboratory conditions, indicating their coagulation capabilities (Table 22).

Table 22: Acidification, coagulation, Beta-galactosidase activity and hydrophobicity of LAB strains isolted from NFM products of Sikkim

NFM Products	Strain code	Acidification and coagulation		Beta-galactosidase activity	Cell surface hydrophobicity (%)
		Average pH (SD)	Coagulation		
Dahi (cow-milk)	DA1	5.18±0.02	+	-	72
	DA3	4.56±0.01	+	-	80
	DA4	5.41±0.01	+	-	75
	DA8	5.90 ±0.03	-	-	77
	DA10	5.17±0.02	+	-	59
	DA11	5.10±0.02	+	-	26
	DA14	5.52±0.02	+	-	28
	DA35	5.31±0.13	+	-	76
	DA41	4.53±0.02	+	-	27
	DA66	4.47±0.35	+	-	79
Soft *chhurpi* (cow-milk)	SC3	5.06±0.01	+	-	87
	SC4	5.10±0.01	+	-	40
	SC5	5.10±0.01	+	-	18
	SC7	4.84±0.01	+	+	47
	SC11	4.77±0.01	+	+	34
	SC17	4.97±0.15	+	-	66
	SC19	4.95±0.03	+	-	44
	SC22	5.21±0.01	+	-	41
	SC26	5.01±0.07	+	-	61
	SC30	5.84±0.82	+	-	27
Dahi (yak-milk)	DY2	5.32±0.02	+	+	48
	DY3	5.41±0.01	+	+	28
	DY14	4.51±0.04	+	-	18
	DY16	5.57±0.02	+	+	54
	DY18	5.32±0.02	+	+	67
	DY19	5.48±0.03	-	+	32
	DY29	4.53±0.01	+	+	43

	DY30	4.48±0.03	+	+	30
	DY36	4.54±0.03	+	+	32
	DY42	4.96±0.01	+	+	6
Mohi (cow-milk)	MH3	5.17±0.02	+	-	67
	MH4	4.94±0.08	+	-	74
	MH9	5.10±0.02	+	-	57
	MH15	5.48±0.08	+	+	59
	MH18	5.18±0.02	+	+	80
	MH20	5.33±0.02	+	-	60
	MH22	5.43±0.03	+	+	61
	MH39	5.44±0.12	+	-	61
	MH40	4.54±0.02	+	-	21
Soft *chhurpi* (yak-milk)	YS4:1	5.99 ±0.03	-	+	26
	YS4-3	5.41±0.01	+	-	26
	YS4:4	4.97±0.15	+	+	25
	YS4:7	4.95±0.03	+	-	77
	YS4:8	5.01±0.07	+	-	79
	YS4:9	5.84±0.82	-	+	25
	YS4-10	5.04±0.03	+	-	30
	YS4:11	5.04±0.03	+	-	86
	YS4:14	5.02±0.01	+	-	93
	YS4:15	5.07±0.04	+	-	42
Hard *chhurpi* (yak-milk)	YS7-1	5.48±0.08	+	-	63
	YS7:2	5.96±0.03	-	-	41
	YS7-3	5.01±0.07	+	+	39
	YS7:4	5.44±0.12	+	-	26
	YS7:5	4.51±0.04	+	+	44
	YS7-7	5.18±0.02	+	-	55
	YS7-8	5.07±0.04	+	-	28
	YS7:10	4.53±0.01	+	-	41
	YS7:12	4.48±0.03	+	+	38

	YS7:13	4.54±0.03	+	-	26
Philu (yak-milk)	YS8-1	5.18±0.02	+	-	35
	YS8-3	4.83±0.05	+	-	23
	YS8-4	5.43±0.03	+	+	59
	YS8-5	4.74±0.03	+	+	59
	YS8-7	5.17±0.02	+	-	51
	YS8-8	5.10±0.02	+	+	45
	YS8-10	5.48±0.03	+	-	34
	YS8-11	4.72±0.02	+	-	23
	YS8-13	5.44±0.12	+	-	17
Reference strain	*Lb. plantarum* MTCC 2034	5.10±0.02	+	-	74

Data represent triplicate sets of experiments, (+) positive result, (-) negative result.

Beta-galactosidase Activity

All 68 bacterial strains isolated from NFM products were assessed for enzyme activity of beta-galactosidase and out of which 23 strains showed positive results indicating the activity of the beta-galactosidase enzyme (Table 22). The presence of blue color in MRS agar plates after incubation presumed positive results for expression of the beta-galactosidase enzyme (data not shown).

Hydrophobicity Assay

Only 13 strains (19.1%) of the 68 strains showed maximal hydrophobic activity (>70%) (Table 22). Among these strains, 93% hydrophobicity was shown by *Enterococcus faecalis* YS4-14 isolated from soft *chhurpi* followed by 87% *Lactococcus lactis* subsp. *cremoris* SC3 isolated from soft *chhurpi* (cow-milk) and 86% by *Enterococcus faecalis* YS4-11 isolated from soft-*chhurpi* (yak-milk). Some strains of LAB display higher

percentage of hydrophobicity activity than the reference strain *L. plantarum* MCC 2034 (74%) (Table 22).

Tolerance Tests: Acid (pH 3.), Lysozyme and Bile (0.3%)

A total of 68 representative strains were screened for acid, lysozyme and bile tolerance using viable cell count method (Table 23). Out of 68 LAB strains, 51.4% (35 strains) showed good tolerance at acidic nature (pH 3) after 2 h of incubation (Fig. 27). The experiment was performed in interval period of 0 and 2 h, after incubation good numbers of the bacterial growth was recorded at 0 h whereas only limited bacterial growth was recorded after 2 h of incubation. *Lactococcus lactis* subsp. *hordniae* SC 17 isolated from soft *chhurpi* (cow-milk) showed the highest viable load of 7.0 log cfu/g followed by *Leuconostoc mesenteroides* YS7-8 (6.6 log cfu/g) isolated from hard *chhurpi* (yak-milk) (Table 23).

Table 23: Survival of LAB from NFM products of Sikkim after 2 h at pH 3.0, lysozyme (100mg/l) and bile (0.3%) concentration

NFM Products	Strain Code	Acid tolerance (pH 3.0)		Lysozyme tolerance (100mg/L)		Bile tolerance (0.3%)	
		Log cfu/ml or g					
		0 h	2 h	0 h	2 h	0 h	2 h
Dahi (cow-milk)	DA1	5.15±0.21	NG	6.83±0.03	5.38±0.12	6.67±0.03	6.21±0.36
	DA3	6.62±0.09	NG	4.92±0.10	NG	7.36±0.22	NG
	DA11	5.38±0.55	5.00±0.00	6.53±0.036	5.73±0.05	7.01±0.54	6.42±0.49
	DA14	6.13±0.19	NG	5.75±0.21	5.30±0.42	6.57±0.60	NG
	DA66	7.11±0.03	NG	5.98±0.08	5.82±0.18	7.45±0.10	NG
	DA8	6.72±0.00	5.65±0.06	5.58±0.15	NG	7.44±0.00	7.30±0.00
	DA41	5.77±0.10	NG	5.69±0.00	6.05±0.08	7.18±0.13	NG
	DA4	5.69±0.12	NG	5.66±0.26	NG	5.87±0.04	5.69±0.08
	DA10	5.21±0.05	4.69±0.12	6.11±0.039	5.84±0.08	5.67±0.33	NG
	DA35	5.81±0.47	NG	6.06±0.026	5.84±0.08	5.07±0.85	NG
Mohi (cow-milk)	MH4	5.00±0.00	NG	5.45±0.21	NG	6.55±0.06	5.73±0.36
	MH5	6.46±0.04	5.23±0.33	6.46±0.01	5.58±0.15	5.03±0.85	NG
	MH40	7.12±0.00	NG	6.11±0.22	5.89±0.01	7.26±0.51	5.84±0.21
	MH18	6.12±0.07	5.77±0.01	5.73±0.05	5.38±0.06	6.39±0.10	NG
	MH3	5.15±0.21	NG	6.76±0.69	6.03±0.48	6.66±0.08	6.21±0.64
	MH9	5.19±0.02	NG	6.09±0.02	6.00±0.00	5.50±0.70	5.10±0.17
	MH15	6.15±0.06	NG	5.00±0.00	NG	5.91±0.09	0
	MH20	6.30±0.03	5.53±0.08	5.65±0.06	5.15±0.21	5.95±0.09	0
	MH22	5.99±0.06	NG	5.84±0.08	NG	5.91±0.03	0
Soft *chhurpi* (cow-milk)	SC30	5.40±0.02	NG	4.92±0.10	NG	7.29±0.00	0
	SC3	6.65±0.00	5.53±0.08	5.95±0.06	5.00±0.00	7.53±0.04	6.96±0.01
	SC4	6.54±0.07	NG	5.00±0.00	NG	7.55±0.02	7.11±0.02
	SC17	7.34±0.05	7.01±0.01	5.69±0.12	5.53±0.08	5.83±0.20	5.69±0.30
	SC19	5.46±0.02	NG	6.02±0.02	5.80±0.15	6.73±0.22	6.14±0.09
	SC11	6.33±0.11	NG	6.02±0.02	5.99±0.06	5.78±0.01	4.60±0.00
	SC26	5.17±0.33	NG	5.51±0.26	NG	5.82±0.09	5.26±0.01
	SC7	5.10±0.14	4.80±0.28	7.26±0.00	NG	7.05±0.70	2.14±0.74
	SC5	5.92±0.03	5.32±0.02	5.30±0.00	5.00±0.00	5.99±0.05	NG
	SC22	6.64±0.01	5.30±0.00	4.92±0.10	NG	5.91±0.08	NG
	SC20	6.10±0.07	NG	6.19±0.019	6.10±0.21	5.20±0.34	NG
Dahi (yak-milk)	DY2	6.82±0.08	5.69±0.12	6.20±0.042	5.89±0.07	5.91±0.09	NG
	DY3	6.70±0.07	5.52±0.03	5.88±0.26	5.62±0.82	7.33±0.05	7.31±0.01
	DY42	5.77±0.10	NG	5.69±0.12	5.47±0.51	7.54±0.05	7.18±0.02
	DY29	6.64±0.01	5.62±0.21	5.51±0.26	NG	7.31±0.02	7.11±0.12
	DY30	5.15±0.21	NG	5.80±0.14	5.47±0.60	5.20±0.34	5.23±0.33
	DY14	6.80±0.04	NG	5.77±0.10	5.47±0.80	5.47±0.00	5.40±0.17
	DY19	5.96±0.01	4.88±0.15	4.92±0.10	NG	5.27±0.81	NG
	DY16	5.69±0.00	4.95±0.06	6.81±0.04	6.75±0.55	5.20±0.34	NG
	DY36	6.74±0.03	6.00±0.14	7.27±0.01	7.23±0.05	5.90±0.09	NG
	DY18	6.30±0.07	5.86±0.12	6.03±0.27	5.23±0.33	5.91±0.06	NG
Soft *chhurpi* (yak-milk)	YS4-4	5.12±0.24	NG	5.82±0.18	5.69±0.06	6.35±0.15	6.07±0.10
	YS4-1	5.50±0.14	4.95±0.06	5.00±0.00	NG	6.12±0.07	6.11±0.16
	YS4-3	6.56±0.02	5.45±0.21	5.98±0.12	5.68±0.02	7.91±0.20	6.86±0.03
	YS4-7	6.02±0.10	5.20±0.29	5.00±0.00	NG	7.17±0.04	6.76±0.06
	YS4-8	6.07±0.10	5.07±0.10	6.64±0.85	6.19±0.58	7.36±0.04	7.13±0.02

	YS4-11	6.14±0.04	5.95±0.12	5.301±0.00	5.23±0.16	7.38±0.01	6.99±0.16
	YS4-15	5.36±0.05	5.13±0.13	5.24±0.34	5.00±0.00	7.55±0.00	7.26±0.03
	YS4-9	6.03±0.05	5.60±0.17	5.57±0.38	5.45±0.31	7.80±0.00	7.62±0.00
	YS4-14	6.68±0.05	6.50±0.06	6.25±0.117	6.12±0.73	6.64±0.05	NG
Hard *chhurpi* (yak-milk)	YS7-4	5.35±0.10	4.57±0.38	5.19±0.40	5.07±0.103	6.68±0.01	6.22±0.16
	YS7-1	5.27±0.14	NG	5.22±0.31	4.73±0.369	5.96±0.03	5.91±0.09
	YS7-5	4.5±0.28	NG	4.92±0.10	NG	6.84±0.07	6.51±0.02
	YS7-13	5.69±0.05	4.23±0.33	5.49±0.01	NG	6.64±0.09	6.34±0.12
	YS7-12	4.15±0.21	NG	4.92±0.10	NG	7.37±0.01	7.23±0.00
	YS7-7	5.88±0.10	5.68±0.02	5.15±0.212	5.00±0.00	6.60±0.09	NG
	YS7-2	5.03±0.05	4.84±0.21	5.42±0.06	NG	6.12±0.07	NG
	YS7-3	4.5±0.28	NG	5.38±0.43	NG	6.01±0.09	NG
Philu (yak-milk)	YS7-8	6.76±0.02	6.61±0.02	6.872±0.538	5.87±0.38	6.28±0.07	NG
	YS8-4	5.95±0.06	5.23±0.33	6.06±0.026	5.47±0.00	6.02±0.06	5.87±0.04
	YS8-5	5.10±0.09	5.00±0.14	5.25±0.06	4.95±0.068	6.26±0.04	NG
	YS8-3	6.12±0.07	NG	5.88±0.15	5.37±0.748	7.36±0.00	7.10±0.03
	YS8-8	4.15±0.21	NG	5.37±0.140	5.23±0.32	6.50±0.03	5.93±0.33
	YS8-7	6.51±0.18	NG	5.69±0.12	NG	7.41±0.00	7.04±0.00
	YS8-11	6.32±0.02	NG	6.19±0.151	6.01±0.08	7.23±0.04	NG
	YS8-1	5.31±0.04	4.30±0.42	5.51±0.26	NG	6.64±0.05	NG
	YS8-10	6.05±0.08	NG	6.10±0.14	5.62±0.775	6.50±0.03	NG

Data represent triplicate sets of experiments. Log cfu/ml for *dahi* and *mohi*; log cfu/g for *chhurpi* and *philu.* NG, no growth.

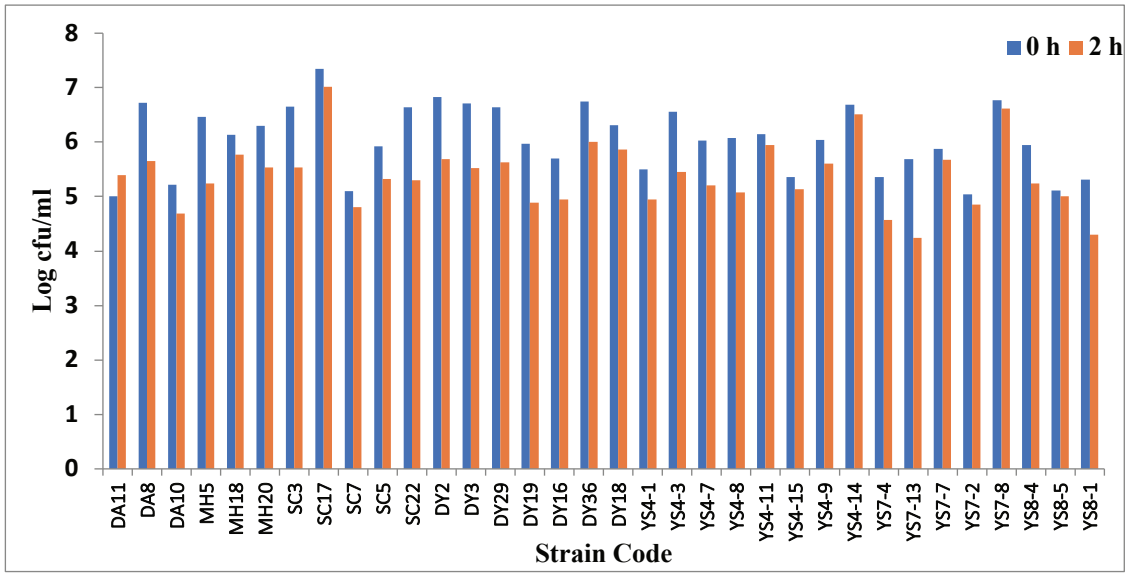


Figure 27: Graphical representation of acid tolerance (pH 3.0) by lactic acid bacteria isolated from naturally fermented milk products of Sikkim.

Similarly, strains were assessed for lysozyme tolerance and 64.7% (44 strains) showed remarkable resistance to lysozyme (100 mg/L) after 2 h of incubation (Fig. 28). *Leuconostoc mesenteroides* DY36 isolated from yak-milk *dahi* showed the highest microbial load of 7.2 log cfu/ml in medium containing lysozyme (Table 23). About 52.9% (36 LAB strains) showed ability to tolerate 0.3% bile salt after 2 h incubation in applied method (Fig. 29). *Leuconostoc mesenteroides* YS4-9 isolated from yak-milk soft *chhurpi* showed the highest survival (7.6 log cfu/g) in 0.3% bile salt tolerance test followed by *Leuconostoc mesenteroides* DY3 (7.3 log cfu/ml) isolated from yak-milk *dahi* (Table 23).

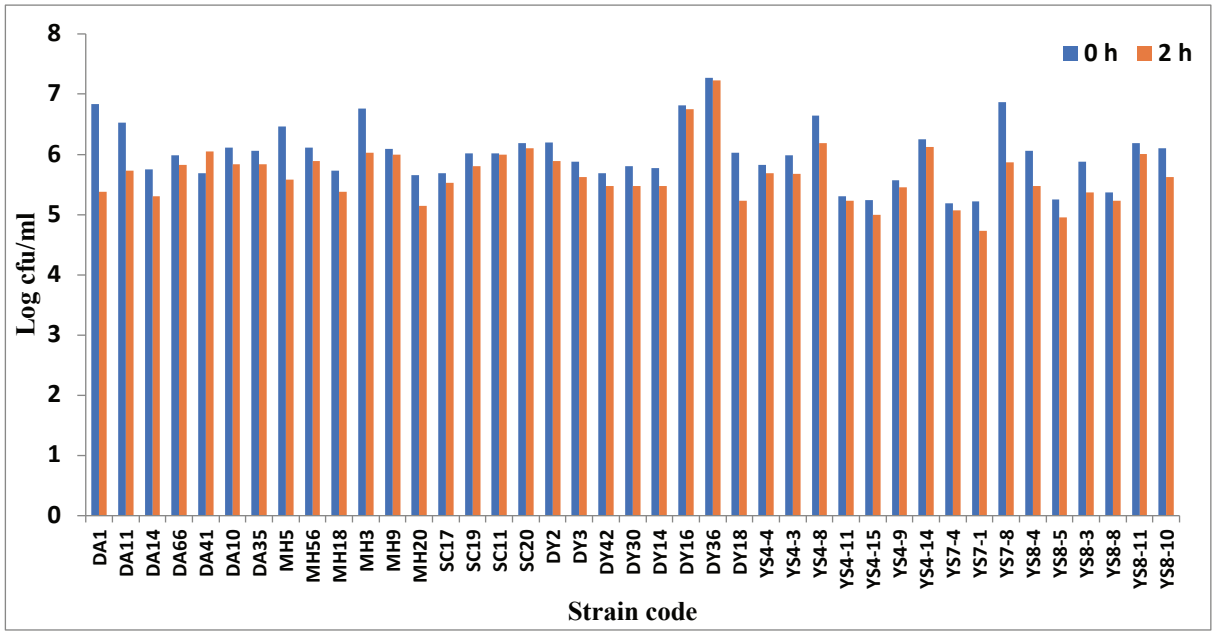


Figure 28: Graphical representation of lysozyme tolerance (100mg/l) by lactic acid bacteria isolated from naturally fermented milk products of Sikkim.

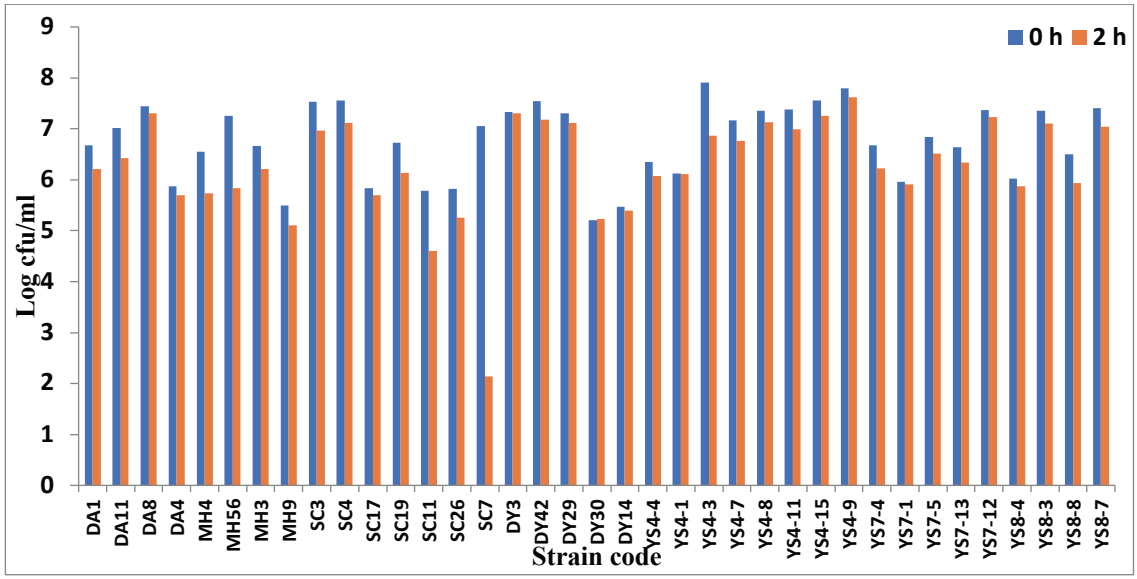


Figure 29: Graphical representation of bile salt tolerance (0.3%) by lactic acid bacteria isolated from naturally fermented milk products of Sikkim.

Bile Salt Hydrolysis Activity

Bile salt hydrolysis activity (BSH) activity was evaluated by growing of strains in different salt to assess their ability to hydrolyze bile salts. viz, sodium taurocholate, sodium cholate and sodium tauroglycocholate. Out of 68 representative strains, only 4 strains were able to hydrolyse sodium tauroglycocholate, however remaining strains were not able to survive in high salt concentration. Further, 31 bacterial strains (45.5%) showed ability to hydrolyse sodium cholate and 28 strains (41%) to hydrolyse sodium tauroglycocholate (Table 24).

Table 24: Bile salt hydrolase activity of LAB strains isolated from NFM products of Sikkim

NFM Products	Strain ID	Bile salt		
		Sodium taurocholate	Sodium cholate	Sodium tauroglycocholate
Dahi (cow-milk)	DA1	-	+	+
	DA3	-	+	+
	DA4	-	+	-
	DA8	-	+	+
	DA10	-	-	-
	DA11	-	+	-
	DA14	-	+	+
	DA35	-	-	-
	DA41	-	+	-
	DA66	-	+	-
Mohi (cow-milk)	MH3	-	+	-
	MH4	-	+	+
	MH9	-	+	-
	MH15	-	-	+
	MH18	-	+	+
	MH22	-	-	-
	MH20	-	+	+
	MH39	-	-	-

	MH40	-	-	-
Soft *chhurpi* (cow-milk)	SC3	-	+	-
	SC4	-	+	-
	SC5	-	-	-
	SC7	-	-	-
	SC11	-	+	+
	SC17	-	+	+
	SC19	-	+	-
	SC22	-	-	-
	SC26	-	+	-
	SC30	-	-	-
Dahi (yak-milk)	DY2	-	+	-
	DY3	-	-	-
	DY14	-	-	-
	DY16	-	+	+
	DY18	-	+	+
	DY19	-	+	+
	DY29	-	+	-
	DY30	-	-	-
	DY36	-	+	-
	DY42	-	-	-
Soft *chhurpi* (yak-milk)	YS4:1	-	-	+
	YS4-3	-	+	+
	YS4:4	-	-	+
	YS4-7	-	-	+
	YS4-8	+	-	-
	YS4-9	+	-	-
	YS4-10	-	-	-
	YS4-11	+	-	-
	YS4-14	-	-	+
	YS4-15	-	-	+
Hard *chhurpi* (yak-milk)	YS7-1	-	+	+
	YS7-2	-	-	-
	YS7-3	-	-	-
	YS7-4	-	-	+
	YS7-7	-	+	+

	YS7-8	-	-	-
	YS7-10	-	-	-
	YS7-12	+	-	+
	YS7-13	-	-	+
Philu (yak-milk)	YS8-1	-	-	+
	YS8-3	-	-	-
	YS8-4	-	+	-
	YS8-5	-	+	+
	YS8-7	-	+	-
	YS8-8	-	-	+
	YS8-10	-	-	-
	YS8-11	-	-	+
	YS8:13	-		-
Reference strains	*Lb. plantarum* MCC 2034	-	-	+
	Lb. plantarum MTCC 1407(T)	-	-	+
	Lb. brevis MCC 2198 (T)	-	-	+

Data represent triplicate sets of experiments, (+) positive result, (-) negative result.

Antimicrobial Activity

Antimicrobial screening of representative bacterial strains was assessed against four pathogenic MTCC cultures viz. *E. coli* MCC2413, *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272 (Table 25). Inhibition zone (mm) was measured after 48 h of incubation. Out of 68 LAB strains, nine strains showed more than 10 mm inhibition zones against all four tested pathogenic microorganisms indicting their strong antimicrobial inhibition (Table 25). These LAB strains are *Leuconostoc mesenteroides* DA1 (cow-milk *dahi*), *Lactococcus lactis* DA8 (cow-milk *dahi*), *Lactococcus lactis* subsp. *tructae* DA66 (cow-milk *dahi*), *Leuconostoc mesenteroides* DY18 (yak-milk

dahi), *Leuconostoc mesenteroides* DY29 (yak-milk *dahi*), *Leuconostoc mesenteroides* SC11 (cow-milk soft *chhurpi*), *Leuconostoc mesenteroides* SC26 (cow-milk soft *chhurpi*), *Lactococcus lactis* subsp. *cremoris* YS8-5 (yak-milk *philu*) and *Lactococcus lactis* subsp. *cremoris* YS8-7 (yak-milk *philu*). 67.6% of LAB isolates showed more antimicrobial inhibition against *Staphylococcus aureus* subsp. *aureus* MTCC 740, 47% against *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, 47% against *Bacillus cereus* MTCC 1272. The bacterial strain showed very less inhibition against *E. coli* MCC2413. Further, *Lactobacillus plantarum* MCC 2034 was considered as positive culture, 8 LAB strains showed good zone of inhibition (ZOI) which was ≥12 mm against *E. coli*. Similarly, 12 strains showed good ZOI ≥12 mm against *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, 21 strains showed efficient ZOI ≥14 mm against *Staphylococcus aureus* subsp. *aureus* MTCC 740 and 23 strains showed good zone of inhibition ≥11 mm against *Bacillus cereus* MTCC 1272 (Table 25).

Table 25: Antimicrobial activity of LAB strains isolated from NFM products of Sikkim against some pathogenic strains

NFM Products	Strain code	Tested microorganisms (mm)			
		***Escherichia coli* MCC 2413**	***Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223**	***Staphylococcus aureus* subsp. *aureus* MTCC 740**	***Bacillus cereus* MTCC 1272**
Dahi (cow-milk)	DA1	10	11	15	13
	DA3	11	10	14	NA
	DA4	NA	11	15	NA
	DA8	11	10	14	10
	DA41	NA	13	14	14
	DA10	11	10	NA	10
	DA11	10	12	14	NA
	DA14	NA	11	13	10
	DA35	11	10	NA	10
	DA66	10	10	14	10
Dahi (yak-milk)	DY2	10	NA	NA	NA
	DY3	NA	NA	NA	NA

	DY14	NA	NA	NA	NA
	DY18	10	13	14	10
	DY19	12	NA	NA	NA
	DY16	12	NA	11	NA
	DY29	10	10	15	13
	DY30	NA	NA	NA	NA
	DY36	12	NA	12	10
	DY42	NA	NA	NA	NA
Mohi (cow-milk)	MH22	NA	NA	12	NA
	MH15	NA	NA	12	NA
	MH4	NA	NA	15	13
	MH18	NA	10	11	14
	MH20	NA	10	15	13
	MH3	NA	10	13	11
	MH9	NA	10	13	14
	MH39	NA	NA	NA	NA
	MH40	NA	NA	NA	NA
Soft *chhurpi* (cow-milk)	SC3	NA	11	15	NA
	SC4	NA	13	18	NA
	SC22	11	NA	12	11
	SC7	NA	NA	11	11
	SC5	NA	NA	12	11
	SC11	14	12	17	11
	SC17	NA	12	14	NA
	SC19	NA	11	14	10
	SC26	14	11	12	12
	SC30	NA	NA	NA	NA
Soft *chhurpi* (yak-milk)	YS4-1	NA	NA	11	NA
	YS4-3	NA	NA	NA	NA
	YS4-4	NA	NA	11	NA
	YS4-9	NA	11	14	12
	YS4-11	NA	NA	NA	11
	YS4-14	NA	NA	NA	11
	YS4-7	NA	NA	13	NA
	YS4-15	NA	NA	12	13
	YS4-10	NA	NA	NA	NA
	YS4-8	NA	NA	11	12
Hard *chhurpi* (yak-milk)	YS7-7	11	NA	13	10
	YS7-1	12	10	13	NA
	YS7-5	11	NA	13	13
	YS7-3	NA	13	13	NA
	YS7-2	NA	13	15	NA
	YS7-4	NA	13	14	12
	YS7-12	NA	13	14	15
	YS7-8	NA	NA	NA	NA
	YS7-13	NA	NA	NA	NA

	YS7-10	NA	NA	NA	NA
Philu (yak-milk)	YS8-1	NA	NA	NA	NA
	YS8-3	NA	NA	12	NA
	YS8-8	12	12	NA	NA
	YS8-10	NA	14	NA	NA
	YS8-4	13	NA	16	11
	YS8-5	10	10	13	10
	YS8-11	NA	NA	13	NA
	YS8-7	10	11	10	15
	YS8-13	NA	NA	NA	NA
	YS8-14	NA	NA	NA	NA
Reference strains	*Lb. plantarum* MCC 2034	12	12	14	11
	Lb. brevis MCC 2198 (T)	10	11	NA	NA
	Lb. fermentum MCC 2760	12	14	NA	12
	Lb. plantarum subspp. *plantarum* MCC 2974	NA	NA	NA	10
Negative control	MRS broth	NA	NA	NA	NA

Data represent triplicate sets of experiments; NA, no antimicrobial activity observed.

PROBIOTIC GENE DETECTION

Evaluation of gene with probiotic markers was assessed for presence of different gene encoding enzymes in LAB strain isolated from NFM products (Table 26). Gene detection was carried out based on *in vivo* determination of acid and bile tolerance, bile salt enzyme hydrolysis, binding capability, bacteriocin production and beta-glucosidase enzyme activity.

Table 26: Evaluation of probiotic LAB strains isolate from NFM products for presence of different gene encoding enzymes

NFM Products	Isolate code	Genes encoding enzymes												
		map A	***mub1***	***msa***	***bsh***	***BGL-1***	**Ir1516**	***agu A***	***apf***	***tdc***	***clp L***	**LBA1446**	**Ir1584**	***mes Y***
Dahi (cow-milk)	DA1	+	+	+	-	+	+	+	-	-	+	+	-	+
	DA3	-	+	-	-	-	+	+	-	-	+	+	-	-
	DA4	-	+	-	-	-	+	+	+	+	+	+	-	-
	DA8	+	+	-	-	-	-	-	+	-	+	+	+	-
	DA10	+	+	+	-	+	+	+	+	+	+	-	-	+
	DA11	+	+	+	+	-	+	+	+	+	+	+	+	+
	DA14	+	+	+	+	+	+	+	-	-	+	+	+	-
	DA35	+	+	+	-	+	+	+	-	-	+	+	-	-
	DA66	-	-	-	-	-	-	-	+	-	+	+	-	-
Soft *chhurpi* (cow-milk)	SC3	+	+	+	-	+	+	+	-	-	+	-	-	-
	SC4	+	+	+	-	-	+	+	-	-	+	+	-	+
	SC5	+	+	+	-	-	+	+	+	+	+	-	-	-
	SC7	+	+	+	-	+	+	-	+	+	+	-	-	+
	SC11	+	+	+	-	-	+	+	+	+	+	+	+	+
	SC17	-	+	+	-	-	+	+	-	-	+	+	-	-
	SC19	-	-	-	-	-	+	+	+	+	+	-	-	-
	SC22	+	+	+	-	+	+	+	+	+	+	-	-	+
	SC26	+	+	+	-	+	+	+	+	+	+	+	+	+
Mohi (cow-milk)	MH3	-	-	-	-	-	-	-	-	-	+	-	-	-
	MH4	-	+	-	-	-	+	+	+	+	+	+	-	-
	MH15	+	+	+	-	+	+	+	-	-	+	+	+	+

	MH18	+	+	-	+	-	+	+	+	+	+	-	-	-
	MH20	+	+	+	+	+	+	+	+	+	+	-	+	+
	MH22	-	-	-	-	-	-	-	-	-	+	-	-	-
	MH40	-	-	-	-	-	-	-	-	-	+	-	-	-
Dahi (yak-milk)	DY2	-	-	-	+	-	-	-	-	-	+	-	-	+
	DY3	-	-	-	+	-	-	-	-	-	-	-	-	+
	DY16	+	+	+	-	+	+	+	+	+	+	+	+	+
	DY18	+	+	+	-	-	+	+	-	-	+	+		+
	DY19	+	+	+	-	+	+	+	-	-	+	-	+	+
	DY29	+	+	+	-	+	+	+	-	-	+	-	-	+
	DY30	-	-	-	-	-	-	-	-	-	+	-	-	+
	DY36	+	+	+	-	+	+	+	+	+	+	+	-	+
	DY42	+	+	+	-	+	+	+	+	+	+	+	-	+
Soft *chhurpi* (yak-milk)	YS4-1	+	+	+	-	-	+	+	+	+	+	-	-	-
	YS4-3	+	+	+	+	-	-	-	+	+	+	+	+	-
	YS4-4	+	+	+	-	-	+	+	+	+	+	+	-	-
	YS4-7	+	+	+	-	-	+	+	-	-	+	+	-	-
	YS4-8	-	-	-	+	-	-	-	-	-	+	+	+	-
	YS4-9	+	+	+	-	+	+	-	-	-	+	-	-	+
	YS4-11	-	+	+	+	-	-	-	+	+	+	+	+	-
	YS4-14	-	-	-	+	-	+	+	+	+	+	-		-
	YS4-15	+	+	+	+	-	+	+	+	+	+	+	+	-
Hard *chhurp*	YS7-1	+	+	+	-	-	-	-	+	+	+	+	+	-
	YS7-2	-	-	-	-	-	-	-	-	-	+	-	-	-

i (yak-milk)	YS7-4	+	+	+	-	+	+	+	+	+	+	-	-	+
	YS7-5	-	-	-	-	-	-	-	-	-	+	-	-	-
	YS7-7	+	+	+	+	+	-	-	+	+	+	-	+	+
	YS7-8	-	-	-	-	-	-	-	-	-	-	-		+
	YS7-12	-	+	-	+	-	-	-	+	+	+	+	+	-
	YS7-13	+	+	+	-	+	-	-	-	-	+	+	+	+
Philu (yak-milk)	YS8-1	+	+	+	-	-	+	+	+	+	+	-	+	-
	YS8-4	-	-	-	-	-	-	-	-	-	+	-	-	-
	YS8-5	+	+	+	-	-	+	+	+	+	+	-	-	-
	YS8-7	+	+	-	-	-	-	-	-	-	+	-	+	-
	YS8-8	+	+	+	+	+	+	+	+	+	+	+	+	-
	YS8-10	+	+	+	-	+	+	+	-	-	+	+		-
Reference strains	*Lb. plantarum* MCC 2034	+	+	+	-	-	+	+	+	-	+	+	+	+
	Lb. plantarum subsp. *plantarum* MCC 2974	-	-	-	-	-	-	-	+	-	-	+	-	+

(+) indicates gene detection; (-) indicates no gene detection.

Detection of genes involved in low pH environment

The genes involved in low-pH survival are *clp L, agu A*, Ir1516*, tdc*, *hdc, odc,* LBA1272, *dlt D*, La995, lr0085, groEL). Some of these genes also have function in bile salt tolerance (Turpin et al. 2011). Approximately 82.3 % of the LAB strains were detected for gene *clp L* encoding for low pH tolerance, 52.9% detected for gene *agu A,* 55.8% of strains showed positive gene detection for Ir1516 which encodes for acid or bile tolerance, and 41.1% detected for gene *tdc* encoding for low pH tolerance (Table 26). The PCR screening with the available primers resulted in single gene products of 158 bp for ATPase (*clpL*) (Fig. 30), 542 bp for agmatine deiminase (*aguA*) (Fig. 31), 143 bp for putative esterase (Ir1516) (Fig. 32), 245 bp for tyrosine decarboxylase (*tdc*) (Fig. 33). No genes were detected in bacterial strains coding for *hdc, odc,* LBA1272, *dlt D*, La995, lr0085 genes. However, a housekeeping gene *groEL*, which is also involved in survival at low pH was not detected in any representative bacterial strains.

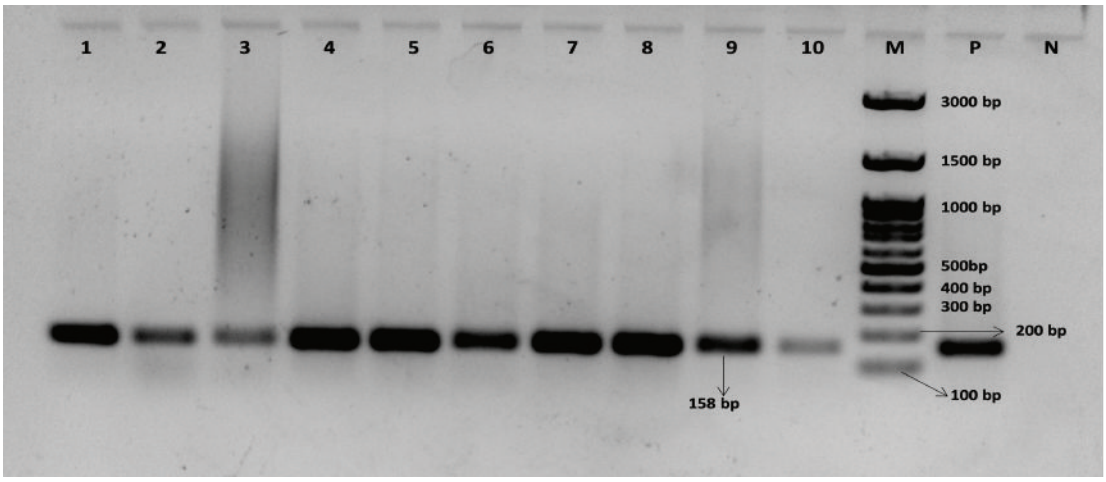


Figure 30: Agarose gel electrophoresis showing PCR amplification of *clp L* gene. Isolate strain: 1-11 (DA1: *Leuconostoc mesenteroides*; DA3: *Lactococcus lactis*; DA4: *Lactococcus lactis*; DA8: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; DA66: *Lactococcus lactis* subsp. *tructae*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC4: *Leuconostoc mesenteroides*), M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative control.

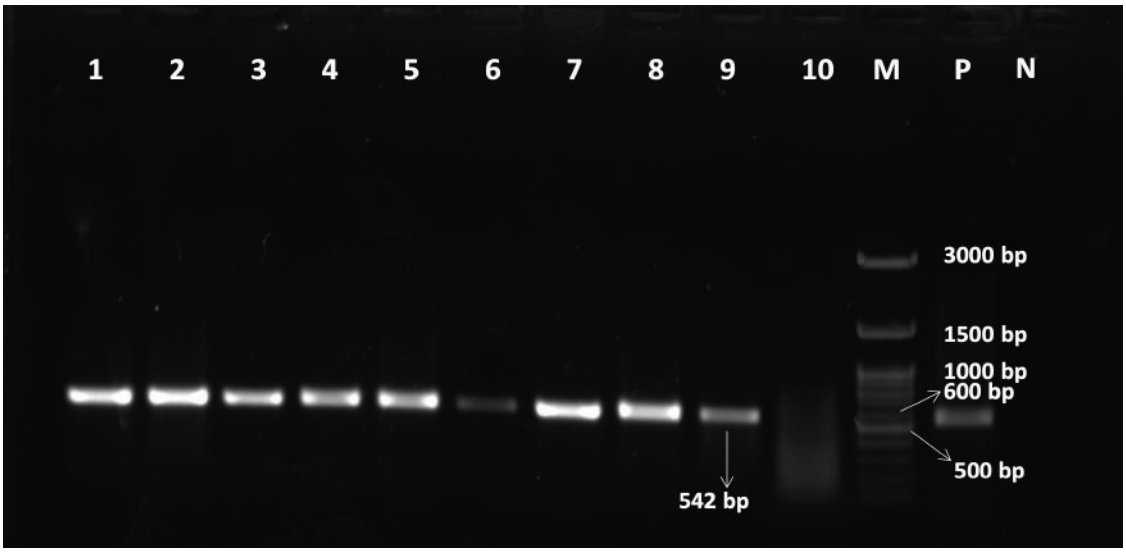


Figure 31: Agarose gel electrophoresis showing PCR amplification of *agu A* gene. Isolate strain: 1-10 (DA1: *Leuconostoc mesenteroides*; DA: *Lactococcus lactis*; DA4: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC4: *Leuconostoc mesenteroides*; SC5: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*). M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative.

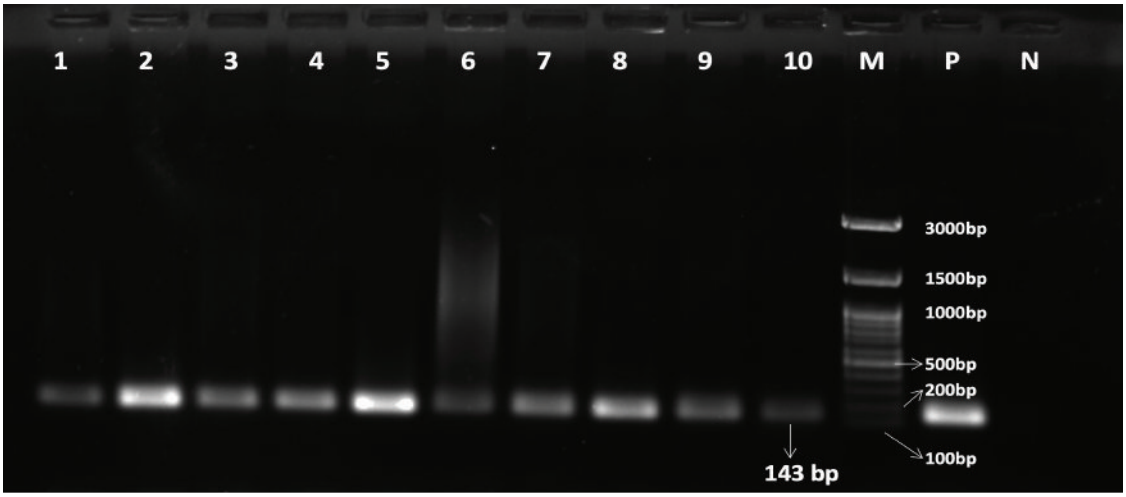


Figure 32: Agarose gel electrophoresis showing PCR amplification of Ir1516 gene. Isolate strain: 1-10; (DA1: *Leuconostoc mesenteroides*; DA3: *Lactococcus lactis*; DA4: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC4: *Leuconostoc mesenteroides*; SC5: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*). M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative.

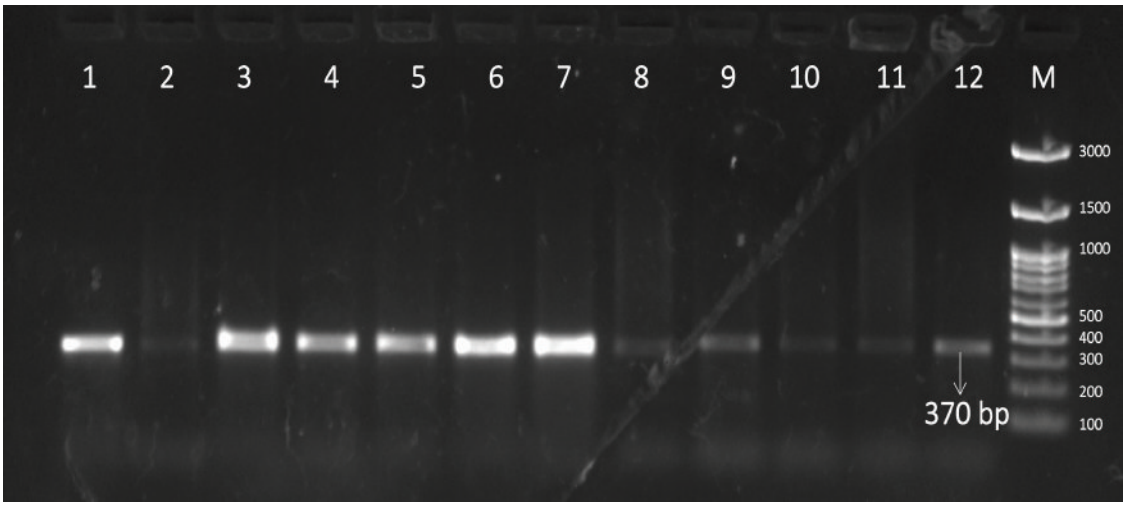


Figure 33: Agarose gel electrophoresis showing PCR amplification of *tdc* gene. Isolate strain: 1-12; (DA4: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; SC5: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*; SC7: *Leuconostoc mesenteroides*; SC11: *Leuconostoc mesenteroides*; SC19: *Lactococcus lactis* subsp. *cremoris*; SC22: *Leuconostoc mesenteroides*; SC26: *Leuconostoc mesenteroides*; MH4: *Lactococcus lactis*), M: 100 bp DNA ladder.

Detection of genes involved in bile salt tolerance

The genes involved in bile tolerances are Ir0085, Ir1584, LBA0552, LBA1429, LBA1446, and *bsh*. About 30.8% strains were detected for Ir1584 gene, 45.5 % of the strains detected for LBA1446 gene and 22% strains detected for *bsh* gene (Table 26). Gene size was detected with 151 bp for gene Ir1584 (major facilitator superfamily permease) (Fig. 34), 275 bp for LBA1446 (multidrug resistance protein) (Fig. 35), 205 bp for *bsh* (bile salt) (Fig. 36). Whereas no bacterial strains were detected for gene Ir0085, LBA0552 and LBA1429.

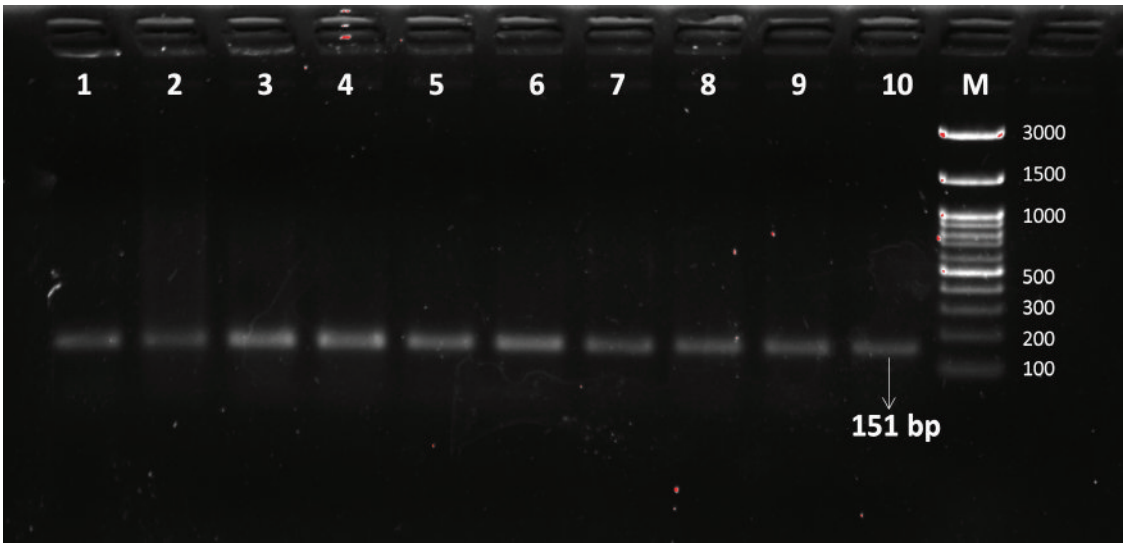


Figure 34: Agarose gel electrophoresis showing PCR amplification of Ir1584 gene. Isolate strain: 1-10 (DA8: *Lactococcus lactis*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; SC11: *Leuconostoc mesenteroides*; SC26: *Leuconostoc mesenteroides*; MH15: *Leuconostoc mesenteroides*; MH20: *Leuconostoc mesenteroides*; DY16: *Leuconostoc mesenteroides*; DY19: *Leuconostoc mesenteroides*; YS4-3: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*), M: 100 bp DNA ladder.

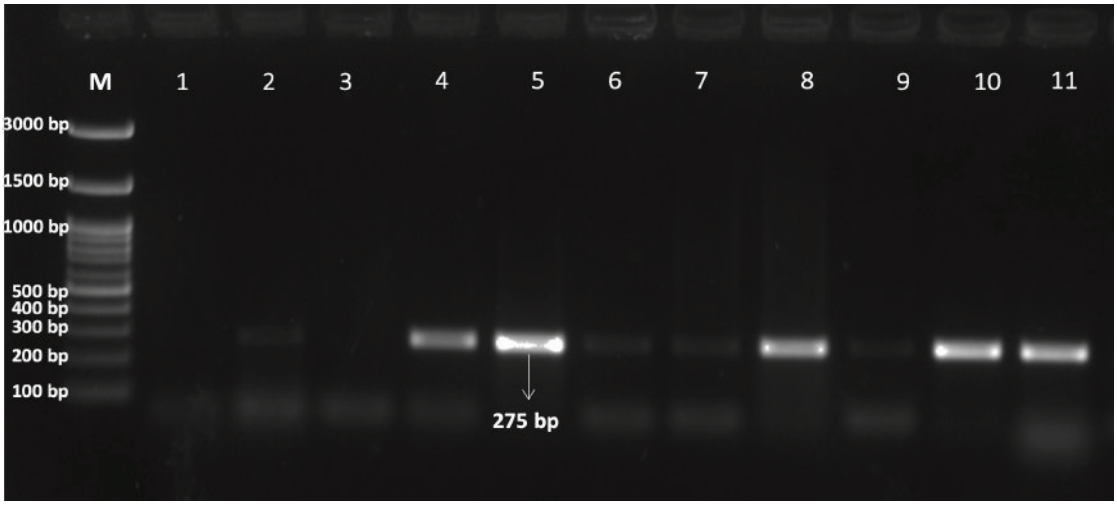


Figure 35: Agarose gel electrophoresis showing PCR amplification of LBA1446 gene. Isolate strain: 1-11 (DA1: *Leuconostoc mesenteroides*; DA3: *Lactococcus lactis*; DA4: *Lactococcus lactis*; DA8: *Lactococcus lactis*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; DA66: *Lactococcus lactis* subsp. *tructae*; SC4: *Leuconostoc mesenteroides;* SC11: *Leuconostoc mesenteroides*; SC17: *Lactococcus lactis* subsp. *hordniae*); M: 100 bp DNAladder.

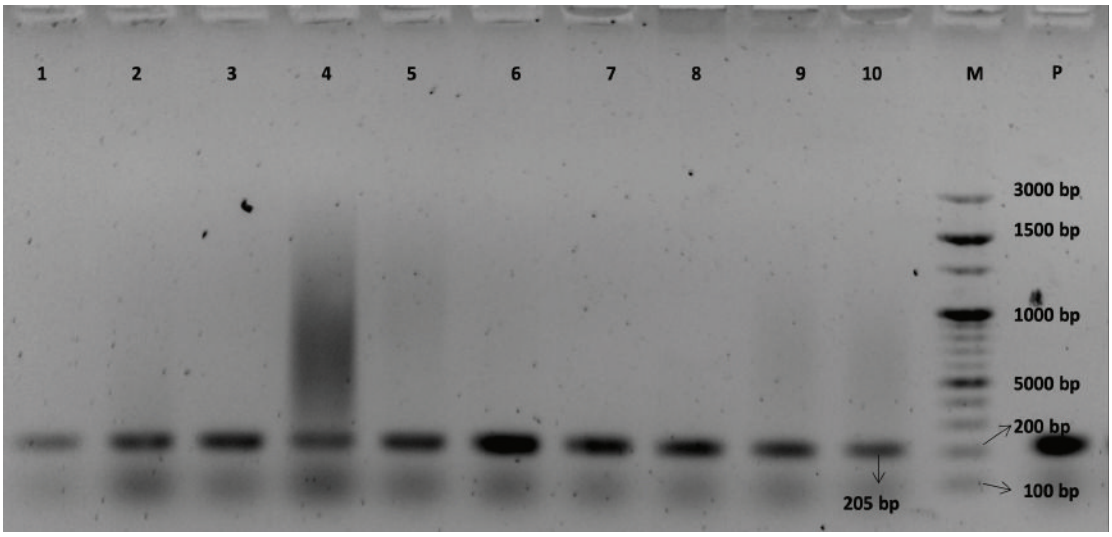


Figure 36: Agarose gel electrophoresis showing PCR amplification of *bsh* gene. Isolate strain: 1-10 (DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; MH4: *Lactococcus lactis*; MH18: *Leuconostoc mesenteroides*; MH20: *Leuconostoc mesenteroides*; DY2: *Leuconostoc mesenteroides*; DY3: *Leuconostoc mesenteroides*; YS4-3: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*; YA4-8: *Enterococcus faecalis*; YS4-11: *Enterococcus faecalis*); M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control.

Detection of genes involved in mucosal binding

Binding related genes such as *mub1, fbp, sor, sbp, msa, agu A* and *apf* were evaluated for mucosal binding. About 55.8% of strains were detected for gene *map A*, 47% of strains detected for gene *apf*, 64.7% of strains for gene *mub 1* and 54.4% of strains for gene *msa* (Table 26). Amplified size was 156 bp for *map A* (mucus adhesin promoting protein) (Fig. 37), 112 bp for gene *apf* (aggregation promoting factor) (Fig. 38), and 150 bp for *mub1* (Mucin-binding protein) (Fig. 39). No bacterial strains were detected for binding gene *fbp, sor*, and *sbp*.

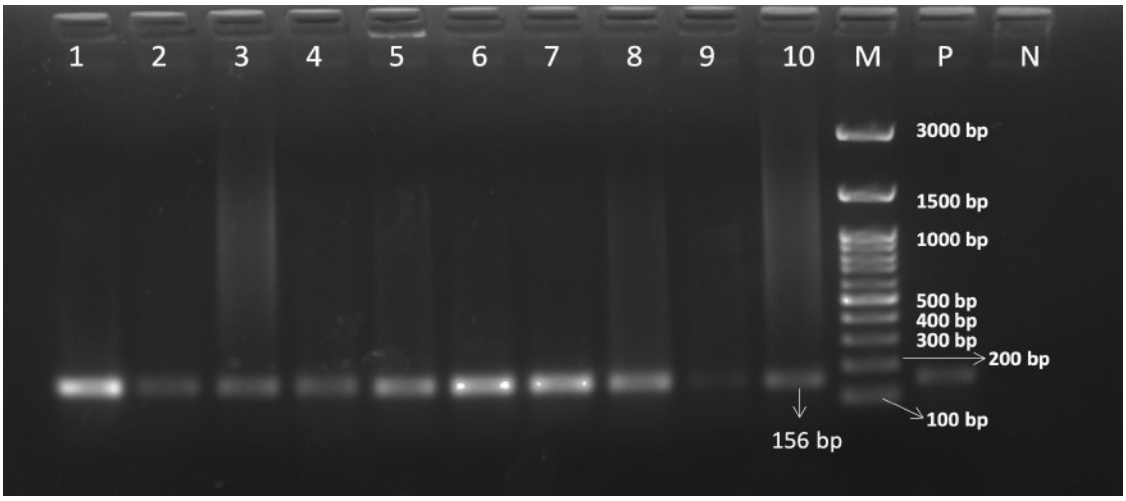


Figure 37: Agarose gel electrophoresis showing PCR amplification of *map A* gene Isolate strain: 1-11(DA1: *Leuconostoc mesenteroides*; DA8: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC4: *Leuconostoc mesenteroides*; SC5: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*; SC7: *Leuconostoc mesenteroides*) M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative.

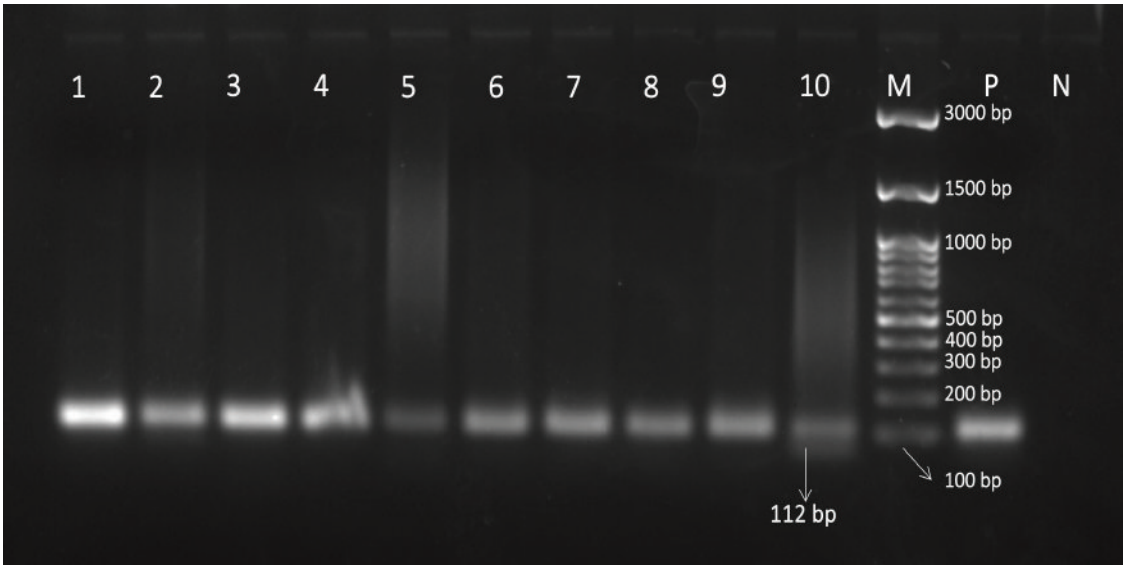


Figure 38: Agarose gel electrophoresis showing PCR amplification of *apf* gene Isolate strain: 1-11 (DA4: *Lactococcus lactis*; DA8: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA66: *Lactococcus lactis* subsp. *tructae;* SC5: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*; SC7: *Leuconostoc mesenteroides*; SC11: *Leuconostoc mesenteroides*), M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative.

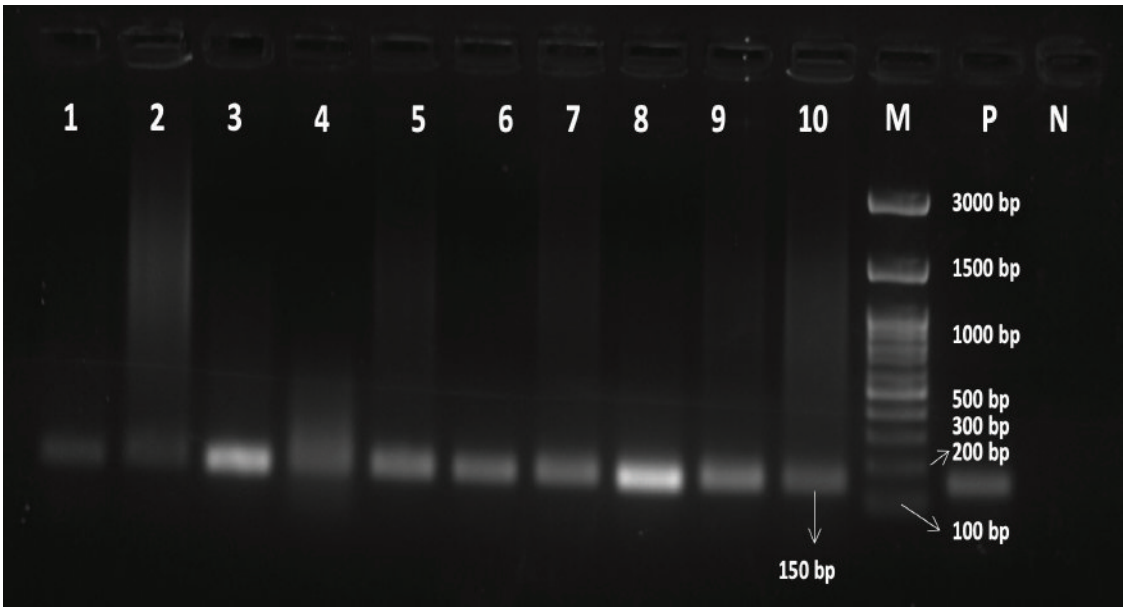


Figure 39: Agarose gel electrophoresis showing PCR amplification of *mub 1* gene. Isolate strain: 1-11 (DA1: *Leuconostoc mesenteroides*; DA3: *Lactococcus lactis*; DA4: *Lactococcus lactis*; DA8: *Lactococcus lactis*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC4: *Leuconostoc mesenteroides*), M: 100 bp DNA ladder; P*: Lb. plantarum* MCC 2034 was used as positive control; N: negative.

Detection of genes involved in bacteriocin production

Bacteriocin producing genes viz. Enterocin AS-48, Nisin, Lactococcin A and Lacticin 481, *Ent B, Ent A,* and *Ent P* and *mes Y* were evaluated for screening of gene for bacteriocin production. About 38.2% of strains were detected for gene *mes Y* (Table 26). The PCR amplification size detected was 182 bp for gene *mes Y* (Mesenteriocin Y) (Fig. 40). No bacterial strains were detected for gene Enterocin AS-48, Nisin, Lactococcin A and Lacticin 481, *Ent B, Ent A,* and *Ent P.*

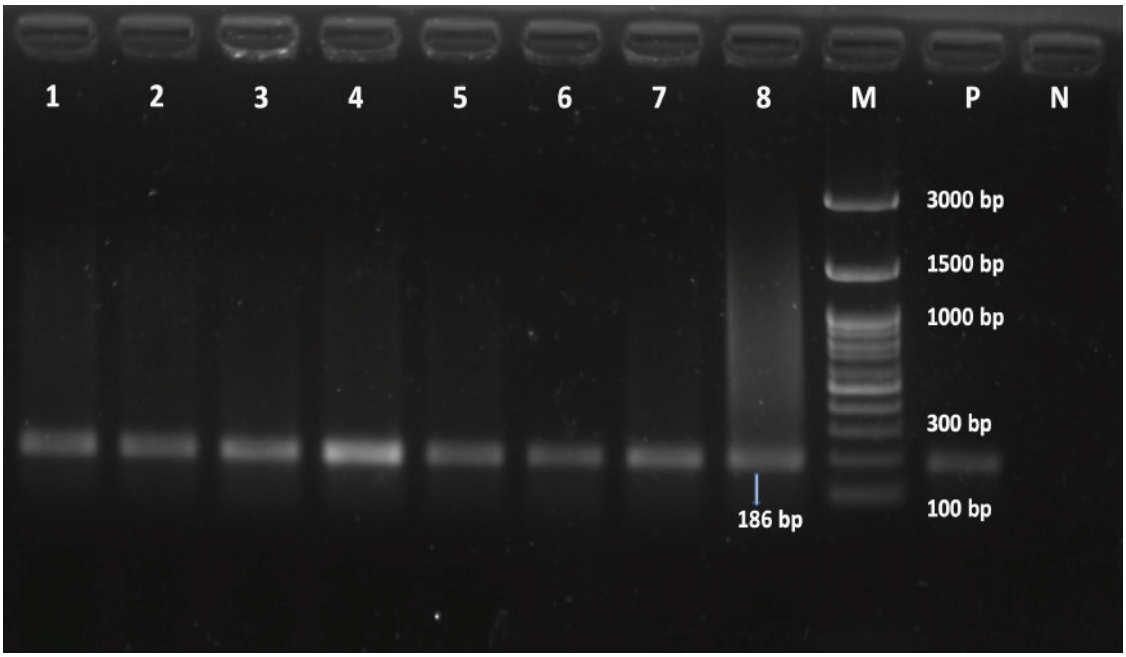


Figure 40: Agarose gel electrophoresis showing PCR amplification of *mes Y* gene. Isolate strain: 1-11(DA1: *Leuconostoc mesenteroides*; DA10: *Leuconostoc mesenteroides*; DA11: *Leuconostoc mesenteroides*; SC4: *Leuconostoc mesenteroides*; SC7: *Leuconostoc mesenteroides*; SC11: *Leuconostoc mesenteroides*; SC19: *Lactococcus lactis* subsp. *cremoris*; SC22: *Leuconostoc mesenteroides*), M: 100 bp DNA ladder; P: *Lb. plantarum* MCC 2034 was used as positive control; N: negative.

Detection of gene involved in beta-glucosidase enzyme

Gene *BGL-1* was evaluated for screening for beta-glucosidase. About 38.2% bacterial strains reported positive for *BGL-1* gene (Table 26). Remaining (61.8%) strains were not detected gene from the bacterial strains. Partial gene sequence was detected having size of 1392 bp for gene *BGL-1* (*B*-glucosidase) (Fig. 41).

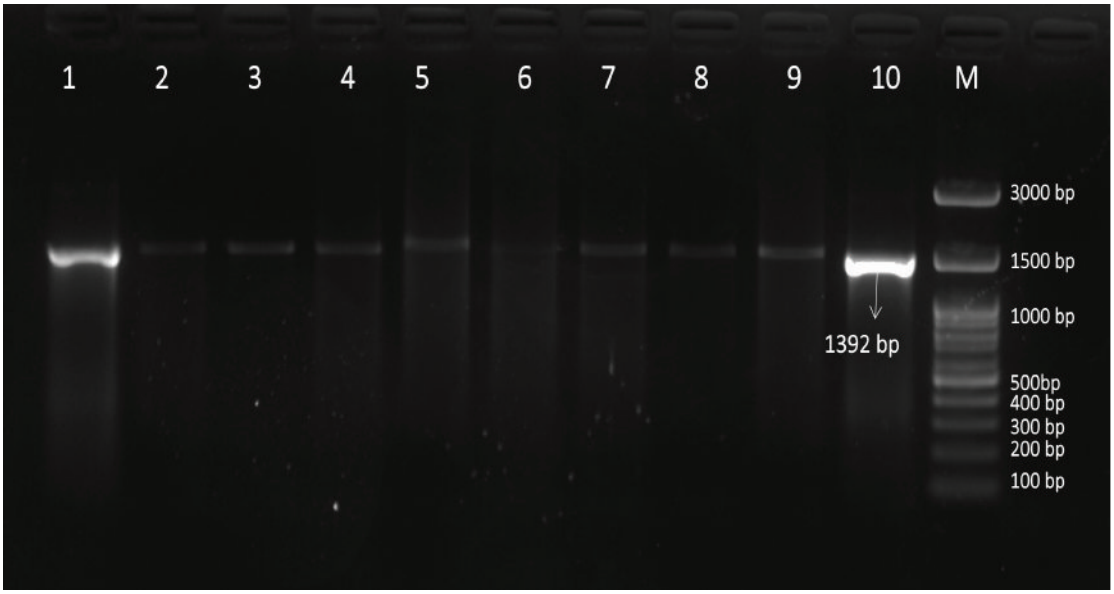


Figure 41: Agarose gel electrophoresis showing PCR amplification of *BGL-1* gene. Isolate strain: 1-10 (DA1: *Leuconostoc mesenteroides*; DA10: *Leuconostoc mesenteroides*; DA14: *Lactococcus lactis*; DA35: *Enterococcus italicus*; SC3: *Lactococcus lactis* subsp. *cremoris*; SC7: *Leuconostoc mesenteroides*; SC22: *Leuconostoc mesenteroides*; SC26: *Leuconostoc mesenteroides*; MH15: *Leuconostoc mesenteroides*; MH20: *Leuconostoc mesenteroides*), M: 100 bp DNA ladder.

Selection of probable probiotic candidates from NFM products of Sikkim

Table 27 summarized the probiotic attributes of LAB strains isolated from naturally fermented milk products of Sikkim showing probiotic characters on the basis of presence of gene encoding for various probiotic properties. Of the 68 representative strains, 53 strains showed at least one *in vivo* determination of probiotic properties as well as gene detection. Nonetheless, 15 strains displayed no probiotic properties. Therefore, on the basis of determination of probiotic attributes supported by gene detection, we selected 20 LAB strains with the high probiotic attributes (Table 28). These strains demonstrated more than three probiotic properties. Among the species, some strains of *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *cremoris* may be considered as good probiotic candidates. These are *Leuconostoc mesenteroides* DA1, DA10, DA11, SC7, SC22, SC26, MH15, MH20, DY16, DY18, DY19, DY29, DY36, DY42 and YS7-4 (15 strains); *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* YS7-7 and YS7-13 (2 strains)*;* and *Lactococcus lactis* subsp. *cremoris* YS7, YS8-8 and YS8-10 (3 strains).

Table 27: Bacterial strains isolated from naturally fermented milk products of Sikkim showing probiotic characters on the basis of gene detection

Bacteria	NFM Product	Milk source	Gene Detected for Probiotic attributes						
			Acid tolerance	Lysozyme tolerance	Bile tolerance	BSH activity	Beta-glucosidase activity	Hydrophobicity	Bacteriocin
Leuconostoc mesenteroides DA1	*Dahi*	Cow	Ir1516, *agu A, clp L,*	ND	LBA1446	-	*BGL -1*	*map A, mub 1, msa,*	*mes Y*
Lactococcus lactis DA3	*Dahi*	Cow	Ir1516, *agu A, clp L,*	ND	LBA1446	-	-	*mub 1, msa*	-
Lactococcus lactis DA4	*Dahi*	Cow	Ir1516, *agu A, tdc, clp L,*	ND	LBA1446	-	-	*mub 1, apf*	-
Lactococcus lactis DA8	*Dahi*	Cow	*clp L*	ND	LBA1446, Ir1584	-	-	*map A, mub 1 apf*	-
Leuconostoc mesenteroides DA10	*Dahi*	Cow	*clp L,* Ir1516, *agu A, tdc*	ND	-	-	*BGL -1*	*map A, mub 1, msa apf*	*mes Y*

Leuconostoc mesenteroides DA11	*Dahi*	Cow	*clp L,* Ir1516 *agu A, tdc*	ND	LBA1446, Ir1584	*bsh*	-	*map A, mub 1, msa apf*	*mes Y*
Lactococcus lactis DA14	*Dahi*	Cow	*clp L,* Ir1516, *agu A*	ND	LBA1446 Ir1584	*bsh*	*BGL*	*map A, mub 1, msa*	-
Enterococcus italicus DA35	*Dahi*	Cow	*clp L,* Ir1516, *agu A,*	ND	LBA1446		*BGL*	*map A, mub 1, msa*	-
Lactococcus lactis DA41	*Dahi*	Cow	-	ND	-	-	-	-	-
Lactococcus lactis subsp. *tructae* DA66	*Dahi*	Cow	*clp L*	ND	LBA1446	-	-	*apf*	-
Lactococcus lactis subsp. *cremoris* SC3	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A,*	ND	-	-	*BGL -1*	*map A, mub 1, msa*	-
Leuconostoc mesenteroides SC4	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A*	ND	LBA1446	-	-	*map A, mub 1, msa*	*mes Y*
Leuconostoc mesenteroides subsp. *jonggajibkimchii* SC5	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A, tdc*	ND	-	-	-	*map A, mub 1, msa apf*	-

Leuconostoc mesenteroides SC7	Soft *chhurpi*	Cow	*clp L* Ir1516, *tdc*	ND	-	-	*BGL-1*	*mapA, mub1, msa apf*	*mesY*
Leuconostoc mesenteroides SC11	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A, tdc*	ND	LBA1446 Ir1584	-	-	*mapA, mub1, msa apf*	*mesY*
Lactococcus lactis subsp. *hordniae* SC17	Soft *chhurpi*	Cow	Ir1516, *agu A*		LBA1446	-	-	*mub1, msa*	
Lactococcus lactis subsp. *cremoris* SC19	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A, tdc*	ND	-	-	-	*apf*	-
Leuconostoc mesenteroides SC22	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A, tdc*	ND	-	-	*BGL-1*	*mapA, mub1, msa apf*	*mesY*
Leuconostoc mesenteroides SC26	Soft *chhurpi*	Cow	*clp L* Ir1516, *agu A, tdc*	ND	LBA1446 Ir1584	-	*BGL-1*	*mapA, mub1, msa apf*	*mesY*
Leuconostoc mesenteroides SC30	Soft *chhurpi*	Cow	-	ND	-	-	-	-	-
Lactococcus lactis MH3	*Mohi*	Cow	*clp L*	ND	-	-		-	-

Lactococcus lactis MH4	*Mohi*	Cow	*clp L agu A, tdc*	ND	LBA1446	*bsh*	-	*mub 1 apf*	-
Lactococcus lactis MH9	*Mohi*	Cow	-	ND	-	-	-	-	-
Leuconostoc mesenteroides MH15	*Mohi*	Cow	*clp L* Ir1516 *, agu A,*	ND	LBA1446 Ir1584	-	*BGL -1*	*map A, mub 1, msa*	*mes Y*
Leuconostoc mesenteroides MH18	*Mohi*	Cow	*clp L* Ir1516 *, agu A, tdc*	ND	-	*bsh*	-	*map A, mub 1 apf*	-
Leuconostoc mesenteroides MH20	*Mohi*	Cow	*clp L* Ir1516 *, agu A, tdc*	ND	Ir1584	*bsh*	*BGL -1*	*map A, mub 1, msa apf*	*mes Y*
Lactococcus lactis MH22	*Mohi*	Cow	*clp L*	ND	-	-	-	-	-
Lactococcus lactis MH39	*Mohi*	Cow	-	ND	-	-	-	-	-
Lactococcus lactis MH40	*Mohi*	Cow	-	ND	-	-	-	-	-
Leuconostoc mesenteroides DY2	*Dahi*	Yak	*clp L*	ND	-	*bsh*	-	-	*mes Y*
Leuconostoc mesenteroides DY3	*Dahi*	Yak	-	ND	-	*bsh*	-	-	*mes Y*
Leuconostoc mesenteroides	*Dahi*	Yak	-	ND	-	-	-	-	-

DY14									
Leuconostoc mesenteroides DY16	*Dahi*	Yak	*clp L* Ir1516, *agu A, tdc*	ND	LBA1446 Ir1584	-	*BGL-1*	*map A, mub 1, msa* *apf*	*mes Y*
Leuconostoc mesenteroides DY18	*Dahi*	Yak	*clp L* Ir1516, *agu A*	ND	LBA1446	-	-	*map A, mub 1, msa*	*mes Y*
Leuconostoc mesenteroides DY19	*Dahi*	Yak	*clp L* Ir1516, *agu A*	ND	Ir1584	-	*BGL-1*	*map A, mub 1, msa*	*mes Y*
Leuconostoc mesenteroides DY29	*Dahi*	Yak	*clp L* Ir1516, *agu A, tdc*	ND	-	-	*BGL-1*	*map A, mub 1, msa*	*mes Y*
Leuconostoc mesenteroides DY30	*Dahi*	Yak	*clp L*	ND	-	-	-	-	*mes Y*
Leuconostoc mesenteroides DY36	*Dahi*	Yak	*agu A, tdc* *clp L*	ND	LBA1446	-	*BGL-1*	*map A, mub 1, msa* *apf*	*mes Y*
Leuconostoc mesenteroides DY42	*Dahi*	Yak	Ir1516, *agu A, tdc* *clp L*	ND	LBA1446	-	*BGL-1*	*map A, mub 1, msa*	*mes Y*

								apf	
Leuconostoc mesenteroides YS4-1			Ir1516, *agu A, tdc* *clp L*	ND	-	-	-	*map A, mub 1, msa* *apf*	-
Leuconostoc mesenteroides subsp. *jonggajibkimchii* YS4-3	Soft *chhurpi*	Yak	*tdc* *clp L*	ND	LBA1446 Ir1584	*bsh*	-	*map A, mub 1, msa* *apf*	-
Enterococcus faecalis YS4-4	Soft *chhurpi*	Yak	Ir1516, *agu A, tdc* *clp L*	ND	LBA1446	-	-	*map A, mub 1, msa* *apf*	-
Enterococcus faecalis YS4-7	Soft *chhurpi*	Yak	Ir1516, *agu A, clp L*	ND	LBA1446	-	-	*map A, mub 1, msa*	-
Enterococcus faecalis YS4-8	Soft *chhurpi*	Yak	*clp L*	ND	LBA1446 Ir1584	*bsh*	-	-	-
Leuconostoc mesenteroides YS4-9	Soft *chhurpi*	Yak	Ir1516, *clp L*	ND	-	-	*BGL-1*	*map A, mub 1, msa*	*mesY*
Enterococcus pseudoavium YS4-10	Soft *chhurpi*	Yak	Ir1516, *agu A, tdc* *clp L*	ND	-	-	-	-	-

Enterococcus faecalis YS4-11	Soft *chhurpi*	Yak	*tdc* *clp L*	ND	LBA1446 Ir1584	*bsh*	-	*mapA, mub1, msa* *apf*	-
Enterococcus faecalis YS4-14	Soft *chhurpi*	Yak	Ir1516, *aguA, tdc clp L*	ND	-	*bsh*	-	*apf*	-
Lactococcus lactis subsp. *cremoris* YS4-15	Soft *chhurpi*	Yak	Ir1516, *aguA, tdc* *clp L*	ND	LBA1446 Ir1584	*bsh*	-	*mapA, mub1, msa* *apf*	-
Lactococcus lactis subsp. *cremoris* YS7-1	Hard *chhurpi*	Yak	Ir1516, *tdc* *clp L*	ND	LBA1446, Ir1584	-	-	*mapA, mub1, msa* *apf*	-
Leuconostoc mesenteroides YS7-2	Hard *chhurpi*	Yak	*clp L*	ND	-	-	-	-	-
Leuconostoc mesenteroides YS7-3	Hard *chhurpi*	Yak	-	ND	-	-	-	-	-
Leuconostoc mesenteroides YS7-4	Hard *chhurpi*	Yak	Ir1516, *aguA, tdc* *clp L*	ND	-	-	*BGL-1*	*mapA, mub1, msa* *apf*	*mesY*
Leuconostoc mesenteroides	Hard *chhurp*	Yak	*clp L*	ND	-	-	-	-	-

subsp. *jonggajibkimchi i* YS7-5	*i*								
Leuconostoc mesenteroides subsp. *jonggajibkimchi i* YS7-7	Hard *chhurp i*	Yak	*tdc* *clp L*	ND	Ir1584	*bsh*	*BGL -1*	*map A, mub 1, msa* *apf*	*mes Y*
Leuconostoc mesenteroides YS7-8	Hard *chhurp i*	Yak	-	ND	-	-	-	-	*mes Y*
Leuconostoc mesenteroides subsp. jonggajibkimchi i YS7-10	Hard *chhurp i*	Yak	-	ND	-	-	-	-	-
Leuconostoc mesenteroides subsp. jonggajibkimchi i YS7-12	Hard *chhurp i*	Yak	*agu A, tdc* *clp L*	ND	LBA1446 Ir1584	*bsh*	-	*mub 1* *apf*	-
Leuconostoc mesenteroides subsp. jonggajibkimchi i YS7-13	Hard *chhurp i*	Yak	*clp L*	ND	LBA1446 , Ir1584	-	*BGL -1*	*map A, mub 1, msa*	*mes Y*
Lactococcus lactis subsp. cremoris YS8-1	*Philu*	Yak	Ir1516 , *agu A, tdc,* *clp L*	ND	Ir1584	-	-	*map A, mub 1, msa* *apf*	-
Lactococcus lactis subsp. *cremoris* YS8-3	*Philu*	Yak	-	ND	-	-	-	-	-

Lactococcus lactis subsp. *cremoris* YS8-4	*Philu*	Yak	*clp L*	ND	-	-	-	-	-
Lactococcus lactis subsp. *cremoris* YS8-5	*Philu*	Yak	Ir1516, *agu A, tdc clp L*	ND	-	-	-	*map A, mub 1, msa apf*	-
Lactococcus lactis subsp. cremoris YS8-7	*Philu*	Yak	*clp L*	ND	Ir1584	-	-	*map A, mub 1*	-
Lactococcus lactis subsp. cremoris YS8-8	*Philu*	Yak	Ir1516, *agu A, tdc clp L*	ND	LBA1446, Ir1584	*bsh*	*BGL -1*	*map A, mub 1, msa apf*	-
Lactococcus lactis subsp. cremoris YS8-10	*Philu*	Yak	Ir1516, *agu A, clp L*	ND	LBA1446	-	*BGL -1*	*map A, mub 1, msa*	-
Lactococcus lactis subsp. *cremoris* YS8-11	*Philu*	Yak	-	ND	-	-	-	-	-
Lactococcus lactis subsp. *cremoris* YS8-13	*Philu*	Yak	-	ND	-	-	-	-	-

(-) indicates no gene detection; ND indicates not determined

Table 28: Selection of probable probiotic bacterial strains from NFM products of Sikkim				
NFM Product	**Milk source**	**Selected Probiotic Strain**	**Probiotic attributes**	**Gene for probiotic properties**
Dahi	Cow	*Leuconostoc mesenteroides* DA1	Bile tolerance, Lysozyme tolerance, Hydrophobicity Antimicrobial activity	Ir1516*, agu A, clp L,* LBA1446 *map A, mub 1, msa, BGL-1, mes Y*
Dahi	Cow	*Leuconostoc mesenteroides* DA10	Acid tolerance, Lysozyme tolerance	*clp L,* Ir1516*, agu A, tdc, map A, mub 1, msa, apf BGL-1, mes Y*
Dahi	Cow	*Leuconostoc mesenteroides* DA11	Acid tolerance Bile tolerance, Lysozyme tolerance	*clp L,* Ir1516*, agu A, tdc* LBA1446 Ir1584, *bsh, map A, mub 1, msa apf, mes Y*
Soft *chhurpi*	Cow	*Leuconostoc mesenteroides* SC7	Acid tolerance Bile tolerance, Beta-glucosidase activity	*clp L* Ir1516*, map A, mub 1, msa BGL-1, mes Y*
Soft *chhurpi*	Cow	*Leuconostoc mesenteroides* SC22	Acid tolerance	*clp L,* Ir1516*, agu A, tdc, BGL-1 map A, mub 1, msa apf, mes Y*
Soft *chhurpi*	Cow	*Leuconostoc mesenteroides* SC26	Bile tolerance Antibacterial activity	*clp L,* Ir1516*, agu A, tdc,* LBA1446, Ir1584, *map A, mub 1, msa apf, BGL-1, mes Y*
Mohi	Cow	*Leuconostoc mesenteroides* MH15	BSH activity, Beta-glucosidase activity	*clp L,* Ir1516*, agu A,* LBA1446, Ir1584, *map A, mub 1, msa, BGL-1, mes Y*
Mohi	Cow	*Leuconostoc mesenteroides* MH20	Acid tolerance Lysozyme tolerance, BSH activity	*clp L,* Ir1516*, agu A, tdc,* Ir1584, *bsh, map A, mub 1, msa apf, BGL-1, mes Y*
Dahi	Yak	*Leuconostoc mesenteroides* DY16	Acid tolerance Bile tolerance, Lysozyme tolerance BSH activity, Beta-glucosidase activity	*clp L* Ir1516*, agu A, tdc,* LBA1446 Ir1584, *map A, mub 1, msa, apf, BGL-1, mes Y*
Dahi	Yak	*Leuconostoc mesenteroides* DY18	Acid tolerance Lysozyme tolerance BSH activity, Beta-glucosidase activity Antimicrobial	*clp L* Ir1516*, agu A,* LBA1446, *map A, mub 1, msa, mes Y*

			activity	
Dahi	Yak	*Leuconostoc mesenteroides* DY19	Acid tolerance BSH activity, Beta-glucosidase activity	*clp L* Ir1516*, agu A,* Ir1584, *map A, mub 1, msa, BGL-1, BGL-1*
Dahi	Yak	*Leuconostoc mesenteroides* DY29	Acid tolerance Beta-glucosidase activity Antimicrobial activity	*clp L* Ir1516*, agu A, tdc, map A, mub 1, msa, BGL-1, mes Y*
Dahi	Yak	*Leuconostoc mesenteroides* DY36	Acid tolerance Lysozyme tolerance Beta-glucosidase activity	*agu A, tdc* *clp L* LBA1446, *map A, mub 1, msa apf, BGL-1, mes Y*
Dahi	Yak	*Leuconostoc mesenteroides* DY42	Bile tolerance, lysozyme tolerance Beta-glucosidase activity	Ir1516*, agu A, tdc clp L,* LBA1446, *map A, mub 1, msa apf, BGL-1, mes Y*
Hard *chhurpi*	Yak	*Lactococcus lactis* subsp. *cremoris* YS7-1	Bile tolerance, lysozyme tolerance BSH activity	Ir1516*, tdc clp L,* LBA1446, Ir1584, *map A, mub 1, msa apf*
Hard *chhurpi*	Yak	*Leuconostoc mesenteroides* YS7-4	Acid tolerance Bile tolerance, Lysozyme tolerance BSH activity	Ir1516*, agu A, tdc clp L, map A, mub 1, msa apf, BGL-1, mes Y*
Hard *chhurpi*	Yak	*Leuconostoc mesenteroides* subsp. *jonggajibkimchii* YS7-7	Acid tolerance Lysozyme tolerance BSH activity	*tdc* *clp L,* Ir1584, *bsh, map A, mub 1, msa apf, BGL-1, mes Y*
Hard *chhurpi*	Yak	*Leuconostoc mesenteroides subsp. jonggajibkimchii* YS7-13	Acid tolerance Bile tolerance, BSH activity	*clp L* LBA1446, Ir1584, *map A, mub 1, msa, BGL-1, mes Y*
Philu	Yak	*Lactococcus lactis* subsp. *cremoris* YS8-8	Bile tolerance, Lysozyme tolerance BSH activity, Beta-glucosidase activity	Ir1516*, agu A, tdc clp L,* LBA1446, *bsh ,* Ir1584, *map A, mub 1, msa apf, BGL-1*
Philu	Yak	*Lactococcus lactis* subsp. *cremoris* YS8-10	Lysozyme tolerance	Ir1516*, agu A, clp L,* LBA1446, *map A, mub 1, msa, BGL-1*

Predictive functionality using PICRUSt2

Overall, predictive functionality based on the 16S rRNA marker gene was categorized into three default levels viz., super-pathway (Level-1), sub-pathway (Level-2) and pathways (Level-1). Level-1 is categorized by six super-pathways: metabolism (78.59%), genetic information processing (11.96%), cellular processes (5.83%), environmental information processing (3.05%), and others with relative abundance below 1% includes organismal systems (0.32%), human diseases (0.25%) (Fig. 42).

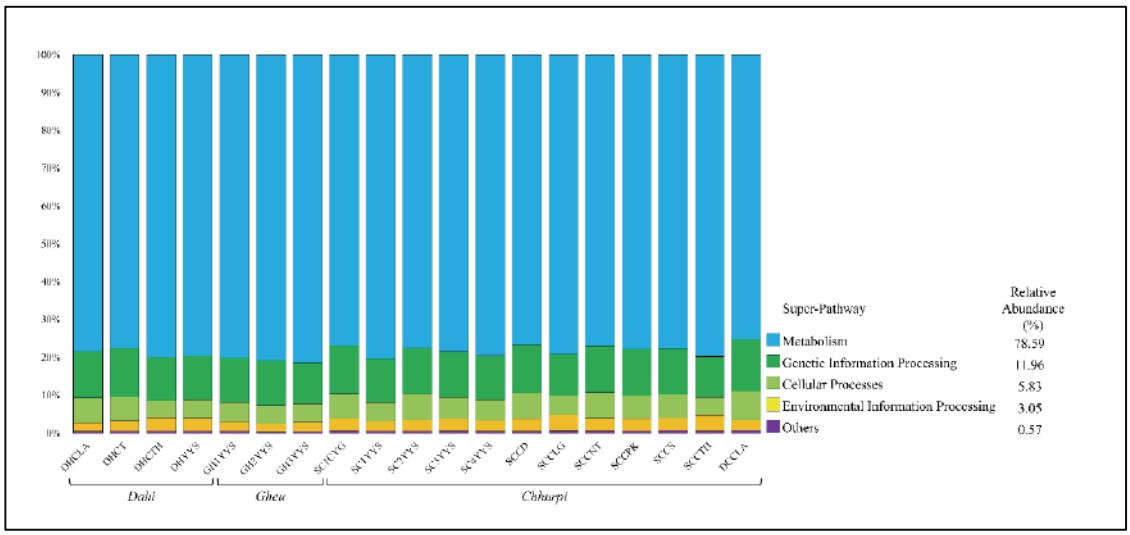


Figure 42: Predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 analysis, distributed at Level-1 (Super-Pathways).

Level-2 is categorized by twenty-nine sub-pathways: carbohydrate metabolism (13.38%), amino acid metabolism (12.8%), metabolism of cofactors and vitamins (12.69%), metabolism of other amino acids (8.77%), metabolism of terpenoids and polyketides (8.53%), lipid metabolism (6.75%), energy metabolism (5.11%), replication and repair (5.01%), xenobiotics biodegradation and metabolism (3.94%), cell motility (3.7%), folding, sorting and degradation (3.47%), glycan biosynthesis and metabolism (3.47%),

translation (2.85%), membrane transport (2.46%), nucleotide metabolism (1.75%), cell growth and death (1.44%), biosynthesis of other secondary metabolites (1.41%), and those pathways >1% comprises of transcription (0.63%), signal transduction (0.58%), cellular community - prokaryotes (0.38%), transport and catabolism (0.31%), environmental adaptation (0.25%), infectious disease: bacterial (0.16%), endocrine system (0.05%), drug resistance: antimicrobial (0.04%), infectious disease: parasitic (0.04%), digestive system (0.01%), neurodegenerative disease (0.01%), immune system (0.01%) (Fig. 43).

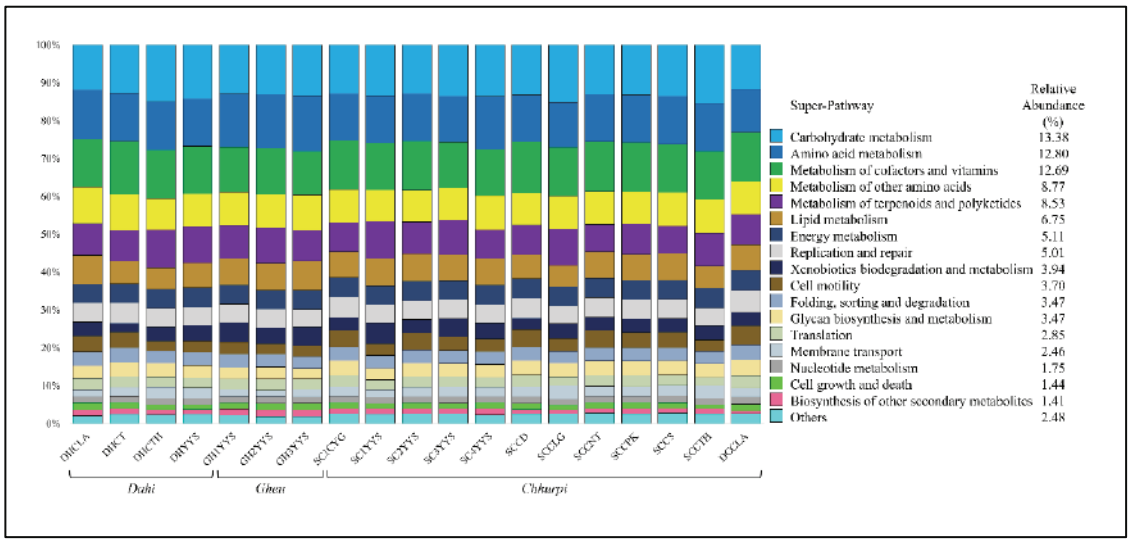


Figure 43: Predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 analysis, distributed at Level-2 (Sub-Pathways).

Level-3 comprised of one hundred and fifty pathways: biosynthesis of ansamycins (3.01%), valine, leucine and isoleucine biosynthesis (2.02%), bacterial chemotaxis (1.93%), fatty acid biosynthesis (1.89%), flagellar assembly (1.77%), D-glutamine and D-glutamate metabolism (1.77%), C5-branched dibasic acid metabolism (1.76%), pantothenate and coa biosynthesis (1.74%), synthesis and degradation of ketone bodies

(1.72%), peptidoglycan biosynthesis (1.61%), D-alanine metabolism (1.57%), biosynthesis of vancomycin group antibiotics (1.52%), lipopolysaccharide biosynthesis (1.51%), aminoacyl-trna biosynthesis (1.45%), sulfur relay system (1.45%), biotin metabolism (1.42%), protein export (1.39%), cell cycle - caulobacter (1.38%), one carbon pool by folate (1.35%), mismatch repair (1.31%), ribosome (1.31%), lysine biosynthesis (1.31%), carbon fixation pathways in prokaryotes (1.29%), pentose phosphate pathway (1.29%), alanine, aspartate and glutamate metabolism (1.28%), lipoic acid metabolism (1.27%), carbon fixation in photosynthetic organisms (1.27%), pyruvate metabolism (1.25%), thiamine metabolism (1.24%), terpenoid backbone biosynthesis (1.23%), homologous recombination (1.23%), citrate cycle (TCA cycle) (1.2%), Folate biosynthesis (1.2%), selenocompound metabolism (1.18%), streptomycin biosynthesis (1.16%), glycine, serine and threonine metabolism (1.15%), glutathione metabolism (1.15%), phenylalanine, tyrosine and tryptophan biosynthesis (1.13%), nicotinate and nicotinamide metabolism (1.1%), cysteine and methionine metabolism (1.1%), vitamin b6 metabolism (1.1%), bacterial secretion system (1.08%), glycolysis/gluconeogenesis (1.05%), butanoate metabolism (1.04%), DNA replication (1.02%), propanoate metabolism (1.02%), and others >1% (35.78%), comprised of valine, leucine and isoleucine degradation (0.96%), histidine metabolism (0.96%), pyrimidine metabolism (0.95%), glyoxylate and dicarboxylate metabolism (0.94%), taurine and hypotaurine metabolism (0.9%), ABC transporters (0.9%), sulfur metabolism (0.89%), riboflavin metabolism (0.87%), drug metabolism - other enzymes (0.87%), fatty acid metabolism (0.85%), base excision repair (0.85%), beta-alanine metabolism (0.84%), purine metabolism (0.79%), tetracycline biosynthesis (0.78%), ubiquinone and other terpenoid-quinone biosynthesis (0.78%), amino sugar and nucleotide sugar metabolism (0.77%), arginine and proline metabolism (0.76%), biosynthesis of

unsaturated fatty acids (0.67%), oxidative phosphorylation (0.65%), glycerophospholipid metabolism (0.64%), rna polymerase (0.63%), cyanoamino acid metabolism (0.63%), starch and sucrose metabolism (0.62%), tryptophan metabolism (0.62%), geraniol degradation (0.61%), porphyrin and chlorophyll metabolism (0.61%), fructose and mannose metabolism (0.61%), rna degradation (0.59%), pentose and glucuronate interconversions (0.59%), nucleotide excision repair (0.58%), nitrogen metabolism (0.58%), two-component system (0.58%), lysine degradation (0.57%), caprolactam degradation (0.54%), galactose metabolism (0.52%), D-arginine and D-ornithine metabolism (0.51%), phenylalanine metabolism (0.5%), phosphotransferase system (pts) (0.48%), nitrotoluene degradation (0.48%), tyrosine metabolism (0.45%), zeatin biosynthesis (0.44%), glycerolipid metabolism (0.42%), methane metabolism (0.42%), vibrio cholerae pathogenic cycle (0.38%), limonene and pinene degradation (0.38%), benzoate degradation (0.36%), ascorbate and aldarate metabolism (0.36%), inositol phosphate metabolism (0.35%), styrene degradation (0.31%), peroxisome (0.31%), polyketide sugar unit biosynthesis (0.3%), aminobenzoate degradation (0.3%), chloroalkane and chloroalkene degradation (0.26%), plant-pathogen interaction (0.25%), other glycan degradation (0.24%), dioxin degradation (0.23%), secondary bile acid biosynthesis (0.22%), biosynthesis of siderophore group non-ribosomal peptides (0.22%), phosphonate and phosphinate metabolism (0.22%), fluorobenzoate degradation (0.18%), chlorocyclohexane and chlorobenzene degradation (0.17%), toluene degradation (0.14%), betalain biosynthesis (0.13%), linoleic acid metabolism (0.13%), epithelial cell signaling in helicobacter pylori infection (0.12%), sphingolipid metabolism (0.11%), glycosaminoglycan degradation (0.1%), naphthalene degradation (0.1%), penicillin and cephalosporin biosynthesis (0.09%), ribosome biogenesis in eukaryotes (0.06%), apoptosis (0.06%), primary bile acid biosynthesis (0.05%), insulin signaling

pathway (0.05%), steroid hormone biosynthesis (0.04%), beta-lactam resistance (0.04%), n-glycan biosynthesis (0.03%), protein processing in endoplasmic reticulum (0.03%), *Staphylococcus aureus* infection (0.03%), RNA transport (0.03%), carotenoid biosynthesis (0.02%), tropane, piperidine and pyridine alkaloid biosynthesis (0.02%), *Vibrio cholerae* infection (0.02%), Chagas disease (American trypanosomiasis) (0.02%), Amoebiasis (0.02%), non-homologous end-joining (0.01%), potein digestion and absorption (0.01%), Alzheimer's disease (0.01%), steroid biosynthesis (0.01%), xylene degradation (0.01%), proteasome (0.01%), NOD-like receptor signaling pathway (0.01%), flavonoid biosynthesis (0.01%), Meiosis - yeast (0.01%), African trypanosomiasis (0.004%), biosynthesis of 12-, 14- and 16-membered macrolides (0.002%), Wnt signaling pathway (0.002%), sesquiterpenoid biosynthesis (0.001%), Basal transcription factors (0.0001%), bacterial invasion of epithelial cells (0.0001%), biosynthesis of type II polyketide backbone (0.00005%), mRNA surveillance pathway (0.00003%), renin-angiotensin system (0.00002%), spliceosome (0.00001%), isoflavonoid biosynthesis (0.000003%) (Fig. 44).

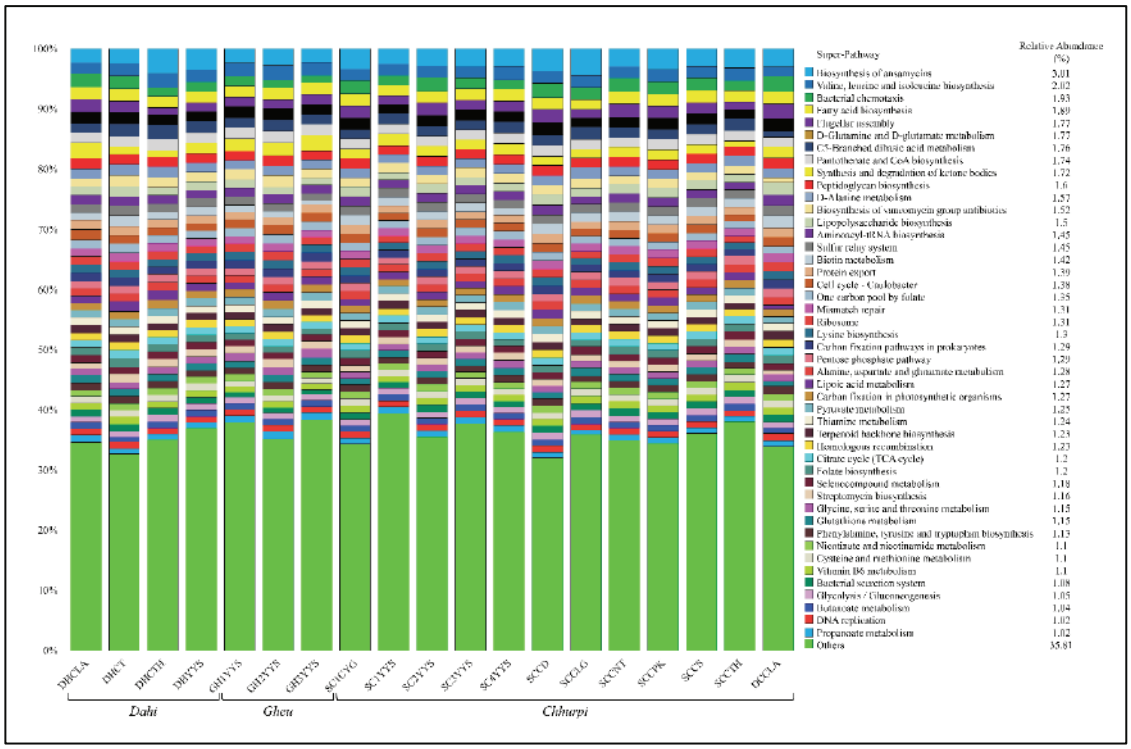


Figure 44: Predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 analysis, distributed at Level-3 (Pathways).

Significant pathways:

At Level-1, Metabolism was observed to be significantly higher in *gheu* in comparison to both *dahi* (p = 0.042) and *chhurpi* (p= 0.001). Significant variation was also observed to be higher in *chhurpi* for cellular processes between gheu (p= 0.002). *Chhurpi* showed significant increase of environmental information processing in comparison with *gheu* (p= 0.0004) (Table 29). At Level-2, Amino acid metabolism is observed to be significantly higher in *gheu* in comparison to both *dahi* (p=0.007) and *chhurpi* (p=0.002). Similarly, we observed a significant increase of biosynthesis of other secondary metabolites in *gheu* as compared to that of *dahi* (p= 0.12) and *chhurpi* (p=0.0002). On the other hand, *chhurpi* showed a significant increase for metabolism of co-factors and

vitamins in respect to *gheu* (p=0.027). Additionally, cell motility was also observed to be significantly higher in *chhurpi* than in *gheu* (p= 0.001), as well as for glycan biosynthesis and metabolism (p=0.021), membrane transport (p= 0.001), and nucleotide metabolism (p=0.011). *Gheu* showed a significant increase for cell growth and death than *chhurpi* (p=0.016) (Table 30).

At Level-3, biosynthesis of ansamycins was observed to be significantly higher in *chhurpi* than in *gheu* (p = 0.021). Similarly, significant increase was also observed in *chhurpi* in comparison to *gheu* for bacterial chemotaxis (p = 0.002), flagellar assembly (p=0.019), lipopolysaccharide biosynthesis (p=0.004), pentose phosphate pathway (p=0.009), thiamine metabolism (p=0.008), homologous recombination (p=0.033), and glycolysis/ gluconeogenesis (p = 0.01). On the other hand, valine, leucine and isoleucine biosynthesis was observed to be significantly higher in *gheu* in comparison to *chhurpi* (p= 0.001). Additionally, *gheu* showed a significant increase to that of *chhurpi* for pantothenate and CoA biosynthesis (p= 0.04), synthesis and degradation of ketone bodies (p = 0.0025), lysine biosynthesis (p= 0.016) and propanoate metabolism (p= 0.001). D-alanine metabolism was observed to be significantly higher in *dahi* in comparison to *chhurpi* (p= 0.048). We observed a significant decrease in biotin metabolism in *gheu* in comparison to both *dahi* (p= 0.028) and *chhurpi* (p= 0.014). Similarly, folate biosynthesis was significantly low in *gheu* in comparison to *dahi* (p= 0.017) and *chhurpi* (p = 0.033). Seleno-compound metabolism was observed to be significantly low in *gheu* than in *dahi* (p = 0.013) and *chhurpi* (p = 0.002). Cysteine, methionine metabolism was also found to be significantly low in *gheu* as compared to *dahi* (p = 0.0003) and *chhurpi* (p = 0.000003). Vitamin B6 metabolism was also observed to be significantly low in *gheu* than in *dahi* (p=0.017) and *chhurpi* (p= 0.012). Additionally, bacterial secretion system was also significantly low in *gheu* in comparison to *dahi* (p= 0.003) and *chhurpi*

(p= 0.0001). In contrast, *gheu* showed significant increase in pyruvate metabolism than both *dahi* (p = 0.035) and *chhurpi* (p = 0.044). Similarly, glycine, serine, threonine metabolism is significantly higher in *gheu* in comparison to *dahi* (p= 0.029) and *chhurpi* (p= 0.041) (Table 31).

Table 29: 16S rRNA-based predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 algorithm and mapped to KEGG database, represented at Super Pathway (Level-1).

Super Pathway (Level-1)	***Dahi***	***Gheu***	***Chhurpi***	***Dahi* vs *gheu***	***Dahi* vs *chhurpi***	**Gheu vs *chhurpi***
Metabolism	78.88±1.12	80.69±0.56	77.97±1.52	0.042	0.243	0.001
Genetic Information Processing	12.04±0.68	11.51±0.51	12.05±0.83	0.293	0.985	0.218
Cellular Processes	5.59±1.05	5.02±0.19	6.12±0.87	0.358	0.416	0.002
Environmental Information Processing	2.98±0.62	2.37±0.17	3.24±0.46	0.143	0.474	0.0004
Others	0.51±0.04	0.41±0.16	0.62±0.1			

Note: Significant pathways shown in colour.

Table 30: 16S rRNA-based predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 algorithm and mapped to KEGG database, represented at Sub Pathway (Level-2).

Sub-Pathway (Level-2)	***Dahi***	***Gheu***	***Chhurpi***	***Dahi* vs *gheu***	***Dahi* vs *chhurpi***	***Gheu* vs *chhurpi***
Carbohydrate metabolism	13.41±1.41	13.12±0.22	13.44±1.01	0.714	0.971	0.339
Amino acid metabolism	12.76±0.27	14.3±0.39	12.44±0.59	0.007	0.159	0.002
Metabolism of cofactors and vitamins	13±0.78	11.86±0.38	12.8±0.4	0.056	0.644	0.027
Metabolism of other amino acids	9.04±0.65	9±0.32	8.62±0.24	0.931	0.294	0.163
Metabolism of terpenoids and polyketides	9.04±0.89	8.72±0.58	8.31±0.86	0.588	0.211	0.377
Lipid metabolism	6.42±0.89	7.29±0.36	6.73±0.51	0.147	0.548	0.084
Energy metabolism	5.08±0.07	5.11±0.06	5.12±0.11	0.563	0.387	0.796
Replication and repair	4.99±0.21	4.75±0.22	5.07±0.34	0.231	0.563	0.111

Xenobiotics biodegradation and metabolism	3.5±0.77	4.78±0.62	3.87±0.71	0.059	0.428	0.102
Cell motility	3.53±0.77	2.93±0.23	3.95±0.66	0.219	0.37	0.001
Folding, sorting and degradation	3.51±0.3	3.29±0.12	3.51±0.23	0.25	0.973	0.065
Glycan biosynthesis and metabolism	3.46±0.17	3.03±0.22	3.59±0.29	0.054	0.284	0.021
Translation	2.87±0.19	2.82±0.15	2.85±0.27	0.725	0.848	0.849
Membrane transport	2.4±0.51	1.94±0.15	2.62±0.38	0.167	0.467	0.001
Nucleotide metabolism	1.71±0.06	1.71±0.02	1.77±0.06	0.924	0.137	0.011
Cell growth and death	1.46±0.28	1.57±0.06	1.4±0.16	0.497	0.677	0.016
Biosynthesis of other secondary metabolites	1.48±0.12	1.78±0.09	1.3±0.21	0.012	0.066	0.0002
Others	2.34±0.17	2±0.17	2.61±0.14			

Note: Significant pathways shown in colour.

Table 31: 16S rRNA-based predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 algorithm and mapped to KEGG database, represented at Pathway (Level-3).

Sub-Pathway (Level-3)	***Dahi***	***Gheu***	***Chhurpi***	***Dahi* vs *gheu***	***Dahi* vs *chhurpi***	***Gheu* vs *chhurpi***
Biosynthesis of ansamycins	3.05±0.9	2.42±0.3	3.15±0.49	0.266	0.833	0.021
Valine, leucine and isoleucine biosynthesis	2.1±0.16	2.28±0.08	1.93±0.16	0.108	0.136	0.001
Bacterial chemotaxis	1.86±0.36	1.38±0.17	2.09±0.31	0.073	0.316	0.002
Fatty acid biosynthesis	1.92±0.13	1.91±0.05	1.88±0.13	0.888	0.629	0.542
Flagellar assembly	1.67±0.41	1.55±0.09	1.86±0.36	0.613	0.43	0.019
D-Glutamine and D-glutamate metabolism	1.79±0.15	1.79±0.1	1.76±0.2	0.946	0.789	0.706
C5-Branched dibasic acid metabolism	1.95±0.36	1.77±0.16	1.69±0.3	0.419	0.248	0.51
Pantothenate and CoA biosynthesis	1.76±0.07	1.83±0.06	1.7±0.07	0.246	0.212	0.04
Synthesis and degradation of ketone bodies	1.65±0.7	2.28±0.26	1.6±0.26	0.169	0.902	0.025
Peptidoglycan biosynthesis	1.65±0.07	1.54±0.08	1.59±0.07	0.123	0.234	0.351
D-Alanine metabolism	1.69±0.11	1.51±0.06	1.55±0.13	0.048	0.082	0.532
Biosynthesis of vancomycin group antibiotics	1.58±0.22	1.74±0.24	1.44±0.22	0.414	0.314	0.138

Lipopolysaccharide biosynthesis	1.5±0.19	1.27±0.07	1.56±0.23	0.088	0.669	0.004
Aminoacyl-tRNA biosynthesis	1.45±0.1	1.43±0.06	1.46±0.15	0.719	0.957	0.648
Sulfur relay system	1.47±0.14	1.32±0.1	1.48±0.09	0.156	0.895	0.088
Biotin metabolism	1.42±0.22	0.92±0.2	1.55±0.22	0.028	0.348	0.014
Protein export	1.41±0.1	1.36±0.06	1.4±0.11	0.44	0.864	0.407
Cell cycle - Caulobacter	1.42±0.25	1.45±0.08	1.34±0.15	0.798	0.586	0.105
One carbon pool by folate	1.4±0.11	1.46±0.1	1.3±0.07	0.524	0.168	0.105
Mismatch repair	1.31±0.06	1.22±0.06	1.33±0.08	0.123	0.603	0.059

Table 31: 16S rRNA-based predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 algorithm and mapped to KEGG database, represented at Pathway (Level-3) (contd.)

Sub-Pathway (Level-3)	***Dahi***	***Gheu***	***Chhurpi***	***Dahi* vs *gheu***	***Dahi* vs *chhurpi***	***Gheu* vs *chhurpi***
Ribosome	1.32±0.1	1.33±0.08	1.3±0.13	0.942	0.655	0.586
Lysine biosynthesis	1.29±0.06	1.37±0.03	1.29±0.09	0.079	0.91	0.016
Carbon fixation pathways in prokaryotes	1.29±0.07	1.41±0.08	1.27±0.08	0.094	0.694	0.068
Pentose phosphate pathway	1.29±0.06	1.2±0.04	1.31±0.08	0.098	0.603	0.009
Alanine, aspartate and glutamate metabolism	1.28±0.05	1.27±0.04	1.28±0.03	0.85	0.867	0.629
Lipoic acid metabolism	1.43±0.25	1.26±0.14	1.22±0.2	0.302	0.197	0.714
Carbon fixation in photosynthetic organisms	1.29±0.08	1.3±0.05	1.25±0.06	0.819	0.444	0.243
Pyruvate metabolism	1.22±0.07	1.35±0.05	1.24±0.06	0.035	0.587	0.044
Thiamine metabolism	1.25±0.06	1.2±0.01	1.25±0.05	0.149	0.85	0.008

Note: Significant pathways shown in colour.

Table 31: 16S rRNA-based predictive gene functionality of naturally fermented milk (NFM) products of Sikkim as inferred by PICRUSt2 algorithm and mapped to KEGG database, represented at Pathway (Level-3). (contd.)

Sub-Pathway (Level-3)	***Dahi***	***Gheu***	***Chhurpi***	***Dahi* vs *gheu***	***Dahi* vs *chhurpi***	***Gheu* vs *chhurpi***
Terpenoid backbone biosynthesis	1.23±0.09	1.27±0.03	1.22±0.1	0.424	0.854	0.156
Homologous recombination	1.25±0.1	1.14±0.05	1.25±0.07	0.103	0.958	0.033
Citrate cycle (TCA cycle)	1.22±0.08	1.18±0.03	1.21±0.04	0.44	0.886	0.253
Folate biosynthesis	1.3±0.13	1±0.09	1.23±0.08	0.017	0.362	0.033
Selenocompound metabolism	1.22±0.06	1.08±0.02	1.19±0.09	0.013	0.459	0.002
Streptomycin biosynthesis	1.24±0.16	1.27±0.11	1.11±0.13	0.768	0.232	0.117
Glycine, serine and threonine metabolism	1.1±0.07	1.37±0.11	1.12±0.07	0.029	0.758	0.041
Glutathione metabolism	1.21±0.06	1.15±0.1	1.13±0.14	0.434	0.137	0.784
Phenylalanine, tyrosine and tryptophan biosynthesis	1.15±0.06	1.15±0.06	1.11±0.08	0.94	0.374	0.434
Nicotinate and nicotinamide metabolism	1.11±0.06	1.07±0.02	1.11±0.02	0.218	0.935	0.052
Cysteine and methionine metabolism	1.11±0.02	0.98±0.02	1.13±0.06	0.0003	0.241	0.000003
Vitamin B6 metabolism	1.13±0.06	0.97±0.05	1.13±0.08	0.017	0.964	0.012
Bacterial secretion system	1.12±0.09	0.82±0.05	1.14±0.11	0.003	0.713	0.0001
Glycolysis / Gluconeogenesis	1.05±0.15	0.99±0.01	1.07±0.09	0.552	0.748	0.01
Butanoate metabolism	1.04±0.15	1.05±0.03	1.04±0.07	0.866	0.949	0.752
DNA replication	1.01±0.07	0.98±0.04	1.03±0.09	0.571	0.561	0.184
Propanoate metabolism	1±0.15	1.21±0.05	0.98±0.1	0.058	0.793	0.001
Others	34.8±1.74	37.2±1.7	35.73±2.02			

Note: Significant pathways shown in colour.

DISCUSSION

Traditional Knowledge and its Importance

An ethnic person of Sikkim prepares naturally fermented milk products using their indigenous or native knowledge for centuries. Some of the dairy products such as *dahi*, *mohi*, *gheu* and *chhurpi* are traditional milk products of the ethnic Nepali community in Sikkim, whereas *chhu, maa* and *philu* are mostly traditionally prepared and consumed by Bhutia, Sherpa, Lepcha and Tibetans in Sikkim. *Somar* is exclusively prepared and eaten by the Sherpas of Sikkim. The practice of using standard starter culture is uncommon, instead, the use of back-slopping by ethnic people of Sikkim is worth documented (Dewan and Tamang 2006; Rai et al. 2016). *Dahi* is most popular fermented milk product in Sikkim, and consumed as refreshing, non-alcoholic and savoury beverage. Besides its importance as beverage, *dahi* is used in many religious, marriage and social functions by every community in Sikkim (Tamang 2005). *Dahi* is considered as sacred food item in many religious and social functions including death and marriages by Hindu and Buddhists populace of Sikkim. *Dahi* is also used as adhesive to make 'tika' with rice and coloured-powder during the greatest Hindu festival called *dashai* and is applied to foreheads by the family elders. It is also mixed with beaten-rice locally called *chiura* and makes an essential food item during the festival such as *ashar ko pandra* signifying the beginning of work in the fields for the farmers (Tamang 2010b). *Dahi* is offered to the bridegroom as a symbol of good luck during marriage and is an essence to solemnize the marriage of Hindu. Tibetans, Bhutia and the Lepcha also use *shyow* (*dahi*) in their religious and social events in marriages and funerals (Tamang 2005). *Shyow* is served exclusively during *shoton* festival of Tibetan (Tamang 2010b). *Mohi* or buttermilk is used as a beverage in meals during many social festivals and religious events. It is offered to guests and visitors in many of the homes as refreshing savoury. *Gheu* is a sacred item in all religious ceremonies of Hindu and Buddhists and is used in the birth,

marriage, death as well as in other prayer as sacred offerings. Culturally *gheu* is tasted to new born baby with honey by father believing to protect from any disease or evils which is custom among the ethnic Nepali in Sikkim. *Gheu* is also used for lighting the lamps for Gods and Goddesses in Hindu temples and Buddhist monasteries. Soft *chhurpi* is served as an important dish as curry and pickle *achar* in the various religious and social festivals. *Chhu* is an important local food and is consumed by the Bhutia as soup along with rice when other foods are not easily available. Hard *chhurpi* is eaten in high altitudes by Bhutia and Sherpa as chewing gum and masticator which gives an extra energy to body by continuous movement of jaws and gum (Rai et al. 2016). *Somar* is consumed mostly by the older generation of the Sherpa and is generally consumed to increase the appetite and to cure digestive problems. Indigenous knowledge of ethnic people of Sikkim in preparation of naturally fermented milks products from milk of cow and yak is very unique in India due to its unique taste, flavour and recipes as well as culinary practices.

Culture-dependent Methods

The pH of naturally fermented milk (NFM) products collected from different places of Sikkim viz. *dahi*, *mohi*, soft *chhurpi*, hard *chhurpi* and *philu,* prepared either from yak and cow milk, was below indicating their acidic in nature. Lactic acid bacteria (LAB) were found dominant microorganisms with $>10^8$ cfu/g in all NFM products of Sikkim, which justified the earlier finding of Dewan and Tamang (2007). LAB was reported as predominant microorganisms with $>10^7$ cfu/ml in *airag* and *tarag,* NFM products of Mongolia (Watanabe et al. 2008). On the basis of phenotypic and biochemical tests, four genera of LAB were tentatively identified viz. *Leuconostoc, Lactococcus, Enterococcus* and *Streptococcus*. However, phenotypic and biochemical parameters for identification of bacteria upto genus/species are not accurate and authentic (Tamang et al. 2016a),

hence, application of 16S rRNA gene sequencing method using PCR products is more reliable and accurate for identify of LAB (Cocolin and Ercolini 2008; Clarridge III 2004; Srinivasan et al. 2015). We used Sanger Sequencing method or Chain-termination DNA (Sanger et al. 1977; Heather and Chain 2016) using 16S rRNA gene sequencing method (Caro et al. 2015) for molecular identification of LAB isolated from NFM products of Sikkim. The 16S rRNA sequencing result showed a complex microbial community with three genera and nine different species and sub-species in NFM products. Identified species were *Leuconostoc mesenteriodes*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteriodes* subsp. *jonggajibkimchii*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *tructae*, *Enterococcus italicus*, and *Enterococcus pseudoavium*. *Leuconostoc* spp. was found predominant in *dahi* samples prepared from yak/cow milk. *Leuconostoc* is dextran producing bacterium (Subathra Devi et al. 2014) and it was reported as the predominant populations in yak milk samples of China (Bao et al. 2012). *Leuconostoc* spp. was reported from in mare milk (Ying et al. 2004) and *Oscypek*, a traditional Polish cheese (Alegria et al. 2012). Hosono et al. (1989) reported that *Leuconostoc* spp. was also dominant bacterium in *dadih*, fermented milk products of Indonesia similar to *dahi* (Hosono et al. 1989). *Lactococcus* and *Enterococcus* are one of the predominant genera present in dairy products (Burgain et al. 2014). Recent culture independent study also revealed the abundance of *Leuconostoc* in naturally fermented milk products of Arunachal Pradesh and Sikkim (Shangpliang et al. 2018). In *mohi* sample the dominant species were *Lactococcus lactis* and *Leuconostoc mesenteriodes*. *Lactobacillus* and *Lactococcus* were the main species reported from *lassi* (Padghan et al. 2015). Prashant et al. (2009) illustrated that *Lactobacillus* was the predominant lactic acid bacterium in *chhurpi* of Arunachal Pradesh; however in *chhurpi* of Sikkim, *Leuconostoc*

mesenteroides was found prominent bacterium followed by *Lactococcus lactis.* One of the reasons for showing different genera of bacteria in *chhurpi* between two Himalayan states might be the difference in environmental condition (Li et al. 2018) and preparation methods.

Among 5 types of milk products, soft *chhurpi* prepared from yak milk exhibited highest species richness number with 6.5 and the lowest species richness number 1 in *dahi* (yak-milk) and *philu* (yak-milk), respectively. Soft *chhurpi* is a cheese-like product with neutral pH, due to its rich protein content that may promote the growth of different types of bacteria (Prashant et al. 2009; Monnet et al. 2012). In soft *chhurpi*, *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *cremoris* were recorded besides that two subspecies *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* and *Lactococcus lactis* subsp. *hordniae* were also identified that have not been reported before from NFM of Sikkim earlier (Dewan and Tamang 2007).

Culture Independent Method

Conventional identification approach is no more an effective tool for authentic identity of microorganisms in foods (Franco-Duarte et al. 2019). Method based on culture relies on the microorganisms being isolated and cultivated, and moreover, species in low numbers are neglected *in vitro* by species in numerical abundance (Hugenholtz and Huber 2003) and also some species may be unculturable (Head et al. 1998). Molecular culture-independent approaches, on the other hand, have proven to be powerful tools in providing a more complete inventory of microbial diversity in food samples (Cocolin and Ercolini 2008; Jianzhonga et al. 2009). Development of new culture-independent technique has proven to be a strong tool for in-depth profiling of the community structure (Lei et al. 2011). The technique of high-throughput sequencing (HTS) offers an overall

insight into the microbial population present in samples (Ercolini 2013; Doyle et al. 2016). In this study, barcoded Illumina MiSeq amplicon sequences of the 16S rRNA gene (V4-V5 region) from samples of NFM products of Sikkim were investigated to profile the bacterial community. *Firmicutes* was the most dominant phylum in NFM of Sikkim; similar observation was reported in other naturally fermented milk products (Zhong et al. 2016; Joishy et al. 2019). *Lactococcus lactis* (>1% abundance) was detected in all samples of NFM products of Sikkim, *Lc. lactis* was also dominant bacterium in fermented milk products of Mongolia (Yu et al. 2011). *Lactobacillus helveticus, Acetobacter lovaniensis, A. pasteurianus, Lactococcus raffinolactis* and *Leuconoctoc mesenteroides,* (>1% abundance) were detected in NFM of Sikkim. In the present culture-independent method, *Lactobacillus helveticus* was detected which had not been recorded earlier in culture-dependent method. *Lb. helveticus* is usually found in dairy products (Moser et al. 2017). Metagenomics-based studies of other milk products of the world such as *kefir*, buttermilk, cheese, etc. also reported the similar diversity of lactic acid bacteria (Delbès et al. 2007; Dobson et al. 2011; Alegría et al. 2012; Quigley et al. 2012; Jayashree et al. 2013; Marsh et al. 2013; Liu et al. 2015; Planý et al. 2016). A relatively high abundance of Proteobacteria-associated *Acetobacteraceae* (acetic acid bacteria) was observed in soft *chhurpi* and *dahi* products in addition to other lactic acid bacteria. Members of *Acetobacteraceae* were also identified in milk related products (Dobson et al. 2011; Liu et al. 2012; Leite et al. 2012; Oki et al. 2014), and showed their dominance in *dahi* than other products due to the effect of heating during the processing steps (Shangpliang et al. 2018). The abundance of *Acetobacteraceae* members was generally found low in *gheu* sample. During the fermentation of *dahi*, we observed an increase in the abundance of *Streptococcaceae* (*Lactococcus*) and then slowly decreased the number in downstream product (Dan et al. 2018). The percentage of

Enterobacteriaceae was found to be relatively high in analysed NFM samples based on the OTUs. *Enterococcus faecalis, Enterococcus faecium* together with the *Lactococcus lactis* subsp. *lactis* in *dahi* of Bhutan were reported earlier (Shangpliang et al. 2017). *Nunu*, African NFM product, is frequently contaminated with *Enterobacteriaceae*, shown by short-read-alignment-based bioinformatics tools, which may be used for high-throughput food safety testing (Walsh et al. 2017). *Staphylococcaceae*, *Bacillaceae, Clostridiaceae* and *Pseudomonadaceae* were detected at >1% abundance in NFM products of Sikkim. *Staphylococcaceae* (*Staphylococcus aureus*) is a common environmental microorganism which is found in raw milk (Jackson et al. 2012), potentially be transferred to milk since milk is a rich source of growth medium for these bacteria (Farrokh et al. 2013). *Pseudomonadaceae* (*Pseudomonas fluorescens)* is usually present in milk and milk products as sources of contaminants (Wiedmann et al. 2000) and *Clostridiaceae* (*Clostridium tyrobutyricum*) is another bacterium found in cheese causing late blowing defect (Panelli et al. 2013). These contaminants were likely associated with the overall process of handling, as samples are naturally fermented milk products, and no controlled process is involved. It is known that contaminations of unwanted or rather non-fermenting bacteria have been acquired from different sources of the production environment (Doyle et al. 2016; Ssajjakambwe et al. 2017). Presence of uncultured bacterium was observed in all samples analyzed at species level obtained from OTUs method, as the database could not assign them to any of closest taxa (Schloss et al. 2011). In addition, limitations on the use of OTUs-based approaches are that the clustering algorithms are computationally intensive, relatively slow and require large memory volumes (Schloss et al. 2009). By HTS method, several unculturable bacteria are detected in fermented milk products (Zhong et al. 2016; Yu et al. 2018). However, the predominance of few species was observed in soft *chhurpi* product and subsequently

a build-up in the *Lactobacillaceae* (*Lactobacillus*) population. *Lactobacillus helveticus* was dominant in soft *chhurpi* (cow-milk) whereas relatively low in *dahi* (yak-milk). A similar result was reported by Watanabe et al. (2008), where *Lb. helveticus* was found predominant LAB component in *Airag* and *Tarag,* naturally fermented milk products of Mangolia. Though we observed a fairly equal distribution among *Acetobacter, Lactococcus, Lactobacillus* and *Leuconostoc* at genus level in 5 NFM products, however, at species level *Lactococcus* was represented only by *Lactococcus lactis, Lactobacillus* was represented by *Lactobacillus helveticus* whereas *Acetobacter* was represented by *A. lovaniensis*, *A. pasteurianus*, *A. syzygii*, *Gluconobacter oxydans* and *Leuconostoc* was *Leuconostoc mesenteriodes* and *Leuconostoc pseudomesenteriodes*. Species of acetic acid bacteria were also reported in fermented milk products (Liu et al. 2012; Haghshenas et al. 2015). Based on an alpha diversity analysis, diversity was observed in bacterial species among the 5 NFM products. However, significance difference was observed only in between *chhurpi* and *dahi* (p=0.0152), whereas there were no significance differences between cow-milk and yak-milk observed in terms of milk sources. Though yak milk has higher contents of fat, protein, lactose, and total solids than that of cow milk (Li et al. 2010), however, it significance differences in composition of microflora in NFM products depending on sources of milk. *Lactobacillus helveticus* was found dominant bacterium in soft *chhurpi* prepared from cow-milk, also reported from fermented cow-milk products (Zhou et al. 2019). *Lactococcus lactis* was the dominant bacterium in soft *chhurpi* prepared from yak-milk, which was also reported a dominant bacterium in fermented yak milk products of China (Bao et al. 2012). In *dahi* (cow milk) *Lactococcus lactis* was dominant, interestingly, *Clostridium tyrobutyricum* was found dominant in *dahi* prepared from raw yak-milk*,* which might have entered through raw yak milk during spontaneous fermentation (Quigley et al. 2013). *Clostridium tyrobutyricum* has

been reported in cheese (D'Incecco et al. 2018) causing a defect called late-blowing by butyric acid fermentation during cheese ripening, resulting into rancid off-flavor formation (Ruusunen et al. 2012).

In NFM products of Sikkim, we detected 4 bacterial genera by phenotypic characterization, 3 genera with 9 species/subspecies by 16S rRNA gene sequencing method (culture-dependent method), and 9 genera with 17 species having relative abundance above 1% by HTS tool. The HTS data showed the bacterial community comprising of *Lactococcus lactis*, *Lactobacillus helveticus*, *Leuconostoc mesenteroides*, *Lactobacillus helveticus, Leuconostoc pseudomesenteroides, Lactococcus raffinolactis, Lactobacillus gasseri, Lactobacillus delbrueckii, Lactococcus piscium, Leuconostoc lactis* and *Lactococcus piscium*. Sequence-based taxonomy has advantages in profiling the bacterial community including both culturable and unculturable in fermented dairy products over the culture-dependent methods (Walsh et al. 2017; Xue et al. 2018; Berhe et al. 2019).

Probiotics Properties

Lactic acid bacteria (LAB) isolated from dairy products have attracted greater attention as a potential food preservative and exhibit numerous probiotic properties including their antimicrobial effect on potential pathogen by the production of bacteriocins, organic acids, ethanol, hydrogen peroxide, diacetyl and reuterine (Jamuna and Jeevaratnam 2004; Melia et al. 2017; Adeyemo et al. 2018). Probiotics are one of the functional properties of fermented foods (Tamang 2015), and some LAB is used as health-promoting organisms (Hwanhlem et al. 2010; Monteagudo-Mera et al. 2012). Probiotics must have the potential to exert a beneficial effect on a host, to withstand high cell counts in foodstuffs and remain viable during a product's shelf life, to withstand transits through the GI tract,

to adhere to the intestinal epithelium cell lining and to colonize the lumen of the intestine, to develop antimicrobial substances through pathogens, technically suitable for industrial processes and should be linked to a marked health benefit (Shewale et al. 2014). In this study, we screened the bacterial strains for β-galactosidase, after incubation 23 bacterial isolates showed green color on MRS agar medium, indicating the presence of beta-galactosidase enzyme (Michlmayr and Kneifel 2014). β- Galactosidase plays an active role in the metabolism of carbohydrates, i.e. the hydrolysis of the glucosidic lactose bond (Al-Zahrani et al. 2019). Beta-glucosidase enzyme has potential applications in the food processing industry; due to low levels of the intestine enzyme, significant portions of the population have become intolerant to lactose and have difficulty in the intake of milk and milk products (Husain 2010). LAB strains were analysed for cell surface hydrophobicity assay, which plays a crucial role in bacterial cell first contact with mucous or epithelial cells (Schillinger et al. 2005). A requirement for pathogen exclusion and immunomodulation is the binding of probiotic bacteria to intestinal cells (Turpin et al. 2012). The peptidoglycan layer of the bacterial cell wall is covered by teichoic acids (lipo), surface proteins and polysaccharides (Sengupta et al. 2013). In addition to properties of the cell surface, physicochemical factors are also reported as non-specific mechanisms that play a sufficient role in bacterial adhesion (Greene and Klaenhammer 1994). Our isolates have demonstrated variable degree of hydrophobicity, out of which, three bacterial strains *Enterococcus faecalis* YS4-14, *Lactococcus lactis* subsp. *cremoris* SC3 and *faecalis* YS4-11 showed good percentage of hydrophobicity which was more than 85%. The large differences in cell surface hydrophobicity could be due to variations in the level of expression of cell surface proteins between strains as well as environmental conditions that could affect surface protein expression (Tomas et al. 2005; De-Vries et al. 2006; Ramiah et al. 2007). Any

strain with a hydrophobic index of more than 70% is regarded as hydrophobic (Nostro et al. 2004). Thus, in the applied method, *Enterococcus faecalis* YS4-14, *Lactococcus lactis* subsp. *cremoris* SC3 and *faecalis* YS4-11 may be considered as a good probiotic candidate. Acid tolerance is another essential requirement for probiotic selection where the bacterial strain has to survive in the upper gastrointestinal tract (GI) (Yadav et al. 2016). These bacteria must survive transit through the stomach and upper part of the intestinal tract until they enter the intestinal tract and exert their probiotic action (Bao et al. 2010). *Lactococcus lactis* subsp. *hordniae* SC 17 and *Leuconostoc mesenteroides* YS7-8 showed good viable cell count (log cfu/ml) after 2 h of exposure at pH 3, which indicates maximum acid tolerance in the applied method (Yadav et al. 2016). *Leuconostoc mesenteroides* DY36 showed remarkable tolerance against lysozyme. Lysozyme enzyme can hydrolyse the cell wall of bacteria (Salton 1958). Similar studies in bacterial resistance to acid, lysozyme and bile have been reported earlier in other fermented milk products (Mathara et al. 2008; Yadav et al. 2016). Tolerance to bile salt is a requirement for metabolic activity and bacterial colonization in the host's small intestine (Havenaar et al. 1992). Bile plays a vital function in the gut's common and non-specific defence mechanisms (Hegyi et al. 2018). The extent of their inhibitory effect is primarily determined by the concentration of bile salts (Charteris et al. 1998). It is claimed that the revealing physiological concentration of human bile varies from 0.35-0.5% (Dunne et al. 2001; Zavaglia et al. 1998; Han et al. 2015) and staying time is tentatively 4 h in the small intestine (Prasad et al. 1998). This study revealed that majority of strains showed good number of cell counts after 2 h incubation at 0.3% bile salt. In our study, 16 LAB strains showed positive for sodium cholate and sodium tauroglycocholate, four strains showed positive for sodium taurocholate however, none of them hydrolysed all the bile salts. The results showed that the substrate specificity of

bile salt hydrolase enzyme (Bsh) enzyme can be affected either by the moiety of amino acids (glycine/taurine) in the conjugate or by other side chains on the moiety of steroids (Gilliland and Speck 1977; du Toit et al. 1998). Glycocholate has been cited as the most prevalent bile salt in the human intestine (Kumar et al. 2012). LAB strain that hydrolyses sodium glycocholate may have more potential to lower the serum cholesterol level if *in vivo* bile salt hydrolysis contributes to lowering serum cholesterol (Brashears et al. 1998). Kumar et al. (2012) reported that Bsh activity in certain species of *Lactobacillus* was the most frequent. Several strains of *Lactobacillus* demonstrated their capacity to deconjugate bile salts into free radicals (Yadav et al. 2016). Therefore, we can predict that LAB strains isolated from NFM products of Sikkim have enzyme properties that may be applicable in the food industry. In addition, LAB has been considered a good alternative for reducing the risk of foodborne diseases and produces antimicrobial metabolites that inhibit the growth of the pathogenic microorganisms responsible for food spoilage (Gutiérrez-Cortés et al. 2017), increase the shelf life and improve the quality of the food (Wei et al. 2006). Antimicrobial evaluation of LAB strains has been assessed using the agar well diffusion method. One of the criteria for probiotic is inhibition of pathogenic growth (Melia et al. 2017) by production of organic acids and low pH (Mobarez et al. 2008; Bezkorovainy 2001). *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 are the most common potential pathogens in developed countries linked to milk products (Jakobsen et al. 2011). Furthermore, *Bacillus cereus* is also identified for many years as agents of foodborne disease (Kramer and Gilbert 1989). Milk has a high chance of contamination by *Salmonella* spp. (Riyaz-Ul-Hassan et al. 2013) as well as other fecal contamination during milking process (Ahmed and Shimamoto 2014). In our investigation, antimicrobial assessment of LAB strains showed different spectra of inhibitions against

tested pathogens. Strain SC11 and SC26 showed maximum inhibition zone (14 mm) against *E.coli* MCC2413, YS8-10 (14 mm) against *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223, SC4 (18 mm) against *Staphylococcus aureus* subsp. *aureus* MTCC740 and YS8-7 (15 mm) against *Bacillus cereus* MTCC1272. LAB strains exhibited varying zone of inhibition from 10-18 mm depending upon the tested pathogens. *Staphylococcus aureus* subsp. *aureus* MTCC740 was most sensitive with 18 mm diameter of ZOI while *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223 and *E. coli* MCC2413 showed least sensitivity with a ZOI 10-14mm. On the other hand *Bacillus cereus* MTCC1272 showed ZOI of 10-15mm. The standard probiotic culture *Lb. plantarum* MCC2034 also showed almost similar trend of inhibition against tested pathogens. A similar study was conducted by Nigam et al. (2012) where LAB isolate produced highest zone of inhibition against *E. coli* (13 mm), *S. aureus* (14 mm) and *B. cereus* (20 mm). Kos et al. (2008) and Ridwan et al. (2008) also reported antagonistic activity of probiotic strains against common pathogen *S. aureus, P. aeroginosa, Y. Enterocolitica, E.coli* and *L. monocytogenes*. LAB strain showed high inhibition against *Staphylococcus aureus* subsp. *aureus* MTCC740 whereas a very low inhibition was recorded against *E. coli* MTCC1272, inhibitions were mostly seen against Gram-positive tested pathogens than against Gram-negative in dairy products (Silva et al. 2018).

Detection of probiotic genes

Evaluation of probiotic and other functional traits by *in vitro* method is not accurate, especially when large numbers of bacteria need to be tested simultaneously (Turpin et al. 2011). On the other hand, advances in our knowledge of LAB genetic diversity and the

growing number of sequenced LAB genomes are easier to analyses the functional properties of LAB strains at the molecular level (Lebeer et al. 2008; Kaushik et al. 2009; Turpin et al. 2010). There has been limited study of molecular screening of bacterial genomes and metagenomes for genes involved in role of interest for various food and health applications (Turpin et al. 2011). In our study, we evaluated screening of a large set of genes involved in probiotic function. The genes were selected for gastrointestinal survival and were present in many probiotic strains. Genes involved in survival of low pH such as *hdc, tdc, odc, agu*, LBA1272, *dltD*, La995, *gtf, clpL* and Ir1516 were analyzed. Four genes *tdc, agu, clpL* and Ir1516 were detected in 41.1%, 52.9%, 82.3% and 55.8%, respectively. However, gene *hdc, odc*, LBA1272, *dltD*, La995, *gtf* were not detected in the tested isolates. Turpin et al. (2011) reported that in their analysis the *hdc, odc, gtf* genes are less detected. Stack et al. (2010) reported *gtf* gene was detected only in *Lb. fermentum*. The glycosyltransferase gene codes are enzymes that help in survival and said to be involved in the development of beta-glucan (Stack et al. 2010). In our study, *groEL* a housekeeping gene also involved in the survival of pH was not detected. However, Turpin et al. (2011) identified *Lb. fermentum*, *P. pentosaceus, Lb. plantarum, P. acidilactici, Lb. paraplantarum, Lb. salivarius* and *Leuconostoc mesenteriodes* which carry that genome. To determine the binding capability of bacteria, 7 different binding related genes (*mapA, mub1, msa, apf, fbp, sor,* and *sbp)* were analysed. Variability of genes *mub, map* and *msa* detection was observed. The gene coding for the mucus binding protein has been documented to be associated with adhesion to HT29 cells, Caco-2 cells, mucus and mucin in different LAB strains (Roos and Jonsson 2002; Buck et al. 2005; van Pijkeren et al. 2006). The *msa* and *mub* genes contain MucBP domains (Turpin et al. 2012). Moreover, *msa* gene was the gene related to binding that was detected more than 50% in our collection. Its detection rate of (54.4%) in our study was higher than previous

rate of (40%) in *L. plantarum* (Zago et al. 2011). No gene for *fbp, sor*, and *sbp* was detected. A range of pathogenic species has been reported to have the non-essential gene *fpbA*, which codes for a protein that binds fibronectin among the other genes found in the entire bacterial set (Rosenkrantz et al. 2013). Analysis of its sequence alignment indicated that it is found in a number of LAB species (Gil et al. 2004; Michail and Abernathy 2002). This indicates that LAB and pathogens may share similar binding mechanisms involving proteins with similar characteristics, suggesting that certain LAB are capable of inhibiting pathogenic adhesion to intestinal cells simply through competition (Turpin et al. 2012).

BSH activity was generally observed in residents of the GIT where there are bile salts (Foley et al. 2019). Genes *bsh* tend to be traveling horizontally to other bacteria, where *L. plantarum* Lp9, originating from buffalo milk, was found to have functional BSH activity (Kaushik et al. 2009). Bacterial genes related to bile salt tolerance such as *bsh*, LBA1432, LBA1679, LBA1446, Ir0085, Ir1584, LBA0552, LBA1429 were analysed in this study. Among other genes LBA1446 and Ir1584 were widely distributed among bacterial collection with 45.5% and 29.4%. Only 22% of bacterial strain showed positive detection for *bsh* gene. Remaining genes LBA1432, LBA1679, Ir0085, LBA0552, and LBA1429 were less frequently (0%) detected in tested bacterial strains. Similar case has been reported previously for Ir0085 gene where the distribution was detected negligible (Turpin et al. 2011). These genes are codes for major facilitator superfamily permease, which may have a crucial function in bile tolerance (Ruiz et al. 2013). Other criteria for probiotic selection are the production of antimicrobial components which eliminate potential pathogenic microorganism (Prabhurajeshwar and Chandrakanth 2019). Bacteriocins are a group of antimicrobial compounds produced by LAB that are ribosomally synthesized peptides to inhibit the growth of similar or closely related

bacterial strains, either in the same species or across genera (Tagg et al. 1976; Bowdish et al. 2005; Cotter et al. 2005; Goel et al. 2020). We analyzed genes related to antimicrobial peptide production *Ent A, Ent B, Ent P,* Enterocin AS-48, *Nisin*, Lactococcin A, Lacticin 481 and *mes Y*, out of which only the gene *mesY* for mesenteriocin Y were detected. Mesenteriocin Y is an anti-listeria bacteriocin developed exclusively by *Leuconostoc mesenteriodes* (Hechard et al. 1992). *Lactococus lactis* subsp. *cremoris* 9B4 and *L. lactis* subsp. *lactis* DPC938 has been reported to produce *Lactoccins A, B* and *M* (Van Belkum et al. 1989; Morgan et al. 1995). Bravo et al. (2009) also reported that *nisin* and lacticin 481 was produced by *L. lactis* spp. *lactis* and *L. lactis* spp. *cremoris* originated from raw ewe's milk. Enterocin encoding genes *EntA, Ent B, Ent P* are most common encoding genes for enterocin A, B and P, respectively (Ogaki et al. 2016). Their analysis revealed that high antimicrobial activity was exhibited by *Enterococcus* spp. The high frequency of bacteriocin production was shown by *E. faecium* followed by *E. feacalis* and other *Enterococcus* spp. (du Toit et al. 2000; Ogaki et al. 2016). Various enterocin exhibited inhibition against several foodborne pathogens including *Listeria monocytes, Listeria strains, Bacillus* sp. and clinical enterococci (Furlaneto-Maia et al. 2020). However, no enterocin encoding genes were detected among bacterial strains in this study. While antibacterial activity was observed in the plate medium it may be due to the development of certain other antibacterial compounds such as lactic acid, acetic acid, or H_2O_2. These enzymes are produced by probiotic bacteria and can remove glycoside moiety from the glycosylated flavonoids in soybean products (Otieno and Shah 2007). In this analysis we tested the gene encoding β-glucosidases for various LAB species. Enzyme β-glucosidases play a significant role in the food industry. These enzymes are produced by probiotic bacteria and may remove glycosylated flavonoids in soybean products (Otieno and Shah 2007). In earlier study,

Bifidobacterium and *Lactobacillus* strains have been identified with β-glucosidase enzymes (Di Gioia et al. 2014; Gaya et al. 2016). Studies on β-glucosidase have extensively done in camel milk (Ibrahim 2018), wine (Mtshali et al. 2010) and milk and cheese (Gheytanchi et al. 2010). This result indicates that LAB isolates derived from NFM products of Sikkim have potential enzyme source that may be important to food industry. This research therefore provides the basis for selecting possible strains for further evaluation of gene expression and its enzyme activity.

Predictive Gene Functionality

Predictive gene functionality of 16S rRNA gene using PICRUSt2 is a very useful approach to get a glimpse of the potential functionality of a sample and is widely used microbial ecology studies (Douglas et al. 2019; Ortiz-Estrada et al. 2019). We observed a high rate of metabolism in the naturally fermented milk products of Sikkim; findings that are quite similar with some of the similar studies in other milk products where high metabolic signatures, particularly, carbohydrate, amino acid, energy metabolism and some other minor metabolic activities (Zhang et al. 2017; Zhu et al. 2018; Chen et al. 2020). *Dahi* and *chhurpi* are casein-based products, whereas *gheu* is fat-based product (Rai et al. 2016; Shangpliang et al. 2018), hereby, we observed a significant variation of metabolism between the two groups. The dominance of acetic acid bacterial groups in *gheu* contributes to significant difference to that of the casein-based products, *dahi* and *chhurpi*. Various significant metabolic pathways were also observed which were significantly higher in *chhurpi* than in *gheu*, particularly, biosynthesis of ansamycins, lipopolysaccharide biosynthesis, pentose phosphate pathway, thiamine metabolism, homologous recombination, glycolysis/gluconeogenesis pathways. On the other hand, *gheu* showed a significant increase than *chhurpi* in metabolic pathways associated with valine, leucine and isoleucine biosynthesis, pantothenate and CoA biosynthesis, synthesis

and degradation of ketone bodies, lysine biosynthesis, propanoate metabolism; some of the metabolic signatures corresponding to higher lipid metabolism as *gheu* is fat-based part of the milk separated of the whole milk before its fermentation. These various metabolic pathways are important health promoting benefits to consumers (Pimentel et al. 2018). Interestingly, the casein-based products, *dahi* and *chhurpi* showed a significant increase than *gheu*, particularly in the pathways associated with biotin metabolism, folate biosynthesis, seleno compound metabolism, cysteine and methionine metabolism, and vitamin B6 metabolism. Some LAB synthesis vitamins in fermented milk products (Homayouni Rad et al. 2016; Linares et al. 2017). Contrastingly, pathways associated with pyruvate metabolism, glycine, serine and threonine metabolism were shown to be significantly higher in the fat-based *gheu* in comparison to both the casein-based *dahi* and *chhurpi*, since glucose enhances amino acid biosynthesis and metabolism (Ye et al. 2018). The dominance of lactic acid bacterial group (Shangpliang et al. 2018) and no significantly observed human disease signatures were present in the NFM products of Sikkim, probably metabolism and diseases profiles are modulated by fermented milk products (Chen et al. 2020). Kyoto Encyclopaedia of Genes and Genomes (KGGE) database (Kanehisa and Goto 2000) predicted some lactic acids bacteria present in NFM products of Sikkim have functional features and health promoting benefits to consumers, and should be a good source for mining probiotic bacteria for functional foods development.

CONCLUSION

The present study focused on the diversity of lactic acid bacteria naturally fermented milk products of Sikkim revealed by culture-dependent and culture-independent techniques and also their probiotic attributes and predictive gene functionality. This is the first time in-depth study of bacterial community in naturally fermented milk products of Sikkim through high-throughput sequencing (HTS) approach. Relatively high percentage of unculturable bacteria was also detected in NFM products. Probiotic attributes present in NFM products were screened both by plate assays and also by encoding genes detection using PCR-technique. On the basis of *in vitro* screening and gene detection we have selected 20 probable probiotic strains. Kyoto Encyclopaedia of Genes and Genomes (KGGE) database predicted metabolic pathways and functionality in NFM products of Sikkim with health promoting benefits to consumers. Further studies on selective cultivation of dominant bacteria, the cultivation of probiotic starter cultures and the optimization of processing methods can lead to the industrialisation of indigenous food products.

SUMMARY

The major objectives of this PhD Thesis were to document native skill and knowledge of ethnic people of Sikkim for preservation of perishable fresh milk of cow and yak into different dairy products by spontaneous or natural fermentation using 'back-slopping method' and their microbiology and probiotics properties. Different types of naturally fermented milk (NFM) products of Sikkim viz. *dahi* (cow-milk), *dahi* (yak-milk), soft *chhurpi* (cow-milk), soft *chhurpi* (yak-milk), *mohi* (cow-milk), hard *chhurpi* (yak-milk), and *philu* (yak-milk) were well documented. The pH of NFM products collected from different places of Sikkim were mild acidic in nature. Lactic acid bacteria (LAB) were found dominant microorganisms with $>10^8$ cfu/g in all NFM products of Sikkim. A total of 272 LAB strains were isolated from 22 NFM samples. *Leuconostoc, Lactococcus, Enterococcus* and *Streptococcus* were tentatively identified on the basis of phenotypic characterization and biochemical tests. The 16S rRNA gene sequencing result based on Sanger method showed a complex microbial community with three genera and nine different species and sub-species viz. *Leuconostoc mesenteriodes*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteriodes* subsp. *jonggajibkimchii*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *tructae*, *Enterococcus italicus* and *Enterococcus pseudoavium*. In sample-wise distribution of lactic acid bacteria, *Lactococcus lactis* and *Leuconostoc mesenteriodes* were dominated in *dahi* (cow-milk). In the other hand, *Leuconostoc* was found dominant in the *dahi* sample (yak-milk). In soft *chhurpi* (cow-milk), the genus *Leuconostoc mesenteroides* was found dominant followed by *Lactococcus lactis* subsp. *cremoris*. Additionally, two subspecies *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* and *Lactococcus lactis* subsp. *hordniae* were identified that have not been reported before. In soft *chhurpi* (yak-milk), *Enterococcus faecalis* was most dominant followed by *Leuconostoc mesenteroides*. In *mohi* sample (cow-milk), *Lactococcus lactis* was

predominant followed by *Leuconostoc mesenteriodes*. In hard *chhurpi* (yak-milk), *Leuconostoc mesenteroides* dominated the sample and *Lactococcus lactis* subsp. *cremoris* was predominant bacteria in *philu* prepared from yak-milk. In terms of abundance and species richness, soft *chhurpi* prepared from yak milk exhibited highest species richness where as lowest species richness were recorded in *dahi* (yak-milk) and *philu* (yak-milk).

We applied high-throughput sequencing method to profile the bacterial community in NFM products of Sikkim. A total of 19 NFM samples were analysed and the analysis revealed *Firmicutes* was the dominant phylum followed by phylum *Proteobacteria*. The overall diversity, family *Streptococcaceae* was dominant followed by *Lactobacillaceae*, *Leuconostocaceae*, *Staphylococcaceae*, *Bacillaceae* and *Clostridiaceae* respectively. Similarly, under phylum *Proteobacteria*, family *Acetobacteraceae, Pseudomonadaceae* and *Enterobacteriaceae* was reported. A relative abundance of uncultured bacteria (9.8%) were also recorded. In this study, the overall diversity of NFM products at species level was dominated by *Lactococcus lactis*, *Lactobacillus helveticus*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactococcus piscium*, *Lactococcus raffinolactis*, *Lactobacillus delbrueckii*, *Leuconostoc lactis* and *Lactobacillus gasseri*. In the other hand, species belonging to the acetic acid bacteria such as *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Acetobacter syzygii* and *Hafnia alvei* were found. Interestingly, a relatively high abundance of *Lactobacillus helveticus* was found in soft *chhurpi* (cow-milk) which is not reported in earlier studies. Nonetheless, we found a fairly equal distribution among *Acetobacter, Lactococcus, Lactobacillus* and *Leuconostoc* at genus level in different NFM products of Sikkim. Analysis of alpha diversity revealed that significance

difference was observed between two samples, *chhurpi* and *dahi* (p=0.0152), however, there were no significance differences observed between milk sources.

We screened the probable probiotics attributes of LAB strains isolated from NFM products of Sikkim. About 33.8% of bacterial isolates showed positive result for beta-galactosidase enzyme activity on MRS plates. LAB isolates demonstrated variable degree of hydrophobicity assay, among them three bacterial strains *Enterococcus faecalis* (YS4-14), *Lactococcus lactis* subsp. *cremoris* (SC3) and *Enterococcus faecalis* (YS4-11) which showed hydrophobicity more than 85%. For acid tolerance, two strains *Lactococcus lactis* subsp. *hordniae* (SC17) and *Leuconostoc mesenteroides* (YS7-8) showed maximum acid tolerance in the study. Similarly, strain *Leuconostoc mesenteroides* (DY36) showed good tolerance to lysozyme environment. For the bile hydrolysis analysis, 16 LAB strains were capable of hydrolyzing both sodium cholate and sodium tauroglycocholate; however, only four strains were capable of hydrolyzing sodium taurocholate. Bile plays a vital role in gut defence mechanisms. Antimicrobial activity assessment of LAB strains showed varying zone of inhibition depending upon the tested pathogens, two strains *Leuconostoc mesenteroides* (SC11) and *Leuconostoc mesenteroides* (SC26) showed maximum inhibition zones against *E.coli* MCC2413, strain *Lactococcus lactis* subsp. *cremoris* (YS8-10) showed good inhibition against *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223, *Leuconostoc mesenteroides* (SC4) against *Staphylococcus aureus* subsp. *aureus* MTCC740 and *Lactococcus lactis* subsp. *cremoris* (YS8-7) against *Bacillus cereus* MTCC1272. Tested pathogen *Staphylococcus aureus* subsp. *aureus* MTCC740 was found most sensitive towards antimicrobial compounds while *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC3223 and *E. coli* MCC2413 showed less sensitivity. The probiotic culture *Lb. plantarum* MCC2034 also showed similar type of inhibition against tested

pathogens. Therefore, we can predict that LAB strains isolated from NFM products of Sikkim may have enzyme properties that may be applicable in the food industry.

Genes involved in survival of low pH such as *tdc, agu, clpL* and Ir1516 were detected in 41.1%, 52.9%, 82.3% and 55.8%, respectively. However, gene *hdc, odc*, LBA1272, *dltD*, La995, *gtf,* and *groEL* were not detected. For the binding capability, variability of genes *mub, map* and *msa* was detected. Binding gene *msa* was detected more than 50% in the study. However, no other binding genes *fbp, sor*, and *sbp* were detected. Generally, BSH activity was observed in gastrointestinal tract where bile salts are present. Bacterial genes *bsh*, LBA1432, LBA1679, LBA1446, Ir0085, Ir1584, LBA0552, and LBA1429 were analysed in this study. LBA1446, Ir1584 and *bsh* were widely distributed among bacterial strains with 45.5%, 29.4% and 22%, respectively. However, genes LBA1432, LBA1679, Ir0085, LBA0552, and LBA1429 were not detected in tested bacterial strains. For bacteriocin producing gene *Ent A, Ent B, Ent P,* Enterocin AS-48, *Nisin*, Lactococcin A, Lacticin 481 and *mes Y* were evaluated. Gene *mesY* were less frequently detected, it codes for Mesenteriocin Y, which is an anti-listeria bacteriocin produced by *Leuconostoc mesenteriodes*. However, no enterocin encoding genes were detected in the present study. Additionally, the gene encoding β-glucosidases for various LAB species were also detected in the study. Previous studies in β-glucosidase activity were mainly reported from milk and milk products. Based on screening of probiotic properties and gene detection, 20 probable probiotic strains of LAB were selected. The probiotic screening result indicates that LAB isolates derived from NFM products of Sikkim have potential probiotic properties that may be used in food industry. This research therefore provides the basic information for selecting possible strains for further evaluation of gene expression and its enzyme activity.

We also analysed the gene sequence obtained from high-through sequencing result using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICTRUSts) software. Based on Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, various predictive metabolic pathways and functional profiles were inferred from OTUs of bacteria from naturally fermented milk products of Sikkim. NFM samples showed high metabolic signatures, mainly, carbohydrate, amino acid, energy metabolism and some other minor metabolic activities. Casein-based products (*dahi* and *chhurpi)*, and fat-based product (*gheu*) revealed a significant variation of metabolism among the two groups. Acetic acid dominance in *gheu* contributes to significant difference to that of the casein-based products. In *chhurpi*, significantly higher metabolic pathways were also observed particularly, biosynthesis of ansamycins, lipopolysaccharide biosynthesis, pentose phosphate pathway, thiamine metabolism, homologous recombination, glycolysis/gluconeogenesis pathways. Similarly, *gheu* showed a significant increase in metabolic pathways associated with valine, leucine and isoleucine biosynthesis, pantothenate and CoA biosynthesis, synthesis and degradation of ketone bodies, lysine biosynthesis, and propanoate metabolism. Interestingly, the casein-based products, *dahi* and *chhurpi* showed a significant increase than *gheu*, particularly in the pathways associated with biotin metabolism, folate biosynthesis, seleno compound metabolism, cysteine and methionine metabolism, and vitamin B6 metabolism. It is said that some LAB synthesis vitamins in fermented milk products. Contrastingly, pathways associated with pyruvate metabolism, glycine, serine and theonine metabolism was shown to be significantly. In addition, KGGE database predicted some lactic acids bacteria present in NFM products of Sikkim have functional features and health promoting benefits to consumers, and should be a good source for mining probiotic bacteria for functional foods development.

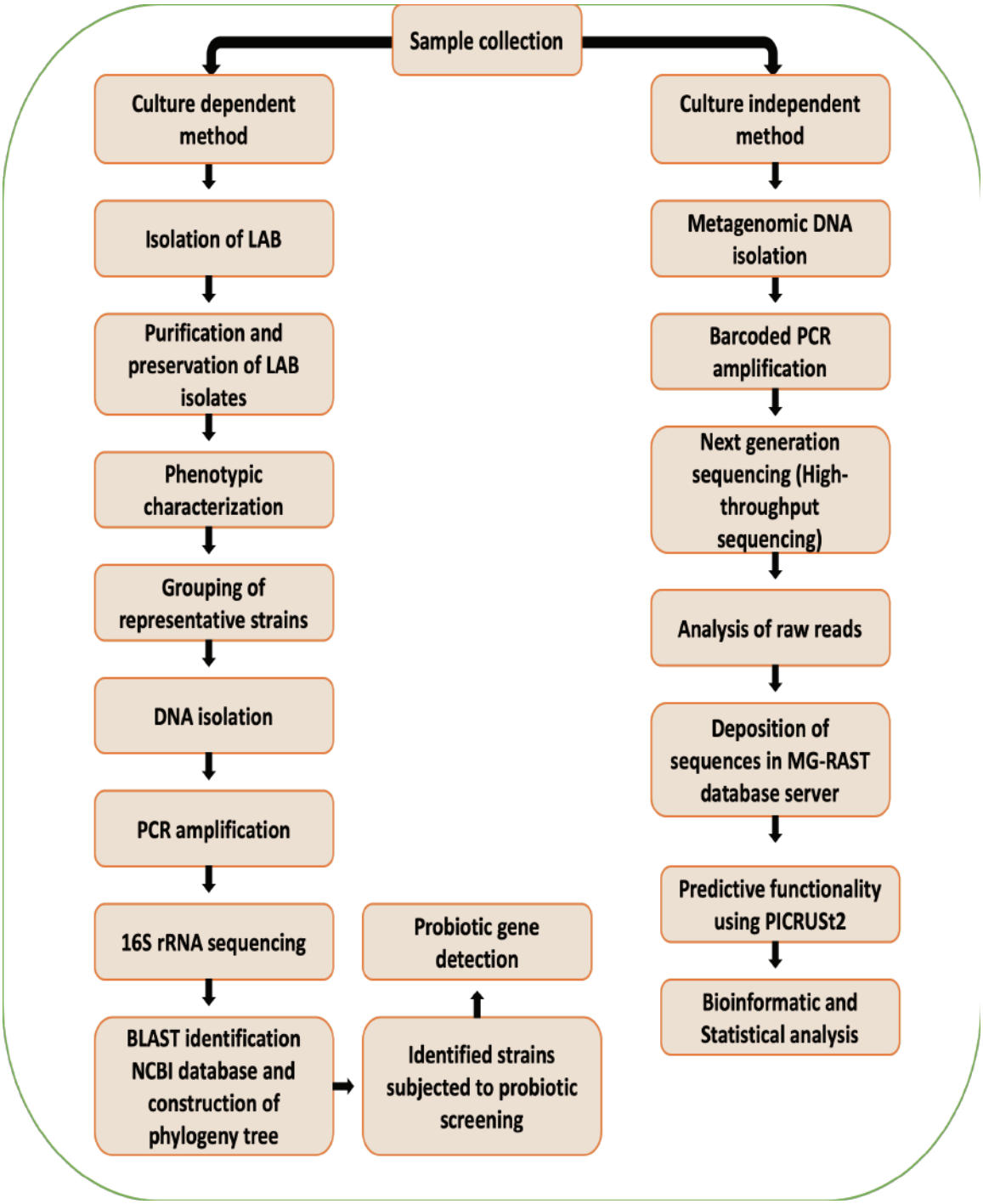
This study has provided the complete information on lactic acid bacterial community of NFM products of Sikkim, evaluated by culture-dependent technique (phenotypic characterization and 16S rRNA); culture-independent methods (NGS techniques by high-throughput amplicon sequencing) and screening of their probiotic attributes. The application of culture-independent NGS methods to research the microbial ecology of fermented foods is of great importance in understanding the products, where Illumina sequencing was shown to be one of the effective tools in this analysis.

This is the first study on insight analysis of the bacterial diversity of Sikkim's naturally fermented milk products by culture-independent method as well as on the screening of probiotic properties of isolated LAB strains from NFM products. This study may provide basic information on composition of indigenous microflora that may be available in the NFM products and may be used to promote the development of starter culture for industrial application. Moreover, data generated from this study can be used as reference data base for the future research.

Highlights of findings

- Based on 16S rRNA gene sequencing result, *Leuconostoc mesenteriodes*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteriodes* subsp. *jonggajibkimchii*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *tructae*, *Enterococcus italicus* and *Enterococcus pseudoavium* were isolated from NFM products of Sikkim.
- Based on high-throughput sequencing data; *Firmicutes* was the dominant phylum followed by phylum *Proteobacteria*. At species level, the following bacteria were detected in NFM samples: *Lactococcus lactis*, *Lactobacillus helveticus*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactococcus piscium*, *Lactococcus raffinolactis*, *Lactobacillus delbrueckii*, *Leuconostoc lactis* and *Lactobacillus gasseri*, *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Acetobacter syzygii* and *Hafnia alvei*.
- On the basis of *in vitro* screening and gene detection 20 probable probiotic strains were selected among *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Enterococcus faecalis*.
- KGGE database predicted metabolic pathways and functionality in NFM products of Sikkim with some health promoting benefits to consumers.

Schematic Diagram and Pictorial Presentation of Complete PhD Work



BIBLIOGRAPHY

Abd-Alla, E.A.M., El-Shafei, K., Ibrahim, G.A. and Sharaf, O.M. (1996). Changes in microflora and biogenic amines of some market processed cheeses during storage. *Egyptian Journal of Dairy Science* 24: 217-226.

Abd-ed-Malek, Y. and Demerdash, M. (1977). Microbiology of *kishk*. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Abd-El-Malek, Y. and Demerdash, M. (1970). Studies on the microbiology of fermented milks in Egypt. I. Sour milk, In: *Proceedings of the 2nd Conference of Microbiology*. Society of Applied Microbiology, Dokki, Cairo, Egypt, pp.151-152.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E. and Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology* 127(3): 200-208.

Achaya, K.T. (1998). *A Historical Dictionary of Indian Food*. Oxford University Press, Delhi.

Adams, M.R., Moss, M.O. and McClure, P. (1995). Food microbiology. The Royal Society of Chemistry. Science Park, Cambridge, pp.121-122.

Adeyemo, S.M., Agun, T.F. and Ogunlusi, E.D. (2018). Antimicrobial activity of lactic acid bacteria isolated from '*Pupuru*': an African fermented staple against food borne-pathogens. *Journal of Molecular Biology and Biotechnology* 3:1-5.

Agarwal, K.N. and Bhasin, S.K. (2002). Feasibility studies to control acute diarrhea in children by feeding fermented milk preparations actimel and Indian *Dahi. European Journal of Clinical Nutrition* 56(4): S56–S59.

Agustinah, W., Warjoto, R. E. and Canti, M. (2019). Yogurt making as a tool to understand the food fermentation process for nonscience participants. *Journal of Microbiology and Biology Education* 20(1): 20.1.15. doi.org/10.1128/jmbe.v20i1.1662.

Ahmed, A., Amer, A. and Sakr, M. (2016). Physical, chemical and microbiological properties of laban rayeb. *Alexandria Journal of Veterinary Sciences* 51: 269. doi.10.5455/ajvs.230003.

Ahmed, A.M. and Shimamoto, T. (2014). Isolation and molecular characterization of *Salmonella enterica, Escherichia coli* O157: H7 and *Shigella* spp. from meat and dairy products in Egypt. *International Journal of Food Microbiology* 168: 57-62.

Ahmed, Z., Wang, Y., Ahmad, A., Tariq Khan, S., Nisa, M., Ahmad, H., and Afreen, A. (2013). *Kefir* and health: a contemporary perspective. *Critical Reviews in Food Science and Nutrition* 53(5): 422-434.

Akabanda, F., Owusu-Kwarteng, J., Glover, R.L.K. and Tano-Debrah, K. (2010). Microbiological characteristics of Ghanaian traditional fermented milk product, *Nunu*. *Nature and Science* 8(9): 178-187.

Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Glover, R.L.K., Nielsen, D.S. and Jespersen, L. (2013). Taxonomic and molecular characterization of lactic acid bacteria and yeasts in *nunu*, a Ghanaian fermented milk product. *Food Microbiology* 34(2): 277-283.

Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Parkouda, C. and Jespersen, L. (2014). The use of lactic acid bacteria starter culture in the production of nunu, a spontaneously fermented milk product in Ghana. *International Journal of Food Science* 2014:721067. doi:10.1155/2014/721067.

Alegría, Á., Szczesny, P., Mayo, B., Bardowski, J. and Kowalczyk, M. (2012). Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and independent approaches. *Applied and Environmental Microbiology* 78(6): 1890-1898.

Al-Zahrani, K., Allam, R., Amasha, R., Jastaniah, S. and Aly, M. (2019). Isolation and identification of *Lactobacillus plantarum* KM5 from camel milk for β-galactosidase production. *Journal of Pharmacy and Biological Sciences* 14(2): 27-35

Amir, A., McDonald, D., Navas-Molina, J.A., Kopylova, E., Morton, J.T., Xu, Z.Z. and Knight, R. (2017). Deblur rapidly resolves single-nucleotide community sequence patterns. *MSystems* 2(2). e00191-16.doi: 10.1128/mSystems.00191-16.

Amiri, Z.R., Khandelwal, P. and Aruna, B.R. (2010). Development of acidophilus milk via selected probiotics and prebiotics using artificial neural network. *Advances in Bioscience and Biotechnology* 1: 224-231.

Anderson, M.J. (2017). Permutational Multivariate Analysis of Variance (PERMANOVA). In: *Wiley StatsRef: Statistics Reference Online* (Eds: Balakrishnan, N., Colton, T., Everitt, B., Piegorsch, W., Ruggeri, F. and Teugels, J.L.), pp. 1-15. Wiley, Hoboken, New Jersey.

Androuet, P. (1976). *Encyclopedia of Cheese*. Omnibus Press, New York.

Aneja, R.P. (2007). East-West fusion of dairy products. In: Gupta S (ed) Dairy India, 6th edn. Dairy India Yearbook, a Dairy India Publication, New Delhi, (pp 51-53).

Aneja, R.P., Mathur, B.N., Chandan, R.C. and Banerjee, A.K. (2002). Technology of Indian milk products: handbook on process technology modernization for professionals, entrepreneurs and scientists. Dairy India Yearbook, New Delhi.

Anon (2003). Buttermilk: a remedy for many diseases. *Indian Diaryman* 55: 21-23.

Archer, A.C. and Halami, P.M. (2015). Probiotic attributes of *Lactobacillus fermentum* isolated from human feces and dairy products. *Applied Microbiology and Biotechnology* 99(19): 8113-8123.

Ashar, M.N. and Prajapati, J.B. (2001a). Role of probiotic cultures and fermented milks in combating blood cholesterol. *Indian Journal of Microbiology* 41(2): 75-86.

Ashar, M.N. and Prajapati, J.B. (2001b). Serum cholesterol level in human fed with acidophilus milk. *Indian Journal of Microbiology* 41(4): 257-263.

Auclair, J. and Accolas, J.P. (1974). Koumiss (koumiss, coomys). In: *Encyclopedia of Food Technology* (Eds: Johnson, A.H. and Peterson, M.S.), (pp. 537-538). CT: AVI, Westport.

Aukrust, T. and Blom, H. (1992). Transformation of Lactobacillus strains used in meat vegetable fermentation. *Food Research International* 25(4): 253-261.

Ayşe, K., Mehmet, Ö., Ibrahim, H.K. and Sibel Bayıl, O. (2019). Identification and isolation of the yeasts in tradition- al yogurts collected from villages in Gaziantep, Turkey. *Biomedical Journal of Science and Technology* 13(5). doi: 10.26717.BJSTR.2019.13.002478.

Azcarate-Peril, M.A., Altermann, E., Hoover-Fitzula, R.L., Cano, R.J. and Klaenhammer, T.R. (2004). Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. *Applied and Environmental Microbiology* 70(9): 5315-5322.

Balamurugan, R., Chandragunasekaran, A.S., Chellappan, G., Rajaram, K., Ramamoorthi, G. and Ramakrishna, B.S. (2014). Probiotic potential of lactic acid bacteria present in home made curd in southern India. *Indian Journal of Medical Research* 140(3): 345-355.

Balaraman, N. and Golay, M.M. (1991) *Livestock Production in Sikkim*. Sikkim Science Society, Gangtok.

Bao, Q., Yu, J., Liu, W., Qing, M., Wang, W., Chen, X., Wang, F., Li, M., Wang, H., Lv, Q. and Zhang, H. (2012). Predominant lactic acid bacteria in traditional fermented yak milk products in the Sichuan Province of China. *Dairy Science and Technology* 92(3): 309-319.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y. and Zhang, H. (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control* 21(5): 695-701.

Barbera, P., Kozlov, A.M., Czech, L., Morel, B., Darriba, D., Flouri, T. and Stamatakis, A. (2019). EPA-ng: massively parallel evolutionary placement of genetic sequences. *Systematic Biology* 68(2): 365–369.

Barbieri, F., Montanari, C., Gardini, F. and Tabanelli, G. (2019). Biogenic amine production by lactic acid bacteria: a review. *Foods (Basel, Switzerland)* 8(1): 17. doi.org/10.3390/foods8010017.

Basson, P. (1981). Women and traditional food technologies. Changes in rural Jordan. *Ecology and Food Nutrition* 11(1): 17-23.

Batra, L.R. and Millner, P.D. (1975). Asian fermented foods and beverages. *Development in Industrial Microbiology* 17: 117-128.

Berhe, T., Ipsen, R., Seifu, E., Kurtu, M.K., Fugl, A. and Hansen, E.B. (2019). Metagenomic analysis of bacterial community composition in *Dhanaan*: Ethiopian traditional fermented camel milk. *FEMS Microbiology Letters* 366(11), fnz128. doi.org/10.1093/femsle/fnz128

Besancon, X., Smet, C., Chabalier, C., Rinemale, M., Reverbel, J.P., Ratomehenina, R. and Galzy, P. (1992). Study of surface yeast flora of Roquefort cheese. *International Journal of Food Microbiology* 17(1): 9-18.

Bettache, G., Fatma, A., Miloud, H. and Mebrouk, K. (2012). Isolation and identification of lactic acid bacteria from Dhan, a traditional butter and their major technological traits. *World Applied Sciences Journal* 17(4): 480-488.

Bezkorovainy, A. (2001). Probiotics: determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition* 73(2): 399-405.

Bhandekar, V., Atkare, V.G., Kadu, K. and Meshram, A. (2018). Physico-chemical quality of Paneer sold in Nagpur city. *International Journal of Chemical Studies* 6(4): 2517-2519

Bintsis T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS Microbiology* 4(4): 665-684.

BIS (1980a) IS: 9617. *Specification for dahi*. Bureau of Indian Standards, Manak Bhawan, New Delhi.

BIS (1980b) IS: 9532. *Indian Standard Specification for chakka and shrikhand*. Bureau of Indian Standards, Manak Bhawan, New Delhi.

Boghra V.R. and Mathur, O.N. (2000). Physico-chemical status of major milk constituents and minerals at various stages of shrikhand preparation. *Journal of Food Science and Technology* 37(2): 111-115.

Bowdish, D.M., Davidson, D.J., Scott, M.G. and Hancock, R.E. (2005). Immunomodulatory activities of small host defense peptides. *Antimicrobial Agents and Chemotherapy* 49(5): 1727-1732.

Bozoudi, D., Agathokleous, M., Anastasiou, I., Papademas, P. and Tsaltas, D. (2017). Microbiological characteristics of *Trachanas*, a traditional fermented dairy product from Cyprus. *Journal of Food Quality*. doi.org/10.1155/2017/8749316

Brashears, M.M., Gilliland, S.E. and Buck, L.M. (1998). Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *Journal of Dairy Science* 81(8): 2103-2110.

Bravo, D., Rodriguez, E. and Medina, M. (2009). Nisin and lacticin 481 coproduction by *Lactococcus lactis* strains isolated from raw ewes' milk. *Journal of Dairy Science* 92(10): 4805-4811.

Buck, B.L., Altermann, E., Svingerud, T. and Klaenhammer, T.R. (2005). Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology* 71(12): 8344-8351.

Bull, M., Plummer, S., Marchesi, J. and Mahenthiralingam, E. (2013). The life history of *Lactobacillus acidophilus* as a probiotic: a tale of revisionary taxonomy, misidentification and commercial success. *FEMS Microbiology Letters* 349(2): 77-87.

Burgain, J., Scher, J., Francius, G., Borges, F., Corgneau, M., Revol-Junelles, A.M., Cailliez-Grimal, C. and Gaiani, C. (2014). Lactic acid bacteria in dairy food: surface characterization and interactions with food matrix components. *Advances in Colloid and Interface Science* 213: 21-35.

Cai, L. (1985). Domestic Animal Breeds and Their Ecological Characteristics in China, ed. by Pieliu, C. China Agricultural Press, Beijing, (pp. 45-49).

Campana, R., van Hemert, S. and Baffone, W. (2017). Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathogens* 9: 12. doi.org/10.1186/s13099-017-0162-4.

Campbell-Platt, G. (1987). *Fermented Foods of the World: A Dictionary and Guide*. Butterworths, England.

Campbell-Platt, G. (1994). Fermented foods: a world perspective. *Food Research International* 27(3): 253-257.

Cao, Y., Gan, W., Ye, Z., Yu, H., Huang, B., Jiang, Y. and Bian, L. (2004). Investigation on yak milk products and their traditional process craft of Daocheng country. In Proceedings of Fourth International Congress on Yak, Research Institute of Animal Science and Veterinary Medicine of Ganzi Prefecture Chengdu, PR China (pp. 4-67).

Caplice, E. and Fitzgerald, G.F. (1999). Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology* 50(1-2): 131-149.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7(5): 335-6.

Cappuccino, J.G. and Sherman, N. (2008). *Microbiology: A laboratory Manual*. Peason Benjamin Cummings. San Francisco, US.

Caro, I., Bécares, G., Fuentes, L., Garcia-Armesto, M.R., Rúa, J., Castro, J.M., Quinto, E.J. and Mateo, J. (2015). Evaluation of three PCR primers based on the *16S rRNA* gene for the identification of lactic acid bacteria from dairy origin. *CyTA-Journal of Food* 13(2): 181-187.

Carpino, S., Rapisarda, T., Belvedere, G., Papademas, P., Neocleous, M., Scadt, I., Pasta, C. and Licitra, G. (2010). Effect of dehydration by sun or by oven on volatiles and aroma compounds of Trachanas. *Dairy Science and Technology* 90(6): 715-727.

Carr, S. (1981) *Cheese*. Mitchell Beazley, London.

Celano, G.V., Cafarchia, C. and Tiecco, G. (1996). Formation of biogenic amines in Pecorino Pugliese cheese. *Industrie Alimentari* 35(346): 249-252.

Chandan, R.C. (1989). *Yogurt: Nutritional Health Properties*. National Yogurt Association, Virgnia.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology* 84(5): 759-768.

Chatterjee, T.K., Sarkar, S. and Biswas, G. (1994). Quality characteristics of some marketed indigenous milk products: major constituents and mineral composition of rabri. *Journal of Food Science and Technology* 31(5): 426-427.

Chen, C., Zhao, S., Hao, G., Yu, H., Tian, H. and Zhao, G. (2017). Role of lactic acid bacteria on the yogurt flavour: A review. *International Journal of Food Properties* 20(1): 316-330.

Chen, X., Zheng, R., Liu, R. and Li, L. (2020). Goat milk fermented by lactic acid bacteria modulates small intestinal microbiota and immune responses. *Journal of Functional Foods* 65: 103744.

Chin Wen, L., Hsiao Ling, C. and Jeruei, L (1999). Identification and characterization of lactic acid bacteria and yeasts isolated from *Kefir* grains in Taiwan. *Australian Journal of Dairy Technology* 54(1): 14-18.

Choi, S.H. (2016). Characterization of airag collected in Ulaanbaatar, Mongolia with emphasis on isolated lactic acid bacteria. *Journal of Animal Science and Technology* 58: 1-10.

Choksi, N. and Desai, H. (2012). Isolation, identification and characterization of lactic acid bacteria from dairy sludge sample. *Journal of Environmental Research and Development* 7: 234-244.

Clarridge III, J.E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* 17(4): 840-862

Cocolin, L. and Ercolini, D. (2008). *Molecular Techniques in the Microbial Ecology of Fermented Foods*. Springer, New York.

Colombo, M., Castilho, N., Todorov, S.D. and Nero, L.A. (2018). Beneficial properties of lactic acid bacteria naturally present in dairy production. *BMC Microbiology* 18(1): 219. doi.org/10.1186/s12866-018-1356-8

Corredig, M. and Dalgleish, D.G. (1997). Isolates from industrial buttermilk: emulsifying properties of materials derived from the milk fat globule membrane. *Journal of Agriculture and Food Chemistry* 45(12): 4595-4600.

Corredig, M., and Dalgleish, D.G. (1998a). Buttermilk properties in emulsions with soybean oil as affected by fat globule. *Journal of Food Science* 63(3): 476–480.

Costantini, A., Cersosimo, M., Prete, V.D. and Garcia-Moruno, E. (2006). Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. *Journal of Food Protection* 69(2): 391-396.

Cotter, P.D., Hill, C. and Ross, R.P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology* 3(10): 777-788.

Csapo, J., Stefler, J., Martin, T.G., Makray, S. and Csapó-Kiss, Z. (1995). Composition of mares' colostrum and milk: Fat content, fatty acid composition and vitamin content. *International Dairy Journal* 5(4): 393-402.

Csapó-Kiss, Z., Stefler, J., Martin, T.G., Makray, S. and Csapó, J. (1995). Composition of mares' colostrum and milk: Protein content, amino acid composition and contents of macro and micro-elements. *International Dairy Journal* 5(4): 403-415.

Czech, L. and Stamatakis, A. (2019). Scalable methods for analyzing and visualizing phylogenetic placement of metagenomic samples. *PloS One* 14(5): doi.org/10.1371/journal.pone.0217050

Daglioğlu, O. (2000). *Tarhana* as a traditional Turkish fermented cereal food. Its recipe, production and composition. *Food/Nahrung* 44(2): 85-88.

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

Danova, S., Petrov, K., Pavlov, P. and Petrova, P. (2005). Isolation and characterization of Lactobacillus strains involved in *koumiss* fermentation. *International Journal of Dairy Technology* 58(2): 100-105.

de Man, J.C., Rogosa, D.M. and Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. *Journal of applied Bacteriology* 23(1): 130-135.

de Palencia, P.F., Werning, M.L., Sierra-Filardi, E., Dueñas, M.T., Irastorza, A., Corbí, A.L. and López, P. (2009). Probiotic properties of the 2-substituted (1, 3)-β-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. *Applied and Environmental Microbiology* 75(14): 4887-4891.

De Roos, N.M. and Katan, M.B. (1998). Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: A review of papers published between 1988 and 1998. *American Journal of Clinical Nutrition* 71(2): 405-411.

De Vries, M.C., Vaughan, E.E., Kleerebezem, M. and de Vos, W.M. (2006). *Lactobacillus plantarum*—survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal* 16(9): 1018-1028.

De Vuyst, L., Moreno, M.F. and Revets, H. (2003). Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. *International Journal of Food Microbiology* 84(3): 299-318.

De, S. (1980). Outlines of Dairy Technology. New Delhi: Oxford University Press.

Delbès, C., Ali-Mandjee, L. and Montel, M.C. (2007). Monitoring bacterial communities in raw milk and cheese by culture-dependent and-independent 16S rRNA gene-based analyses. *Applied in Environmental Microbiology* 73(6): 1882-1891.

Deosarkar S.S., Khedkar C.D. and Kalyankar S.D. (2016). Butter: Manufacture. In: *The Encyclopedia of Food and Health* vol. 1, (Eds. Caballero, B., Finglas, P., and Toldrá, F.), pp. 529-534. Oxford: Academic Press.

Devshete, N.G., Hembade, V.R., Mandwade, K.G. and Burbade, R. (2013). Preparation of omega-3 enriched probiotic Shrikhand using walnut powder. *International Journal of Processing and Post Harvest Technology* 4: 74-78.

Dewan, S. and Tamang, J.P. (2006). Microbial and analytical characterization of *Chhu*, a traditional fermented milk product of the Sikkim Himalayas. *Journal of Scientific and Industrial Research* 65:747-752.

Dewan, S. and Tamang, J.P. (2007). Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. *Antonie van Leeuwenhoek* 92: 343-352.

Di Gioia, D., Strahsburger, E., de Lacey, A.M.L., Bregola, V., Marotti, I., Aloisio, I., Biavati, B. and Dinelli, G. (2014). Flavonoid bioconversion in Bifidobacterium pseudocatenulatum B7003: A potential probiotic strain for functional food development. *Journal of Functional Foods* 7: 671-679.

Dillehay, D.L., Webb, S.J., Schmelz, E.M. and Merill. A.H. (1994). Dietary sphingomyelin inhibits 1, 2-dimethylhydrazine-induced colon cancer in CF1 mice. *Journal of Nutrition* 124(5): 615–620.

D'Incecco, P., Pellegrino, L., Hogenboom, J.A., Cocconcelli, P.S. and Bassi, D. (2018). The late blowing defect of hard cheeses: behaviour of cells and spores of *Clostridium tyrobutyricum* throughout the cheese manufacturing and ripening. *LWT Food Science and Technology* 87: 134-141.

Diosma, G., Romanin, D.E., Rey-Burusco, M.F., Londero, A. and Garrote, G. (2014). Yeasts from kefir grains: isolation, identification, and probiotic characterization. *World Journal of Microbiology and Biotechnology* 30: 43–53.

Dobson, A., O'Sullivan, O., Cotter, P.D., Ross, P. and Hill, C. (2011). High-throughput sequence-based analysis of the bacterial composition of *kefir* and an associated *kefir* grain. *FEMS microbiology letters* 320(1): 56-62.

Dönmez, N., Kısadere, I., Balaban, C. and Kadiralieva, N. (2014). Effects of traditional homemade koumiss on some hematological and biochemical characteristics in sedentary men exposed to exercise. *Biotechnic and Histochemistry* 89(8): 558-563.

Douglas, G.M., Maffei, V.J., Zaneveld, J., Yurgel, S.N., Brown, J.R., Taylor C.M., Huttenhower, C. and Langille, M.G. (2019). PICRUSt2: An improved and extensible approach for metagenome inference. *BioRxiv* 672295.

Doyle, C.J., Gleeson, D., O'Toole, P.W. and Cotter, P.D. (2016). High-throughput sequencing highlights the significant influence of seasonal housing and teat preparation on the raw milk microbiota. *Applied and Environmental Microbiology* doi:10.1128/AEM.02694-16.

Doyle, M.P. and Beuchat, L.R. (2007). Food Microbiology. Fundamentals and frontiers. 3rd ed. Center for food safety, The University of Georgia, Griffin, GA 30223–1797. American Society for Microbiology, ASM press 1752 N Street, N.W. Washington, DC 20036-2804, USA.

du Toit, M., Franz, C.M.A.P., Dicks, L.M.T., Schillinger, U., Haberer, P., Warlies, B., Ahrens, F. and Holzapfel, W.H. (1998). Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. *International Journal of Food Microbiology* 40(1-2): 93-104.

du Toit, M.D., Franz, C.M.A.P., Dicks, L.M.T. and Holzapfel, W.H. (2000). Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces. *Journal of Applied Microbiology* 88(3): 482-494.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C. and Kiely, B. (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *The American Journal of Clinical Nutrition* 73(2): 386s-392s.

Economidou, P.L. (1975). *Studies on Greek trahanas-a wheat fermented milk food.* M.S. Thesis, Cornwell University, USA.

Economidou, P.L. and Steinkraus, K.H. (1977). Greek trahanas. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Ehrmann, M.A., Kurzak, P., Bauer, J. and Vogel, R.F. (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *Journal of Applied Microbiology* 92(5): 966-975.

El-Gendy, S.M. (1983). Fermented foods of Egypt and the Middle East. *Journal of Food Protection* 46(4): 358-367.

Elling, J.L., Duncan, S.E., Keenan, T.W., Eigel, W.N. and Boling, J. (1996). Composition and Microscopy of Reformulated Creams from Reduced-Cholesterol Butteroil. *Journal of Food Science* 61(1): 48-53.

El-Sadek, G.M., Zawahry, M.R., Mahmoud, S.A.Z. and ABD, E.M. (1958). Chemical composition of Egyptian *Kishk*. *Indian Journal of Dairy Science* 11: 67-75.

El-Samaragy, Y.A. (1997). Labneh or Yoghurt cheese: A review. *Egyptian Journal of Dairy Science* 25(2): 165-178.

Ercolini, D. (2013). High-throughput sequencing and metagenomics: Moving forward in the culture-independent analysis of food microbial ecology. *Applied and Environmental Microbiology* 79: 3148-3155.

Falardeau, J., Keeney, K., Trmčić, A., Kitts, D. and Wang, S. (2019). Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility. *Food Microbiology* 83: 48-58.

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., Thevenot, D., Condron, R., De Reu, K., Govaris, A. and Heggum, K. (2013). Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. *International Journal of Food Microbiology* 162(2): 190-212.

Fenster, K., Freeburg, B., Hollard, C., Wong, C., Rønhave Laursen, R. and Ouwehand, A.C. (2019). The production and delivery of probiotics: A review of a practical approach. *Microorganisms 7*(3):83. doi.org/10.3390/microorganisms7030083.

Feng, P., Weagant, S.D., Grant, M.A., Burkhardt, W., Shellfish, M. and Water, B. (2002). Enumerations of *Escherichia coli* and the coliform bacteria. In: Bacteriological Analytical Manual. U.S Food and Drug Administration. Maryland, United States.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. *International Journal of Environmental Research and Public Health* 11(5): 4745-4767.

FIL-IDF (1981). *Consumption statistics for milk and milk products, 1979*. IDF Document 131.

Foley, M.H., O'Flaherty, S., Barrangou, R. and Theriot, C.M. (2019). Bile salt hydrolases: gatekeepers of bile acid metabolism and host-microbiome crosstalk in the gastrointestinal tract. *PLoS Pathogens* 15(3): e1007581. doi.org/10.1371/journal.ppat.1007581.

Franco-Duarte, R., Černáková, L., Kadam, S., Kaushik, K.S., Salehi, B., Bevilacqua, A., Corbo, M.R., Antolak, H., Dybka-Stępień, K., Leszczewicz, M., Relison Tintino, S., Alexandrino de Souza, V.C., Sharifi-Rad, J., Coutinho, H., Martins, N. and Rodrigues, C.F. (2019). Advances in chemical and biological methods to identify microorganisms-from past to present. *Microorganisms* 7(5):130. doi.org/10.3390/microorganisms7050130.

Franz, C.M.A.P., Huch, M., Mathara, J.M., Abriouel, H., Benomar, N., Reid, G., Galvez, A. and Holzapfel, W.H. (2014). African fermented foods and probiotics. *International Journal of Food Microbiology* 190: 84–96.

Furlaneto-Maia, L., Ramalho, R., Rocha, K.R. and Furlaneto, M.C. (2020). Antimicrobial activity of enterocins against *Listeria* sp. and other food spoilage bacteria. *Biotechnology Letters* 42: 797–806.

Gadallah, M.G.E. and Hassan, M.F.Y. (2019). Quality properties of Kishk (a dried fermented cereal-milk mixture) prepared from different raw materials. *Journal of the Saudi Society of Agricultural Sciences* 18(1): 95-101.

Galloway, J.H. and Crawford, R.J.M. (1985). Cheese fermentations. In: *Microbiology of Fermented Foods* Vol. I, (Ed: Wood, B.J.B.) pp. 111-165. Elsevier Applied Science Publishers, London.

Garcia-Gonzalez, N., Prete, R., Battista, N. and Corsetti, A. (2018). Adhesion properties of food-associated *Lactobacillus plantarum* strains on human intestinal epithelial cells and modulation of IL-8 release. *Frontiers in Microbiology* 9: 2392. doi: 10.3389/fmicb.2018.02392.

Garrote, G.L., Abraham, A.G. and De Antoni, G.L. (1997). Preservation of *kefir* grains, a comparative study. *LWT-Food Science and Technology* 30(1): 77-84.

Gaya, P., Peirotén, Á., Medina, M. and Landete, J.M. (2016). Isoflavone metabolism by a collection of lactic acid bacteria and bifidobacteria with biotechnological interest. *International Journal of Food Sciences and Nutrition* 67(2): 117-124

Gebreselassie, N., Abay, F. and Beyene, F. (2016). Biochemical and molecular identification and characterization of lactic acid bacteria and yeasts isolated from

Ethiopian naturally fermented buttermilk. *Journal of Food Science and Technology* 53(1): 184-196.

Georgala, A. (2012). The nutritional value of two fermented milk/cereal foods named 'Greek *Trahanas'* and 'Turkish *Tarhana'*: A Review. *Journal of Nutritional Disorder and Therapy* 11(3): 2161-0509.

Ghayal, G., Jha, A., Kumar, A., Gautam, A.K. and Rasane, P. (2015). Effect of modified atmospheric packaging on chemical and microbial changes in dietetic rabri during storage. *Journal of Food Science and Technology* 52(3):1825–1829.

Gheytanchi, E., Heshmati, F., Shargh, B.K., Nowroozi, J. and Movahedzadeh, F. (2010). Study on β-galactosidase enzyme produced by isolated lactobacilli from milk and cheese. *African Journal of Microbiology Research* 4(6): 454-458.

Ghosh, J. and Rajorhia, G.S. (1990). Selection of starter culture for production of indigenous fermented milk product (*Misti dahi*). *Lait* 70(2): 147-154.

Ghosh, J. and Rojorhia, G.S. (1987). Chemical, microbiological and sensory properties of *misti dahi* sold in Calcutta. *Asian Journal of Dairy Science* 6: 11-16.

Ghosh, T., Beniwal, A., Semwal, A. and Navani, N.K. (2019). Mechanistic Insights into probiotic properties of lactic acid bacteria associated with ethnic fermented dairy products. *Frontiers in Microbiology* 10:502. doi: 10.3389/fmicb.2019.00502.

Gil, R., Silva, F.J., Peretó, J. and Moya, A. (2004). Determination of the core of a minimal bacterial gene set. *Microbiology and Molecular Biology Reviews* 68(3): 518-537.

Gilliland, S.E. (1985). *Bacterial starter Cultures for Foods*. CRC Press, Boca Raton, Florida.

Gilliland, S.E. and Speck, M.L. (1977). Use of the Minitek system for characterizing lactobacilli. *Applied in Environmental Microbiology* 33(6):1289-1292.

Gocmen, D., Gurbuz, O., Rouseff, R.L., Smoot, J.M. and Dagdelen, A.F. (2004). Gas chromatographic-olfactometric characterization of aroma active compounds in sun-dried and vacuum-dried *tarhana*. *European Food Research and Technology* 218(6): 573-578.

Goel, A., Halami, P.M. and Tamang, J.P. (2020). Genome analysis of *Lactobacillus plantarum* isolated from some Indian fermented foods for bacteriocin production and probiotic marker genes. *Frontiers in Microbiology* 11**,** 40. doi.org/10.3389/fmicb.2020.00040.

Goh, Y.J. and Klaenhammer, T.R. (2010). Functional roles of aggregation-promoting like factor in stress tolerance and adherence of Lactobacillus acidophilus NCFM. *Applied and Environmental Microbiology* 76(15): 5005-5012.

Gomes, A.M.P. and Malcata, F.X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science and Technology* 10(4-5): 139-157.

Grahame, W.G. (1996). Methods for preservation and extension of shelf life. *International Journal of Food Microbiology* 33(1): 51-64.

Greene, J.D. and Klaenhammer, T.R. (1994). Factors involved in adherence of lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology* 60(12): 4487-4494.

Guetouache, M. and Guessas, B. (2015). Characterization and identification of lactic acid bacteria isolated from traditional cheese (*Klila*) prepared from cow's milk. *African Journal of Microbiology Research* 9(2): 71-77.

Guo, L, Xu, W.L, Li, C.D, Ma, Y., Guo, Y.S., Qian, J.P. and Zhu, J.J. (2020). Production technology, nutritional, and microbiological investigation of traditionally fermented mare milk (Chigee) from Xilin Gol in China. *Food Science and Nutrition* 8: 257– 264.

Gupta, R.C., Mann, B., Joshi, V.K. and Prasad, D.N. (2000). Microbiological, chemical and ultrastructural characteristics of *misti doi* (sweetened dahi). *Journal of Food Science and Technology* 37(1): 54-57

Gutiérrez-Cortés, C., Suárez, H., Buitrago, G. and Díaz-Moreno, C. (2017). Isolation and evaluation of the antagonist activity of lactic acid bacteria in raw cow milk. *Agronomía Colombiana* 35(3): 357-364.

Haghshenas, B., Nami, Y., Abdullah, N., Radiah, D., Rosli, R., Barzegari, A. and Yari Khosroushahi, A. (2015). Potentially probiotic acetic acid bacteria isolation and identification from traditional dairies microbiota. International *Journal of Food Science and Technology* 50(4): 1056-1064.

Halász A., Baráth, A., Simon-Sarkadi, L. and Holzapfel, W.H. (1994). Biogenic amines and their production by microorganisms in food. *Trends in Food Science and Technology* 5(2): 42-49.

Hammer, Ø., Harper, D.A. and Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4(1): 9. http://folk.uio.no/ohammer/past.

Han, J., Liu, Y., Wang, R., Yang, J., Ling, V. and Borchers, C.H. (2015). Metabolic profiling of bile acids in human and mouse blood by LC–MS/MS in combination with phospholipid-depletion solid-phase extraction. *Analytical Chemistry* 87(2): 1127-1136.

Hao, Y., Zhao, L., Zhang, H. and Zhai, Z. (2010). Identification of the bacterial biodiversity in koumiss by denaturing gradient gel electrophoresis and species-specific polymerase chain reaction. *Journal of Dairy Science* 93(5): 1926–1933.

Harrigan, W.F. (1998). *Laboratory Methods in Food Microbiology*. Gulf Professional Publishing.

Harris, B.J.R., Dorminey, D.E., Smith, W.A., Horn, H.H. and Wilcox, C.J. (1992). Effects of feather meal at two protein concentrations and yeast culture on production parameters in lactating dairy cows. *Journal Dairy Sciences* 75(12): 3524-3530.

Hartles, P., van Hooidonk, J. and la Riviere, J.W.M. (1977). Kefir. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Harun-ur-Rashid, M., Togo, K., Ueda, M., Miyamoto, T. (2006). Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk *Dahi* in Bangladesh. *World Journal of Microbiology and Biotechnology* 23(1):125–133.

Hasisurong, A. and Manglai. (2003). Koumiss and its value in medicine. *Zhongguo Zhong Yao Za Zhi* 28: 11-14.

Havenaar, R., Ten Brink, B. and Huis, J.H. (1992). Selection of strains for probiotic use. *In Probiotics* (pp. 209-224). Springer, Dordrecht. ISBN: 978-94-010-5043-2

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* 35(1): 1-21.

Heather, J.M. and Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics* 107(1): 1-8.

Héchard, Y., Dérijard, B., Letellier, F. and Cenatiempo, Y. (1992). Characterization and purification of mesentericin Y105, an anti-Listeria bacteriocin from Leuconostoc mesenteroides. *Microbiology* 138(12): 2725-2731.

Hegyi, P., Maléth, J., Walters, J.R., Hofmann, A.F. and Stephen J. Keely, S.J. (2018). Guts and Gall: Bile acids in regulation of intestinal epithelial function in health and disease. *Physiological Reviews* 98(4): 1983-2023.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S. and Calder, P.C. (2014). The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology* 11(8): 506-514.

Holt, J.G., Krieg, N.R., Sfneath, T.H.A., Stanley, J.T. and William, S.T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th Edition. Baltimore, MD.

Holzapfel, W.H., Haberer, P., Geisen, R., Bjorkroth, J. and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of Clinical Nutrition* 73(2): 365S–373S.

Holzapfel, W.H., Harberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J.H.J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology* 41: 85–101.

Holzapfel, W.H.N. and Wood, B.J. (2012). *The genera of lactic acid bacteria* (Vol.2). Springer Science & Business Media.

Homayouni Rad, A., Yari Khosroushahi, A., Khalili, M. and Jafarzadeh, S. (2016). Folate bio-fortification of yoghurt and fermented milk: a review. *Dairy Scieince and Technology* 96**:** 427–441.

Hosono, R., Nishimoto, S. and Kuno, S. (1989). Alterations of life span in the nematode *Caenorhabditis elegans* under monoxenic culture conditions. *Experimental Gerontology* 24(3): 251-264.

Huang, S., Rabah, H., Ferret-Bernard, S., Le Normand, L., Gaucher, F., Guerin, S., Nogret, I., Le Loir, Y., Chen, X.D., Jan, G. and Boudry, G. (2019). Propionic fermentation by the probiotic *Propionibacterium freudenreichii* to functionalize whey. *Journal of Functional Foods* 52: 620-628.

Hugenholtz, P. and Huber, T. (2003). Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *International Journal of Systematic and Evolutionary Microbiology* 53(1): 289-293.

Husain, Q. (2010). B-galactosidases and their potential applications. A review. *Biochemistry and Life Sciences* 30(1): 41-62.

Hutkins, R. (2018). *Microbiology and Technology of Fermented Foods*, 2nd Edition. Wiley Press, London, ages 624. ISBN: 978-1-119-02744-7.

Hwanhlem, N., Watthanasakphuban, N., Riebroy, S., Benjakul, S., H-Kittikun, A. and Maneerat, S. (2010). Probiotic lactic acid bacteria from Kung-Som: Isolation, screening, inhibition of pathogenic bacteria. *International Journal of Food Science and Technology* 45(3): 594–601.

Ibrahim, A.H. (2018). Enhancement of β-galactosidase activity of lactic acid bacteria in fermented camel milk. *Emirates Journal of Food and Agriculture* 30(4): 256-267.

Idoui, T., Boudjerda, J., Leghouchi, E. and Karam, N.E. (2009). Lactic acid bacteria from" Sheep's Dhan", a traditional butter: Isolation, identification and major technological traits. *Grasas Y Aceites* 60(2):177-183.

International Dairy Federation (1988). *Whole Milk: Determination of Milkfat, Protein and Lactose Content: Guide for the Operation of Mid-infra-red Instruments*. The Federation.

Ishii, S. and Y, Konagaya. (2002). Beneficial role of koumiss intake of Mongolian nomads. *Journal of Japan Society of Nutrition and Food Science* 55:281-285.

Jackson, E.E., Erten, E.S., Maddi, N., Graham, T.E., Larkin, J.W., Blodgett, R.J., Schlesser, J.E. and Reddy, R.M. (2012). Detection and enumeration of four foodborne pathogens in raw commingled silo milk in the United States. *Journal of Food Protection* 75(8): 1382-1393.

Jadhav, S.R., Shandilya, U.K. and Kansal, V.K. (2013). Exploring the ameliorative potential of probiotic *dahi* containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on dextran sodium sulphate induced colitis in mice. *Journal of Dairy Research* 80: 21–27.

Jakobsen, R.A., Heggebø, R., Sunde, E.B. and Skjervheim, M. (2011). *Staphylococcus aureus* and *Listeria monocytogenes* in Norwegian raw milk cheese production. *Food Microbiology* 28(3): 492-496.

Jamison, S. and Brereton, J. (2014). *The Rigveda: the earliest religious poetry of India.* Oxford University Press, New Delhi.

Jamuna, M. and Jeevaratnam, K. (2004). Isolation and partial characterization of bacteriocins from *Pediococcus* species. *Applied Microbiology and Biotechnology* 65(4): 433-439.

Jayashree, S., Pushpanathan, M., Rajendhran, J. and Gunasekaran, P. (2013). Microbial diversity and phylogeny analysis of buttermilk, a fermented milk product, employing 16S rRNA-based pyrosequencing. *Food Biotechnology* 27(3): 213-221.

Jeyaram, K., Romi, W., Singh, T.A., Devi, A.R. and Devi, S.S. (2010). Bacterial species associated with traditional starter cultures used for fermented bamboo shoot production in Manipur state of India. *International Journal of Food Microbiology* 143(1-2): 1-8.

Jianzhonga, Z., Xiaolia, L., Hanhub, J. and Mingshengb, D. (2009). Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiology* 26(8): 770-775.

Joishy, T.K., Dehingia, M. and Khan, M.R. (2019). Bacterial diversity and metabolite profiles of curd prepared by natural fermentation of raw milk and back sloping of boiled milk. *World Journal of Microbiology and Biotechnology* 35, 102. doi.org/10.1007/s11274-019-2677-y.

Jolliffe, I.T. and Cadima, J. (2016). Principal component analysis: a review and recent developments. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 37420150202. doi.org/10.1098/rsta.2015.0202.

Joosten, H.M.L.J., Rodriguez, E. and Nunez, M. (1997). PCR detection of sequences similar to the AS-48 structural gene in bacteriocin-producing enterococci. *Letters in Applied Microbiology* 24(1): 40-42.

Kakisu, E., Irigoyen, A., Torre, P., De Antoni, G.L. and Abraham, A.G. (2011). Physicochemical, microbiological and sensory profiles of fermented milk containing probiotic strains isolated from *kefir*. *Journal of Dairy Research* 78(4): 456-463.

Kanawjia, S.K. and Singh, S. (1996). Sensory and textural changes in paneer during storage. *Buffalo Journal* 12: 329-334.

Kanehisa, M. and Goto, S. (2000). KEGG: Kyoto encyclopaedia of genes and genomes. *Nucleic Acids Research* 28(1): 27-30.

Karche, R.V., Thakare V.M., Bhagat A.V. and Shirsath S.A. (2015). Microbiological quality of cow milk *shrikhand* blended with sapota pulp. *International Journal of Food, Agriculture and Veterinary Sciences* 5(1): 18-22.

Katiyar, S.K., Bhasin, A.K. and Bhatia, A.K. (1991). Traditionally processed and preserved milk products of Sikkimese Tribes. *Science and Culture* 57(10-11): 256-258.

Katoh, K., Misawa, K., Kuma, K.I. and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30(14): 3059–3066.

Kaushik, J.K., Kumar, A., Duary, R.K., Mohanty, A.K., Grover, S. and Batish, V.K. (2009). Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PloS One* 4(12): 1-11.

Keisam, S., Romi, W., Ahmed, G. and Jeyaram, K. (2016). Quantifying the biases in metagenome mining for realistic assessment of microbial ecology of naturally fermented foods. *Scientific Reports* 6: 34155.

Khalafalla, S.M., Fayed, E.O., Aly, A.A. and El-Samragy, Y.A. (1988). Composition and microbiological properties of Laban rayeb produced in Lower Egypt. *Ecology of Food and Nutrition* 21(4): 297-302.

Khan, S.U. and Pal, M.A. (2011). *Paneer* production: A review. *Journal of Food Science and Technology* 48(6): 645-660

Khaskheli, M., Jamali, A., Arain, M.A., Nizamani, A.H., Soomro, A.H. and Arain, H.H. (2008). Chemical and sensory quality of indigenous milk based product '*Rabri'*. *Pakistan Journal of Nutrition* 7(1): 133-136.

Kim, H.Y. (2014). Analysis of variance (ANOVA) comparing means of more than two groups. *Restorative Dentistry & Endodontics* 39:74-77.

Klaenhammer, T.R., Barrangou, R., Buck, B.L., Azcarate-Peril, M.A. and Altermann, E. (2005). Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiology Reviews* 29(3): 393-409.

Koleva, D., Georgieva, R., Nikolova, D. and Danova, S. (2009). Lactic acid microflora of Bulgarian milk products from mountain regions. *Biotechnology and Biotechnological Equipment* 23(1): 856-860.

Koroleva, N.S. (1988). Starters for fermented milks. In: Bulletin 227. Brussels: International Dairy Federation 35-40.

Kos, B., Šušković, J., Beganović, J., Gjuračić, K., Frece, J., Iannaccone, C. and Canganella, F. (2008). Characterization of the three selected probiotic strains for the application in food industry. *World Journal of Microbiology and Biotechnology* 24(5): 699-707.

Kosikowski, F.V. (1977). *Cheese and Fermented Milk Products*, 2nd Edition. Edwards Bros. Inc., Ann Arbor, Michigan.

Kramer, J.M. and Gilbert, R.J. (1989). *Bacillus cereus* and other *Bacillus*. *In Foodborne Bacterial Pathogens* (pp. 21-70). Marcel Dekker New York.

Kumar, M., Ghosh, M. and Ganguli, A. (2012). Mitogenic response and probiotic characteristics of lactic acid bacteria isolated from indigenously pickled vegetables and fermented beverages. *World Journal of Microbiology and Biotechnology* 28(2): 703-711.

Kurmann, J.A., Rasic, J.L. and Kroger, M. (1992). *Encyclopedia of fermented fresh milk products: an international inventory of fermented milk, cream, buttermilk, whey, and related products.* Springer Science and Business Media. New York: Van Nostrand Reinhold.

La Rivière, J.L. (1963). Studies on *kefir* grain. In Journal of General Microbiology 31(1): 5. Marlborough House, Basingstoke Rd, Spencers Woods, Reading Rg7 1ae, Berks, England: Soc General Microbiology.

La Riviere, J.W.M., Kooiman, P. and Schmidt, K. (1967). *Kefiran*, a novel polysaccharide produced in the *kefir* grain by *Lactobacillus brevis*. *Archives of Microbiology* 59(1-3): 269-278.

Lane, D.J. (1991). 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Goodfellow, M., Eds., *Nucleic Acid Techniques in Bacterial Systematic*, John Wiley and Sons, New York, 115-175.

Laxminarayana, H. and Shankar, P.A. (1980). Fermented milk in human nutrition. *Indian Dairyman* 32(2): 121-129.

Laxminarayana, H., Nambudripad, V.K.N., Laxmi, N.V., Anantaramaiah, S.H., Sreenivasamurty, V. and Iya, K.K. (1952). Studies on *dahi*. III. Taxonomy of lactic acid bacteria of *dahi*. *Indian Journal of Vetinary Science and Animal Husbandry* 22: 13-25.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reveiws* 72(4): 728-764.

Lei, L., Duan, Y., Zhang, K.Q. and Yang, J. (2011). Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Applied Microbiology and Biotechnology* 93: 993-1003.

Leisner, J.J., Millan, J.C., Huss, H.H. and Larsen, L.M. (1994). Production of histamine and tyramine by lactic acid bacteria isolated from vacuum-packed sugar-salted fish. *Journal of Applied Bacteriology* 76(5): 417-423.

Leite, A.M., Mayo, B., Rachid, C.T., Peixoto, R.S., Silva, J.T., Paschoalin, V.M.F. and Delgado, S. (2012). Assessment of the microbial diversity of Brazilian *kefir* grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiology* 31(2): 215-221.

Li, H., Ma, Y., Dong, A., Wang, J., Li, Q., He, S. and Maubois, J.L. (2010). Protein composition of yak milk. *Dairy Science and Technology* 90(1): 111-117.

Li, N., Wang, Y., You, C., Ren, J., Chen, W., Zheng, H. and Liu, Z. (2018). Variation in raw milk microbiota throughout 12 months and the impact of weather conditions. *Scientific Reports* 8(1): 1-10.

Linares, D.M., Gómez, C., Renes, E., Fresno, J.M., Tornadijo, M.E., Ross, R.P. and Stanton, C. (2017). Lactic acid bacteria and bifidobacteria with potential to design natural biofunctional health-promoting dairy foods. *Frontiers in Microbiology* 8, 846. doi: 10.3389/fmicb.2017.00846

Lisko, D.J., Johnston, G.P. and Johnston, C.G. (2017). Effects of dietary yogurt on the healthy human gastrointestinal (GI) microbiome. *Microorganisms* 5(1):6. doi.org/10.3390/microorganisms5010006.

Liu, S.N., Han, Y. and Zhou, Z.J. (2011). Lactic acid bacteria in traditional fermented Chinese foods. *Food Research International* 44(3): 643-651.

Liu, W., Xi, X., Sudu, Q., Kwok, L., Guo, Z., Hou, Q., Menhe, B., Sun, T. and Zhang, H. (2015). High-throughput sequencing reveals microbial community diversity of Tibetan naturally fermented yak milk. *Annals of Microbiology* 65:1741-1751.

Liu, W.J., Sun, Z.H., Zhang, Y.B., Zhang, C.L., Yang, M., Sun, T.S., Bao, Q.H., Chen, W. and Zhang, H.P. (2012). A survey of the bacterial composition of *kurut* from Tibet using a culture-independent approach. *Journal of Dairy Science* 95(3): 1064-1072.

Liu, Zheng, W.Y., Kwok, L.Y., Sun, Z., Zhang, J., Guo, Z., Hou, Q., Menhe, B. and Zhang, H. (2015). High-throughput sequencing for the detection of the bacterial and fungal diversity in Mongolian naturally fermented cow's milk in Russia. *BMC Microbiology*15:45. doi 10.1186/s12866-015-0385-9.

Ljungh, A. and Wadstrom T (2009). *Lactobacillus* Molecular Biology. Caister Academic Press. ISBN: 1904455417

Loretan, T., Mostert, J.F. and Viljoen, B.C. (2003). Microbial flora associated with South African household *kefir*. *South African Journal of Science* 99(1): 92-94.

Louca, S., Polz, M.F., Mazel, F., Albright, M.B., Huber, J.A., O'Connor, M.I., Ackermann, M., Hahn, A.S., Srivastava, D.S., Crowe, S.A. and Doebeli, M. (2018). Function and functional redundancy in microbial systems. *Nature Ecology and Evolution* 2(6): 936–943.

Lucas, P.M., Blancato, V.S., Claisse, O., Magni, C., Lolkema, J.S. and Lonvaud-Funel, A. (2007). Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. *Microbiology* 153(7): 2221-2230.

Mahmoud, S.A.Z. (1977). Chemical and microbiological composition of Egyptian *kishk*. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Malacarne, M., Martuzzi, F., Summer, A. and Mariani, P. (2002). Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *International Dairy Journal* 12(11): 869-877.

Mane, R., Padghan, P., Patil, S. and Patil, R. (2017). Studies on physico-chemical properties of *shrikhand* from buffalo milk blended with sweet corn milk. *Asian Journal of Dairy and Food Research* 36(4): 295-297.

Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A. and Smid, E.J. (2017). Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology* 44: 94–102.

Marsh, A.J., O'Sullivan, O., Hill, C., Ross, R.P. and Cotter, P.D. (2013). Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. *PloS One* 8: e69371. doi.org/10.1371/journal.pone.0069371.

Marshall, V.M. (1982). Flavour compounds in fermented milks. *Perfumer and Flavorist* 7(2): 27-34.

Martínez, M.I., Rodríguez, E., Medina, M., Hernández, P.E. and Rodríguez, J.M. (1998). Detection of specific bacteriocin-producing lactic acid bacteria by colony hybridization. *Journal of Applied Microbiology* 84(6): 1099-1103.

Mathara, J.M., Schillinger, U., Guigas, C., Franz, C., Kutima, P.M., Mbugua, S.K., Shin, H.K. and Holzapfel, W.H. (2008). Functional characteristics of *Lactobacillu*s spp. from traditional *Maasai* fermented milk products in Kenya. *International Journal of Food Microbiology* 126(1-2): 57-64.

Mathara, J.M., Schillinger, U., Kutima, P.M., Mbugua, S.K. and Holzapfel, W.H. (2004). Isolation, identification and characterization of the dominant microorganisms of *kule naoto*: the Maasai traditional fermented milk in Kenya. *International Journal of Food Microbiology* 94: 269-278.

Mathur, B.N. (1991). Indigenous milk products of India: The related research and technological requirements. *Indian Dairyman* 43(2): 61-74.

Mayo, B., Ammor, M.S., Delgado, S. and Alegría, A. (2010). Fermented milk products. In: *Fermented Foods and Beverages of the World* (Eds. Tamang, J.P. and Kailasapathy, K.), pp. 263-288. CRC Press, Taylor & Francis Group, New York.

McCarty, C. (1998). CHROMASPRO 1.34: Free program. Available from URL: http://www.technelysium.com.au/chromas.html.

Melia, S., Purwati, E., Yuherman, J., Aritonang, S.N. and Silaen, M. (2017). Characterization of the antimicrobial activity of lactic acid bacteria isolated from buffalo milk in West Sumatera (Indonesia) against *Listeria monocytogenes*. *Pakistan Journal of Nutrition* 16(8): 645-650.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A. and Wilkening, J. (2008). The metagenomics RAST server-a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9(1): 386. doi:10.1186/1471-2105-9-386

Michail, S. and Abernathy, F. (2002). *Lactobacillus plantarum* reduces the in vitro secretory response of intestinal epithelial cells to enteropathogenic *Escherichia coli* infection. *Journal of Pediatric Gastroenterology and Nutrition* 35(3): 350-355.

Michlmayr, H. and Kneifel, W. (2014). β-Glucosidase activities of lactic acid bacteria: mechanisms, impact on fermented food and human health. *FEMS Microbiology Letters* 352(1): 1-10.

Miguel, M.G.C.P, Cardoso, P.G., Magalhães, K.T. and Schwan, R.F. (2011). Profile of microbial communities present in tibico (sugary *kefir*) grains from different Brazilian States. *World Journal of Microbiology and Biotechnology* 27(8): 1875-1884.

Misra, A.K. (1992). Commercial production of dahi. *Indian Dairyman.* 44: 501-503.

Mital, B.K. (1977). Indian fermented milks. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Moayednia, N. and Mazaheri, A.F. (2012). The shifts of acidophilus milk at the refrigerator. *Journal of Food Biosciences and Technology* 2: 65-70.

Mobarez, A.M., Hosseini Doust, R., Sattari, M. and Mantheghi, N. (2008). Antimicrobial effects of bacteriocin like substance produced by L. acidophilus from traditional yoghurt on *P. aeruginosa* and *S. aureus*. *Journal of Biological Sciences* 8(1): 221-224.

Mogilevsky, S. (1977). Kefir. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Mohanan, K.R., Shankar, P.A. and Laxminarayana, H. (1984). Microflora of *dahi* prepared under household conditions of Bangalore. *Journal of Food Science and Technology* 21: 45-46.

Mohania, D., Kansal, V.K., Kruzliak, P. and Kumari, A. (2014). Probiotic *dahi* containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* modulates the formation of aberrant crypt foci, mucin-depleted foci, and cell proliferation on 1, 2-dimethylhydrazine-induced colorectal carcinogenesis in wistar rats. *Rejuvenation Research* 17(4): 325-333.

Monnet, C., Back, A. and Irlinger, F. (2012). Growth of aerobic ripening bacteria at the cheese surface is limited by the availability of iron. *Applied and Environmental Microbiology* 78(9): 3185–3192.

Monteagudo-Mera, A., Rodríguez-Aparicio, L., Rúa, J., Martínez- Blanco, H., Navasa, N., García-Armesto, M.R. and Ferrero, M.Á. (2012). In vitro evaluation of physiological probiotic properties of different lactic acid bacteria strains of dairy and human origin. *Journal of Functional Foods* 4(2): 531–541.

Morcos, S.R., Hegazi, S.M. and El-Damhoughy, S.T. (1973). Fermented foods in common use in Egypt I The nutritive value of *kishk*. *Journal of Science of Food and Agriculture*. 24(10):1153-1156.

Morgan, S., Ross, R.P. and Hill, C. (1995). Bacteriolytic activity caused by the presence of a novel lactococcal plasmid encoding lactococcins A, B, and M. *Applied in Environmental Microbiology* 61(8): 2995-3001.

Moser, A., Berthoud, H., Eugster, E., Meile, L. and Irmler, S. (2017). Detection and enumeration of *Lactobacillus helveticus* in dairy products. *International Dairy Journal* 68: 52-59.

Moslemy, M., Fard, R., Hosseini, S.M., Homayouni, A. and Mortazavian, A. (2015). A review: incorporation of Propionibacteria in fermented milks as a probiotic. *Critical Reviews in Food Science and Nutrition* 56. doi.10.1080/10408398.2013.766584.

Mourad, G. and Bettache, G. (2018). Characterization of lactic acid bacteria isolated from traditional butter produced in Djelfa province of Algeria. *Biosciences Biotechnology Research Asia* 15(3): 737.

Mtshali, P.S., Divol, B., Van Rensburg, P. and Du Toit, M. (2010). Genetic screening of wine-related enzymes in Lactobacillus species isolated from South African wines. *Journal of Applied Microbiology* 108(4): 1389-1397.

Mu, Z., Yang, X. and Yuan, H. (2012). Detection and identification of wild yeast in *Koumiss*. *Food Microbiology* 31(2): 301-308.

Mulder, H., and Walstra, P. (1974). The milk fat globule. Centre for Agricultural Publishing and Documentation, Wageningen, the Netherlands.

Nalbantoglu, U., Cakar, A., Dogan, H., Abaci, N., Ustek, D, Sayood, K. and Can, H. (2014). Metagenomic analysis of the microbial community in kefir grains. *Food Microbiology* 41: 42-51.

Negussie, G., Abrahamsen, R.K., Fekadu, B. and Narvhus, J. (2012). A survey on spontaneously fermented buttermilk in Northern Ethiopia. *African Journal of Food Science and Technology* 3(3): 78-89

Nguyen, T.D.T., Kang, J.H. and Lee, M.S. (2007). Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology* 113(3): 358-361.

Nigam, A., Kumar, A., Madhusudan, H.V. and Bhola, N. (2012). In-vitro screening of antibacterial activity of lactic acid bacteria against common enteric pathogens. *Journal of Biomedical Sciences* 1(4). doi: 10.3823/1010

Nostro, A., Cannatelli, M.A., Crisafi, G., Musolino, A.D., Procopio, F. and Alonzo, V. (2004). Modifications of hydrophobicity, in vitro adherence and cellular aggregation of Streptococcus mutans by Helichrysum italicum extract. *Letters in Applied Microbiology* 38(5): 423-427.

Oberman, H. (1985). Fermented Milks. In: *Microbiology of Fermented Foods* Vol I (Ed. Wood B.J.B.), (pp. 167-195). Elsevier Applied Science Publishers, London.

Obi, C.N., Olugbue, V.U.and Mpamugo, C.P. (2016). Yoghurt production from powdered milk using mixed lactic acid bacteria starter cultures. *Saudi Journal of Pathology and Microbiology* 1(2): 42-49.

Ogaki, M.B., Rocha, K.R., Terra, M.R., Furlaneto, M.C. and Maia, L.F. (2016). Screening of the enterocin-encoding genes and antimicrobial activity in *Enterococcus* species. *Journal of Microbiology and Biotechnology* 26(6):1026-1034.

Oki, K., Dugersuren, J., Demberel, S. and Watanabe, K. (2014). Pyrosequencing analysis of the microbial diversity of Airag, Khoormog and Tarag, traditional fermented dairy products of Mongolia. *Bioscience of Microbiota, Food and Health* 33(2): 53-64.

Olasupo, N.A., Schillinger, U. and Holzpafel, W.H. (2001). Studies on some technological properties of predominant lactic acid bacteria isolated from Nigerian fermented foods. *Food Biotechnology* 15: 157-167. doi: 10.1081/FBT-100107627

Ortiz-Estrada, Á.M., Gollas-Galván, T., Martínez-Córdova, L.R. and Martínez-Porchas, M. (2019). Predictive functional profiles using metagenomic 16S rRNA data: a novel approach to understanding the microbial ecology of aquaculture systems. *Reviews in Aquaculture* 11(1):234-245.

Otieno, D.O. and Shah, N.P. (2007). Endogenous β-glucosidase and β-galactosidase activities from selected probiotic micro-organisms and their role in isoflavone biotransformation in soymilk. *Journal of Applied Microbiology* 103(4): 910-917.

Ouwehand, A.C., Kirjavainen, P.V., Shortt, C. and Salminen, S. (1999). Probiotics: mechanisms and established effects. *International Dairy Journal* 9(1): 43-52.

Özdemir, G.B., Oryaşın, E., Bıyık, H.H., Özteber, M. and Bozdoğan, B. (2011). Phenotypic and genotypic characterization of bacteriocins in enterococcal isolates of different sources. *Indian Journal of Microbiology* 51(2): 182-187.

Ozdemir, S., Gocmen, D. and Kumral, A.Y. (2007). A traditional Turkish fermented cereal food: *Tarhana*. *Food Review International* 23(2):107-121.

Ozden, A. (2008). Other fermented dairy products: bioyogurt-probiotic yogurt. *Guncel Gastroenteroloji* 12(3): 169-181.

Padghan, P.V., Mann, B., Rajeshkumar, Sharma, R. and Kumar, A. (2015). Studies on bio-functional activity of traditional *lassi*. *Indian Journal of Traditional Knowledge* 1: 124-131.

Pal, P.K., Hossain, S.A. and Sarkar, P.K. (1996). Optimisation of process parameters in the manufacture of *churpi*. *Journal of Food Science and Technology* 33(3): 219-223.

Panelli, S., Brambati, E., Bonacina, C. and Feligini, M. (2013). Detection of *Clostridium tyrobutyricum* in milk to prevent late blowing in cheese by automated ribosomal intergenic spacer analysis. *Journal of Food Science* 78(10): 1569-1574.

Paraskevopoulou, A., Athanasiadis, I., Blekas, G., Koutinas, A.A., Kanellaki, M. and Kiosseoglou, V. (2003). Influence of polysaccharide addition on stability of a cheese whey kefir-milk mixture. *Food Hydrocolloids* 17(5): 615-620.

Parker, M., Zobrist, S., Donahue, C., Edick, C., Mansen, K., Hassan Zade Nadjari, M., Heerikhuisen, M., Sybesma, W., Molenaar, D., Diallo, A.M., Milani, P. and Kort, R. (2018). Naturally fermented milk from northern Senegal: bacterial community composition and probiotic enrichment with *Lactobacillus rhamnosus*. *Frontier in Microbiology* 9:2218. doi: 10.3389/fmicb.2018.02218.

Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30(21): 3123–3124.

Patel, P., Patel, V. and Subhash, R. (2015). Probiotic *lassi* preparation and sensory evaluation using *L. paracasei* immobilized dry fruit pieces. *International Journal of Fermented Foods* 4 (1-2): 23-33.

Patidar, S.K. and Prajapati, J.B. (1988). Standardization and evaluation of *lassi* prepared using *Lb. acido*philus and *S. thermophiles*. *Journal of Food Science and Technology* 35(5): 428-431.

Patidar, S.K. and Prajapati, J.B. (1998). Standardization and evaluation of lassi prepared using *Lactobacillus acidophilus* and *Streptococcus thermophilus*. *Journal of Food Science and Technology* 35(5): 428-431.

Patil, M.M., Pal, A., Anand, T. and Ramana, K.V. (2010). Isolation and characterization of lactic acid bacteria from curd and cucumber. *Indian Journal of Biotechnology* 9: 166-172.

Patrignani, F., Lanciotti, R., Mathara, J.M., Guerzoni, M.E. and Holzapfel, W.H. (2006). Potential of functional strains, isolated from traditional Maasai milk, as starters for the production of fermented milks. *International Journal of Food Microbiology* 107(1): 1–11.

Paul, I., Athmaselvi, K.A. and Geetha, P. (2016). Physicochemical, sensorial and microbiological properties of mishti dahi supplemented with flax lignan during storage and its antidiabetic activity. Bioscience, Biotechnology Research Asia 13(2). doi.org/10.13005/bbra/2155

PFA (2010). Prevention of food adulteration rules, 1954 (amended up to 2009). Universal Law Publishing Company Pvt Ltd, New Delhi, pp 165-166.

Pfeiler, E.A. and Klaenhammer, T.R. (2009). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* 75(18): 6013-6016.

Pfeiler, E.A., Azcarate-Peril, M.A. and Klaenhammer, T.R. (2007). Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. *Journal of Bacteriology* 189(13): 4624-4634.

Pimentel, G., Burton, K. J., von Ah, U., Bütikofer, U., Pralong, F. P., Vionnet, N., Portmann, R. and Vergères, G. (2018). Metabolic footprinting of fermented milk consumption in serum of healthy men. *Journal of Nutrition* 148(6): 851–860.

Planý, M., Kuchta, T., Šoltýs, K., Szemes, T., Pangallo, D. and Siekel, P. (2016). Metagenomic analysis of Slovak bryndza cheese using next-generation 16S rDNA amplicon sequencing. *Nova Biotechnologica et Chimica* 15(1): 23-34.

Plessas, S., Trantallidi, M., Bekatorou, A., Kanellaki, M., Nigam, P. and Koutinas, A.A. (2007). Immobilization of *kefir* and *Lactobacillus casei* on brewery spent grains for use in sourdough wheat bread making. *Food Chemistry* 105(1): 187-194.

Prabhurajeshwar, C. and Chandrakanth, K. (2019). Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic Lactobacilli strains isolated from commercial yoghurt. *Clinical Nutrition Experimental* 23: 97-115.

Prakash, O. (1961). *Food and Drinks in Ancient India*. Munshi Ram Monoharlal Publication, Delhi.

Prasad, J. (1997). Animal husbandry and dairy science (A guide for civil service preliminary examination). Kalyani Publishers, New Delhi, India, (pp. 383).

Prasad, J., Gill, H., Smart, J. and Gopal, P.K. (1998). Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. *International Dairy Journal* 8(12): 993-1002.

Prasant, Tomar, S.K., Singh, R., Gupta, S.C., Arora, D.K., Joshi, B.K. and Kumar, D. (2009). Phenotypic and genotypic characterization of lactobacilli from *Churpi* cheese. *Dairy Science and Technology* 89(6): 531-540.

Qian, Y., Long, X., Pan, Y., Li, G. and Zhao, X. (2018). Isolation and identification of lactic acid bacteria (*Lactobacillus plantarum* YS2) from yak yogurt and its probiotic properties. *Biomedical Research* 29(4). doi: 10.4066/biomedicalresearch.29-17-3418.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41(D1): D590-D596.

Quigley, L., O'Sullivan, O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F. and Cotter, P.D. (2012). High-throughput sequencing for detection of subpopulations of bacteria not

previously associated with artisanal cheeses. *Applied in Environmental Microbiology* 78(16): 5717-5723.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., R. Ross, R.P., Fitzgerald, G.F. and Cotter, P.D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews* 37(5): 664–698.

Rai, R., Shangpliang, HNJ and Tamang, J.P. (2016). Naturally fermented milk products of the Eastern Himalayas. *Journal of Ethnic Foods* 3(4): 270-275.

Raju, P.N. and Pal, D. (2009). The physico-chemical, sensory, and textural properties of *misti dahi* prepared from reduced fat buffalo milk. *Food and Bioprocess Technology* 2(1): 101-108.

Ramakrishnan, C.V. (1977). The use of fermented foods in India. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Ramakrishnan, C.V. (1979). Studies on Indian fermented foods. *Baroda Journal of Nutrition* 6: 1-57.

Ramiah, K., Van Reenen, C.A. and Dicks, L.M.T. (2007). Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor EF-Tu and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR. *International Journal of Food Microbiology* 116(3): 405-409.

Ramos, C.L., Thorsen, L., Schwan, R.F. and Jespersen, L. (2013). Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. *Food Microbiology* 36(1): 22-29.

Ranganathan, B., Nambrudipad, V.K.N., Dunani, A.T. and Iya, K.K. (1964). *Bacterial flora of milk and milk products of India*. Indian Council of Agricultural Research, Series No. 37.

Rangappa, K.S. and Achaya, K.T. (1974). *Indian Dairy Products*. Asia Publishing House, New Delhi.

Rao, K.N. and Solanki, D.C. (2007). Fermented dairy products-lab to industry: Research creativity and experiences. In: Souvenir of the 3rd International Conference on Fermented Foods Health Status and Social Well-being (pp. 70-73). Anand (India).

Rasane, P., Kailey, R. and Singh, S.K. (2017). Fermented indigenous Indian dairy products: standards, nutrition, technological significance and opportunities for its processing. *Journal of Pure and Applied Microbiology* 11(2): 1199-1213.

Rasic, J.L. and Kurmann, J.A. (1978). *Yoghurt - Scientific Grounds, Technology, Manufacture and Preparations*. Technical Dairy Publishing House, Copenhagen.

Rathi, S.D., Deshmukh, D.K., Ingle, U.M. and Syed, H.M. (1990). Studies on the physico-chemical properties of freeze-dried dahi. *Indian Journal of Dairy Science* 43(2): 249-251.

Ray, H.P. and Srinivasan, R.A. (1972). Use of microorganisms for production of indigenous fermented milk products (sweetened *dahi*). *Journal of Food Science and Technology* 9: 62-65.

Rezac, S., Kok, C.R., Heermann, M. and Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Frontiers in Microbiology* 9:1785. doi: 10.3389/fmicb.2018.01785.

Ridwan, B.U., Koning, C.J.M., Besselink, M.G.H., Timmerman, H.M., Brouwer, E.C., Verhoef, J., Gooszen, H.G. and Akkermans, L.M.A. (2008). Antimicrobial activity of a multispecies probiotic (Ecologic 641) against pathogens isolated from infected pancreatic necrosis. *Letters in Applied Microbiology* 46(1): 61-67.

Riyaz-Ul-Hassan, S., Verma, V. and Qazi, G.N. (2013). Real-time PCR-based rapid and culture-independent detection of Salmonella in dairy milk–addressing some core issues. *Letters in Applied Microbiology* 56(4): 275-282.

Robinson, R.K. (1990). *Dairy Microbiology*, 2nd Edition. Chapman and Hall, London.

Robinson, R.K. and Tamime, A.Y. (2006). Types of fermented milks. In: *Fermented Milks* (Ed. Tamine, A.Y.), pp. 1-10. John Wiley & Sons, Hoboken.

Rodriguez, J.M., Cintas, L.M., Casaus, P., Horn, N., Dodd, H.M., Hernández, P.E. and Gasson, M.J. (1995). Isolation of nisin-producing *Lactococcus lactis* strains from dry fermented sausages. *Journal of Applied Bacteriology* 78(2): 109-115.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4: e2584.

Romi, W., Ahmed, G. and Jeyaram, K. (2015). Three-phase succession of autochthonous lactic acid bacteria to reach a stable ecosystem within 7 days of natural bamboo shoot fermentation as revealed by different molecular approaches. *Molecular Ecology* 24(13): 3372-3389.

Roos, S. and Jonsson, H. (2002). A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* 148(2): 433-442.

Rosenkrantz, J.T., Aarts, H., Abee, T., Rolfe, M.D., Knudsen, G.M., Nielsen, M.B., Thomsen, L.E., Zwietering, M.H., Olsen, J.E. and Pin, C. (2013). Non-essential genes form the hubs of genome scale protein function and environmental gene expression networks in *Salmonella entericas*ero var *typhimurium*. *BMC Microbiology* 13, 294.doi.org/10.1186/1471-2180-13-294.

Rueda, R., Sabatel, J.L., Maldonado, J., Molina-Font, J.A. and Gil, A. (1998). Addition of gangliosides to an adapted milk formula modifies levels of fecal Escherichia coli in preterm newborn infants. *The Journal of Pediatrics* 133(1): 90-94.

Ruiz, L., Margolles, A. and Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Frontiers in Microbiology* 4, 396. doi.org/10.3389/fmicb.2013.00396

Rul, F. (2017). Yogurt: microbiology, organoleptic properties and probiotic potential. Fermented Foods, Part II: Technological Interventions, CRC Press, p. 525. Food Biology Series, 9781138637849. HAL-01579303.

Ruusunen, M., Surakka, A., Korkeala, H. and Lindström, M. (2012). *Clostridium tyrobutyricum* strains show wide variation in growth at different NaCl, pH, and temperature conditions. *Journal of Food Protection* 75(10):1791-1795.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4): 406-425.

Salton, M.R.J. (1958). The lysis of micro-organisms by lysozyme and related enzymes. *Microbiology* 18(2): 481-490.

Sanders M.E. and in't Veld J.H. (1999). Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. *Antonie van Leeuwenhoek* 76: 293-315.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceeding of the National Academy of Sciences* 74(12): 5463-5467.

Sarkar, A., Mukherjee, A.D., Bera, M.K., Das, A., Juyal, N., Morthekai, R. Deshpande, R.D., Shinde, V.S. and Rao, K.S. (2016). Oxygen isotope in archaeological bioapatites from India: Implications to climate change and decline of Bronze Age Harappan civilization. *Scientific Reports* 6: Article number: 26555. DOI: 10.1038/srep26555

Sarkar, S (2008). Innovations in Indian fermented milk products-a review. *Food Biotechnology* 22(1): 78-97.

Sarkar, S. (2007). Biotechnological innovations in *kefir* production: a review. *British Food Journal* 110(3): 283-295.

Sarkar, S., Kuila, R.K. and Misra, A.K. (1996). Anti-bacterial properties of *shrikhand*. *Indian Journal of Dairy Sciences* 49(4): 270-276.

Sarkar, S.P., Dave, J.M. and Sannabhadti, S.S. (1992). A note on the effect of thermization of *Misti dahi* on the acid producers count. *Indian Journal of Dairy Science* 45(3):131-134.

Schillinger, U., Guigas, C. and Holzapfel, W.H. (2005). In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *International Dairy Journal* 15(12): 1289-1297.

Schillinger, U. and Lücke, F.K. (1987). Identification of lactobacilli from meat and meat products. *Food Microbiology* 4(3): 199-208.

Schloss, P.D. and Westcott, S.L. (2011). Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Applied and Environmental Microbiology* 77(10): 3219-3226.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J. and Sahl, J.W. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied in Environmental Microbiology* 75(23): 7537-7541.

Schmelz, E.M., Dillehay, D.L., Webb, S.K., Reiter, A., Adams, J. and Merill, A.H. (1996). Sphingomyelin consumption supresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1, 2-dimethylhydrazine: Implications for dietary sphingolipids and colon carcinogenis. *Cancer Research* 56(21): 4936-4941.

Schmitz, A. and Riesner, D. (2006). Purification of nuclei acids by selective precipitation with polyethylene glycol 6000. *Analytical Biochemistry* 354(2): 311-313.

Schneller, R., Good, P. and Jenny, M. (1997). Influence of pasteurized milk, raw milk and different ripening cultures on biogenic amine concentrations in semi-soft cheeses during ripening. *Food Research Technology* 204(4): 265-272.

Sengupta, R., Altermann, E., Anderson, R.C., McNabb, W.C., Moughan, P.J. and Roy, N.C. (2013). The role of cell surface architecture of lactobacilli in host-microbe

interactions in the gastrointestinal tract. *Mediators of inflammation.* doi:10.1155/2013/237921.

Seol, D., Jhang, S.Y., Kim, H., Kim, S.Y., Kwak, H.S., Kim, S.H., Lee, W., Park, S., Kim, H., Cho, S. and Kwak, W. (2019). Accurate and strict identification of probiotic species based on coverage of whole-metagenome shotgun sequencing data. *Frontiers in Microbiology* 10: 1683. doi: 10.3389/fmicb.2019.01683.

Sert, D., Mercan, E. and Dertli, E. (2017). Characterization of lactic acid bacteria from yogurt-like product fermented with pine cone and determination of their role on physicochemical, textural and microbiological properties of product. *LWT Food Science and Technology* 78: 70-76.

Shah, N.P. (1994). *Lactobacillus acidophilus* and lactose intolerance: A review. *ASEAN Food Journal* 9: 47-54.

Shah, N.P. (2001). Functional foods from probiotics and prebiotics. *Food Technology* 55 (11): 46-53.

Shah, N.P. (2007). Functional cultures and health benefits. *International Dairy Journal* 17(11): 1262–1277.

Shah, N.P. and Jelen, P. (1990). Survival of lactic acid bacteria and their lactases under acidic conditions. *Journal of Food Science* 55(2): 506-509.

Shangpliang, H.N.J., Rai, R., Keisam, S., Jeyaram, K. and Tamang, J.P. (2018). Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing. *Scientific Reports* 8(1): 1532. doi.10.1038/s41598-018-19524-6.

Shangpliang, H.N.J., Sharma, S., Rai, R. and Tamang, J.P. (2017). Some technological properties of lactic acid bacteria isolated from *Dahi* and *Datshi*, naturally fermented milk products of Bhutan. *Frontiers in Microbiology* 8:116. doi:10.3389/fmicb.2017.00116

Sharma, N.K., Gill, J.P.S., Joshi, D.V. and Kwatra, M.S. (1993). Microflora of Indian fermented milk products (*dahi*). *Indian Journal of Dairy Science* 46: 85-87.

Sharma, S. and Singh, P. (2014). Isolation and characterization of β-galactosidase enzyme producing microbe and optimization of its enzyme activity under different culture condition. *International Journal of Current Microbiology and Applied Sciences* 3(7): 148-155.

Shewale, R.N., Sawale, P.D., Khedkar, C.D. and Singh, A. (2014). Selection criteria for probiotics: A review. *International Journal of Probiotics and Prebiotics* 9.

Shi, L.H., Balakrishnan, K., Thiagarajah, K., Mohd Ismail, N.I. and Yin, O.S. (2016). Beneficial properties of probiotics. *Tropical Life Sciences Research* 27(2): 73-90.

Shiby, V.K. and Mishra, H.N. (2013). Fermented milks and milk products as functional foods: a review. *Critical Reviews in Food Science and Nutrition* 53(5): 482-496.

Shrivastava, S. and Goyal, G.K. (2007). Preparation of *paneer*: A Review. *Indian Journal of Dairy Science* 60(6): 377-388.

Shruti, S. and Kansal, V.K. (2011). Effect of feeding probiotic dahi containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on enzymes that catalyze carcinogen activation and detoxification in rats. *Milchwissenschaft* 66(3): 244-247.

Shuaib, A.C.A. and Azmey, M.S.M. (1977). Fermented foods of Sri Lanka. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, GIAM-V, Bangkok.

Sieuwerts, S. (2016). Microbial interactions in the yoghurt consortium: current status and product implications. *SOJ Microbiology & Infectious Diseases* 4: 1-5.

Silla-Santos, M.H. (2001). Toxic nitrogen compounds produced during processing: biogenic amines, ethyl carbamides, nitrosamines. In: *Fermentation and Food Safety* (Eds: Adams, M.R. and Nout, M.J.R.), pp. 119-140. Aspen Publishers, Inc. Gaithersburg, Md., USA.

Silva, C.C.G., Silva, S.P.M. and Ribeiro, S.C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. *Frontiers in Microbiology* 9, 594. doi:10.3389/fmicb.2018.00594.

Singh, D. and Singh, J. (2014). *Shrikhand*: a delicious and healthful traditional Indian fermented dairy dessert. *Trends in Biosciences* 7: 153-155.

Singh, D., Singh, J., Kumar, S. and Verma, T. (2014). Microbiological evaluation of soy fortified *shrikhand* by using response surface methodology. *International Journal of Applied Biology and Pharmaceutical Technology* 5(1): 1-7.

Singh, R. (2007). Characteristics and technology of traditional Indian cultured dairy products. *Bulletin-International Dairy Federation* 415: 11-20.

Soccol1, C.R., de Souza Vandenberghe, L.P., Spier, M.R., Medeiros, A.B.P., Yamaguishi, C.T., Lindner, J.D.D., Pandey, A. and Thomaz-Soccol1, V. (2010). The potential of probiotics: a review. *Food Technology* 48 (4): 413–434.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. and Massa, S. (2005). A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *Journal of Applied Microbiology* 98(4): 855-861.

Sprong, R.C., Hulstein, M.F.E., and van der Meer, R. (2002). Bovine milk fat components inhibit food-borne pathogens. *International Dairy Journal* 12(2-3): 209-215.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E.L. and Lynch, S.V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One* 10(2). doi.org/10.1371/journal.pone.0117617.

Ssajjakambwe, P., Bahizi, G., Setumba, C., Kisaka, S., Vudriko, P., Atuheire, C., Kabasa, J.D. and Kaneene, J.B. (2017). Milk hygiene in rural south western Uganda: prevalence of mastitis and antimicrobial resistance profiles of bacterial contaminants of milk and milk products. *Veterinary medicine international* 6. doi.org/10.1155/2017/8710758

Stack, H.M., Kearney, N., Stanton, C., Fitzgerald, G.F. and Ross, R.P. (2010). Association of beta-glucan endogenous production with increased stress tolerance of intestinal lactobacilli. *Applied in Environmental Microbiology* 76(2): 500-507.

Steinkraus, K.H. (1983) *Handbook of Indigenous Fermented Food*, Vol. 9. Marcel Dekker, Inc., New York.

Steinkraus K.H. (1996). *Handbook of Indigenous Fermented Food*. 2nd Ed. CRC press, Taylor & Francis Group, New York.

Steinkraus, K.H., Shapiro, K.B., Hotchkiss, J.H. and Mortlock, R.P. (1996). Investigations into the antibiotic activity of tea fungus/kombucha beverage. *Acta Biotechnologica* 16(2-3): 199-205.

Stratton, J.E., Hutkins, R.W. and Taylor, S.L. (1991). Biogenic amines in cheese and other fermented foods. A review. *Journal of Food Protection* 54(6): 460-470.

Subathra Devi, C., Reddy, S. and Mohanasrinivasan, V. (2014). Fermentative production of dextran using *Leuconostoc* spp. isolated from fermented food products. *Frontiers in Biology* 9(3): 244–253.

Sudheendra Ch, V.K., Madhavi, T.V., Gopikrishna, G. and Shah, R.K. (2018). Study of microbial changes in probiotic and synbiotic lassi during storage. *The Pharma Innovation* 7(2): 94-97.

Sukumar, De. (2004). Indian Diary Products, Outlines if Dairy Technology, (Oxford University Press, New Delhi, (pp.463-464).

Sun, Z., Liu, W., Bao, Q., Zhang, J., Hou, Q., Kwok, L., Sun, T. and Zhang, H. (2014). Investigation of bacterial and fungal diversity in *tarag* using high-throughput sequencing. *Journal of Dairy Science* 97(10): 6085-6096.

Sun, Z., Liu, W., Gao, W., Yang, M., Zhang, J., Wang, J., Menghe, B., Sun, T. and Zhang, H. (2010). Identification and characterization of the dominant lactic acid bacteria from *kurut*: the naturally fermented yak milk in Qinghai, China. *Journal of General and Applied Microbiology* 56(1): 1-10.

Sun, Z.H, Liu, W.J., Zhang, J., Yu, J., Gao, W. and Jiri, B. (2010). Identification and characterization of the dominant lactic acid bacteria isolated from traditional fermented milk in Mongolia. *Folia Microbiologica* 55(3): 270-276.

Surono, I.S. (2004). *Probiotics, Fermented Milk and Health*. Indonesian Food and Berevage Entrepreneur association (YAPMMI). Tri Cipta Karya. Jakarta.

Surono, I.S. and Hosono, A. (2011). Fermented Milks: types and standards of identity. In: *Encyclopedia of Dairy Sciences*, Second Edition, vol. 2, (Eds. Fuquay. J.W., Fox, P.F. and McSweeney, P.L.H.), pp. 470–476. Academic Press, San Diego.

Swapna, G. and Chavannavar, S.V. (2013). *Shrikhand*: Value added traditional dairy product. *International Journal of Food and Nutritional Sciences* 2(4): 45-51.

Tagg, J.R.. Dajani. A.S. and Wannamaker, L.W. (1976). Bactcriocins of Gram-positive bacteria. *Bacteriological Review* 40(3): 722-756.

Takeda, S., Yamasaki, K., Takeshita, M., Kikuchi, Y., Tsend-Ayush, C., Dashnyam, B., Ahhmed, A.M., Kawahara, S. and Muguruma, M. (2011). The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. *Animal Science Journal* 82(4): 571–579.

Tamang, J.P. (2005). *Food Culture of Sikkim*. Sikkim Study Series volume IV. Information and Public Relations Department, Government of Sikkim, Gangtok, p. 120.

Tamang, J.P. (2010a). Diversity of fermented foods. In: *Fermented Foods and Beverages of the World* (Eds: Tamang, J.P. and Kailasapathy, K), pp. 41-84. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P. (2010b). *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic Values*. CRC Press, Taylor & Francis Group, New York, pages 295. ISBN: 9781420093247.

Tamang, J.P. (2015). *Health Benefits of Fermented Foods and Beverages*. CRC Press, Taylor & Francis Group, New York, pages 636. ISBN: 978-1-4665-88097.

Tamang, J.P. (2020). History and Culture of Indian Ethnic Fermented Foods and Beverages. In: *Ethnic Fermented Foods and Alcoholic Beverages of India: Science History and Culture* (Ed: Tamang, J.P.), pp. 1-40. Springer Nature, Singapore. ISBN: 978-981-15-1486-9.

Tamang, J.P. and Samuel, D. (2010). Dietary culture and antiquity of fermented foods and beverages. In: *Fermented Foods and Beverages of the World* (Eds: Tamang, J.P. and Kailasapathy, K), pp.1–40. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P., Cotter, P., Endo, A., Han, N.S., Kort, R., Liu, S.Q., Mayo, B., Westerik, N. and Hutkins, R. (2020). Fermented foods in a global age: east meets west. *Comprehensive Reviews in Food Science and Food Safety* 19: 184-217.

Tamang, J.P., Dewan, S., Thapa, S., Olasupo, N.A., Schillinger, U. and Holzapfel, W.H. (2000). Identification and enzymatic profiles of predominant lactic acid bacteria isolated from soft variety chhurpi, a traditional cheese typical of the Sikkim Himalayas. *Food Biotechnology* 14(1-2): 99–112.

Tamang, J.P., Holzapfel, W.H. and Watanabe, K. (2016a). Diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology* 7:377. doi: 10.3389/fmicb.2016.00377.

Tamang, J.P., Shin, D.H., Jung, S.J. and Chae, S.W. (2016b). Functional properties of microorganisms in fermented foods. *Frontiers in Microbiology* 7:578. doi: 10.3389/fmicb.2016.00578.

Tamang, J.P., Tamang, N., Thapa, S., Dewan, S., Tamang, B., Yonzan, H., Rai, A.K., Chettri, R., Chakrabarty, J. and Kharel, N. (2012). Microorganisms and Nutritional value of Ethnic fermented foods and alcoholic beverages of North East India. *Indian Journal of Traditional Knowledge* 11: 7-25.

Tamang, J.P., Thapa, N., Rai, B., Thapa, S., Yonzan, H., Dewan, S., Tamang, B., Sharma, R.M., Rai, A.K., Chettri, R., Mukhopadhyay, B. and Pal, B. (2007). Food consumption in Sikkim with special reference to traditional fermented foods and beverages: a micro-level survey**.** *Journal of Hill Research* 20(1): 1-37.

Tamime, A.Y. (1981). Microbiology of starter cultures. In: *Dairy Microbiology* vol 2 The Microbiology of Milk Products, (Ed: Robinson, R.K.), pp. 113-156, Applied Science, London.

Tamime, A.Y. and McNulty, D. (1999). Kishk-a dried fermented milk/cereal mixture. 4. Microbiological quality. *Le Lait* 79(4): 449-456.

Tamime, A.Y. and O'connor, T.P. (1995). *Kishk*-a dried fermented milk/cereal mixture. *International Dairy Journal* 5(2): 109-128.

Tamime, A.Y. and Robinson, R.K. (1985). *Yogurt – Science and Technology*. Pergamon Press, Oxford.

Tamime, A.Y. and Robinson, R.K. (1999). Yoghurt: science and technology (2nd ed.). Woodhead Publishing, CRC Press.

Tamime, A.Y. and Robinson, R.K. (2007). *Yoghurt Science and Technology*. Woodhead Publishing Ltd., Cambridge.

Tamime, A.Y., Muir, D.D., Khaskheli, M. and Barclay, M.N.I. (2000). Effect of processing conditions and raw materials on the properties of *Kishk* 1. Compositional and microbiological qualities. *LWT-Food Science and Technology* 33(6): 444-451.

Tamine, A.Y. and Robinson, R.K. (1988). *Fermented milks and their future trends. II: Technological aspects. Journal* of Dairy Research 55(2): 281-307.

Tang, H., Ma, H., Hou, Q., Li, W., Xu, H., Liu, W., Sun, Z., Haobisi, H. and Menghe, B. (2020). Profiling of koumiss microbiota and organic acids and their effects on koumiss taste. *BMC Microbiology* 20(1): 85. doi.org/10.1186/s12866-020-01773-z.

Tavakoli, M., Habibi Najafi, M.B. and Mohebbi, M. (2019). Effect of the milk fat content and starter culture selection on proteolysis and antioxidant activity of probiotic yogurt. *Heliyon* 5: e01204. doi: 10.1016/j.heliyon.2019.e01204.

Ten Brink, B., Damink, C., Joosten, H.J. and Huis in't Veld, J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology* 11(1): 73-84.

Thapa, N. and Tamang, J.P. (2020). Ethnic Fermented Foods and Beverages of Sikkim and Darjeeling Hills (Gorkhaland Territorial Administration). In: *Ethnic Fermented Foods and Alcoholic Beverages of India: Science History and Culture* (Ed: Tamang, J.P.), pp. 479-537. Springer Nature, Singapore. ISBN: 978-981-15-1486-9.

Thornley, M.J. (1960). The differentiation of Pseudomonas from other Gram-negative bacteria on the basis of arginine metabolism. *Journal of Applied Bacteriology* 23(1): 7-52.

Tomás, M.S., Wiese, B. and Nader-Macías, M.E. (2005). Effects of culture conditions on the growth and auto-aggregation ability of vaginal *Lactobacillus johnsonii* CRL 1294. *Journal of Applied Microbiology* 99(6): 1383-1391.

Topisirovic, L., Kojic, M., Fira, D., Golic, N., Strahinic, I. and Lozo, J. (2006). Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. *International Journal of Food Microbiology* 112(3): 230-235.

Turpin, W., Humblot, C. and Guyot, J.P. (2011). Genetic screening of functional properties of lactic acid bacteria in a fermented pearl millet slurry and in the metagenome of fermented starchy foods. *Applied in Environmental Microbiology* 77(24): 8722-8734.

Turpin, W., Humblot, C., Thomas, M. and Guyot, J.P. (2010). Lactobacilli as multifaceted probiotics with poorly disclosed molecular mechanisms. *International Journal of Food Microbiology* 143(3): 87-102.

Turpin, W., Humbolt, C., Noordine, M.L., Thomas, M. and Guyot, J.P. (2012). *Lactobacillaceae* and cell adhesion: genomic and functional screening. *PLoS One* 7(5). doi:10.1371/journal.pone.0038034

United States Department of Agriculture (1978). *Cheese Varieties and Descriptions*. USDA, Washington, D.C.

Vale, S.R. and Gloria M.B.A. (1997). Determination of biogenic amine in cheese. *Journal of AOAC International* 80(5): 1006-1012.

Van Belkum, M.J., Hayema, B.J., Geis, A., Kok, J. and Venema, G. (1989). Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid. *Applied and Environmental Microbiology* 55(5): 1187-1191.

van Pijkeren, J.P., Canchaya, C., Ryan, K.A., Li, Y., Claesson, M.J., Sheil, B., Steidler, L., O'Mahony, L., Fitzgerald, G.F., van Sinderen, D. and O'Toole, P.W. (2006). Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. *Applied and Environmental Microbiology* 72(6): 4143-4153.

Vedamuthu, E.R. (1982). Fermented milks. In: *Economic Microbiology*, Vol 7. Fermented Foods, (Ed: Rose, A.H.), Academic Press, London.

Vijayalakshmi, R. and Murugesan, T. (2001). Shelf life and microbiological quality of selected dairy products. *Journal of Food Science and Technology* 38(4): 385-386.

Viswanathan, M.V., Unnikrishnan, P.M., Komatsu, K., Fushimi, H. and Basnet, P. (2003). A brief introduction to Ayurvedic system of medicine and some of its problems. *Indian Journal of Traditional Knowledge* 2: 159-169.

Vlasova, A.N., Kandasamy, S., Chattha, K.S., Rajashekara, G. and Saif, L.J. (2016). Comparison of probiotic lactobacilli and bifidobacteria effects, immune responses

and rotavirus vaccines and infection in different host species. *Veterinary Immunology and Immunopathology* 172: 72–84.

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J. and Roos, S. (2007). The early response to acid shock in Lactobacillus reuteri involves the ClpL chaperone and a putative cell wall-altering esterase. *Applied and Environmental Microbiology* 73 (12): 3924-3935.

Walsh, A.M., Crispie, F., Daari, K., O'Sullivan, O., Martin, J.C., Arthur, C.T., Claesson, M.J., Scott, K.P. and Cotter, P.D. (2017). Strain-level metagenomic analysis of the fermented dairy beverage *nunu* highlights potential food safety risks. *Applied and Environmental Microbiology* 83(16): e01144-17.

Walsh, A.M., Crispie, F., Kilcawley, K., O'Sullivan, O., O'Sullivan, M.G., Claesson, M.J. and Cotter, P.D. (2016). Microbial succession and flavor production in the fermented dairy beverage kefir. *Msystems* 1: e00052-16. doi.org/10.1128/mSystems.00052-16.

Walsh, A.M., Crispie, F., O'Sullivan, O., Finnegan, L., Claesson, M.J. and Cotter, P.D. (2018). Species classifier choice is a key consideration when analysing low-complexity food microbiome data. *Microbiome* 6: 50. doi.org/10.1186/s40168-018-0437-0.

Wang, D., Liu, W., Ren, Y., De, L., Zhang, D., Yang, Y., Bao, Q., Zhang, H. and Menghe, B. (2016). Isolation and identification of lactic acid bacteria from traditional dairy products in Baotou and Bayannur of midwestern Inner Mongolia and q-PCR analysis of predominant species. *Food Science of Animal Resources* 36(4): 499-507.

Wang, J., Chen, X., Liu, W., Yang, M. and Zhang, H. (2008). Identification of Lactobacillus from *koumiss* by conventional and molecular methods. *European Food Research and Technology* 227(5): 1555-1561.

Watanabe, K., Fujimoto, J., Sasamoto, M., Dugersuren, J., Tumursuh, T. and Demberel, S. (2008). Diversity of lactic acid bacteria and yeasts in *Airag* and *Tarag*, traditional fermented milk products of Mongolia. *World Journal of Microbiology and Biotechnology* 24(8): 1313-1325.

Wei, H., Wolf, G. and Hammes, W.P. (2006). Indigenous microorganisms from iceberg lettuce with adherence and antagonistic potential for use as protective culture. *Innovative Food Science and Emerging Technologies* 7(4): 294-301.

Whitehead, K., Versalovic, J., Roos, S. and Britton, R.A. (2008). Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Applied and Environmental Microbiology* 74(6): 1812-1819.

Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R. and Boor, K.J. (2000). Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Applied and Environmental Microbiology* 66(5): 2085-2095.

Witthuhn, R.C., Schoeman, T. and Britz, T.J. (2005). Characterisation of the microbial population at different stages of *kefir* production and *kefir* grain mass cultivation. *International Dairy Journal* 15(4): 383-389.

Wong, P.Y.Y. and Kitts, D.D. (2003). A comparison of the butter milk solids functional properties to non fat dried milk, soy protein isolate, dried egg white, and egg yolk powders. *Journal of Dairy Science* 86(3): 746-754.

Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., Guo, M. and Zhang, H. (2009). Isolation and preliminary probiotic selection of lactobacilli from Koumiss in Inner Mongolia. *Journal of Basic Microbiology* 49(3): 318-326.

Wulijideligen, S., Arakawa, K., Miyamoto, M. and Miyamoto, T. (2013). Interaction between lactic acid bacteria and yeasts in *airag*, an alcoholic fermented milk. *Animal Science Journal* 84(1): 66-74.

Xue, Z., Kable, M.E. and Marco, M.L. (2018). Impact of DNA sequencing and analysis methods on 16S rRNA gene bacterial community analysis of dairy products. *mSphere* 3(5): e00410-18. doi: 10.1128/mSphere.00410-18.

Ya, T., Zhang, Q., Chu, F., Merritt, J., Bilige, M., Sun, T., Du, R. and Zhang, H. (2008). Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from *koumiss* in Inner Mongolia, China. *BMC Immunology* 9(1): 68.

Yadav, R., Puniya, A.K. and Shukla, P. (2016). Probiotic properties of *Lactobacillus plantarum* RYPR1 from an indigenous fermented beverage *Raabadi*. *Frontiers in Microbiology* 7:1683. doi.org/10.3389/fmicb.2016.01683.

Yao, G., Yu, J., Hou, Q., Hui, W., Liu, W., Kwok, L-Y, Menghe, B., Sun, T., Zhang, H. and Zhang, W. (2017). A perspective study of Koumiss microbiome by metagenomics analysis based on single-cell amplification technique. *Frontiers in Microbiology* 8:165. doi: 10.3389/fmicb.2017.00165.

Ye, J.Z., Lin, X.M., Cheng, Z.X., Su, Y.B., Li, W.X., Ali, F.M., Zheng, J. and Peng, B. (2018). Identification and efficacy of glycine, serine and threonine metabolism in

potentiating kanamycin-mediated killing of *Edwardsiella piscicida*. *Journal of Proteomics* 183: 34-44.

Ye, Y. and Doak, T.G. (2009). A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Computational Biology* 5(8).

Yegna Narayan Aiyar, A.K. (1953). Dairying in ancient India. *Indian Dairyman* 5: 77-83.

Ying, A.N. and Adachi, Y. and Ogawa, Y. (2004). Classification of lactic acid bacteria isolated from chigee and mare milk collected in Inner Mongolia. *Animal Science Journal* 75: 245 - 252.

Yu, J., Mo, L., Pan, L., Yao, C., Ren, D. and An, X., Tsogtgerel, T., Zhang, H. and Liu, W. (2018). Bacterial microbiota and metabolic character of traditional sour cream and butter in Buryatia, Russia. *Frontiers in Microbiology* 9, 2496. doi:10.3389/fmicb.2018.02496.

Yu, J., Wang W.H., Menghe, B.L., Jiri, M.T., Wang, H.M., Liu, W.J., Bao, Q.H., Zhang, J.C., Wang, F., Xu, H.Y., Sun, T.S. and Zhang, H.P. (2011). Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. *Journal of Dairy Science* 94(7): 3229–3241.

Zago, M., Fornasari, M.E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., Reinheimer, J. and Giraffa, G. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology* 28(5): 1033-1040.

Zavaglia, A.G., Kociubinski, G., Perez, P. and Graciela, D. (1998). Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. *Journal of Food Protection* 61(7): 865-873.

Zhang, F., Wang, Z., Lei, F., Wang, B., Jiang, S., Peng, Q., Zhang, J. and Shao, Y. (2017). Bacterial diversity in goat milk from the Guanzhong area of China. *Journal of Dairy Science* 100(10): 7812-7824.

Zhang, H., J. Xu, J. Wang, Menghebilige, T. Sun, H. Li. and M. Guo. (2008). A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China. *Food Control* 19(6): 578-586.

Zhang, Y., Du, R., Wang, L. and Zhang, H. (2010). The antioxidative effects of probiotic *Lactobacillus casei* Zhang on the hyperlipidemic rats. *European Food Research and Technology* 231(1): 151-158.

Zhong, Z., Hou, Q., Kwok, L., Yu, Z., Zheng, Y., Sun, Z., Menghe, B. and Zhang, H. (2016). Bacterial microbiota compositions of naturally fermented milk are shaped by both geographic origin and sample type. *Journal of Dairy Science* 99(10): 7832-7841.

Zhou, T., Huo, R., Kwok, L.Y., Li, C., Ma, Y., Mi, Z. and Chen, Y. (2019). Effects of applying *Lactobacillus helveticus* H9 as adjunct starter culture in yogurt fermentation and storage. *Journal of Dairy Science* 102(1): 223-235.

Zhu, Y., Cao, Y., Yang, M., Wen, P., Cao, L., Ma, J., Zhang, Z. and Zhang, W. (2018). Bacterial diversity and community in Qula from the Qinghai–Tibetan Plateau in China. *PeerJ* 6: 6044.

CURRICULUM VITAE

Name: **Ranjita Rai**

Email: skmeseranjurai@gmail.com

Address: **Central Martam, P/O Bermiok Bazar, West Sikkim, 737113.**

Mobile no. **9593779024**

PhD Thesis Title: Diversity of Lactic Acid Bacteria and Their Probiotic Properties in Some Naturally Fermented Milk Products of Sikkim (Supervisors: Professor (Dr.) Jyoti Prakash Tamang).

Qualifications:

Degree	University	Month and year of joining	Month and year of passing	Division/ Grade
M.Phil (Microbiology)	Sikkim University	7/2011	6/2013	First division
M.Sc (Microbiology)	Sikkim University	07/2009	06/2011	Second division
B.Sc	Govt. College Tadong	07/2006	06/2009	Second division

Research Experience: Expertise in Bacterial isolation, Bacterial identification from Culture-dependent method, DNA isolation, PCR reaction, Amplified product purification, Phylogeny tree constructions, and Analysis of bacterial microflora using Culture-independent method (High-throughput Amplicon Sequencing), Probiotic screening, Gene detection and Predictive Functionalities; Bioinformatics Analysis of 16S rRNA Gene Sequencing Data.

Awards

1) Fellowship grant for Junior Research Fellow (JRF) and Senior Research Fellow (SRF), from Dept. of Science and Technology (DST), Govt. of India (September 2014- December 2019).

Oral presentations at International Conferences

1) International Conference on "Nutraceuticals and Chronic Diseases (INCD)" at Indian Institute of Technology, Guwahati, Assam: 23-25 September 2019.
2) International Conference on "Ethnic Fermented Foods and Beverages: Microbiology and Health Benefits" at Sikkim University, Gangtok: 20-21 November 2015.

Workshop/Conferences Attended

1) Participated and presented poster entitled "Bacterial diversity of naturally fermented milk products of Sikkim with some probiotic potentials" at "6th AIST International Imaging Workshop & DAILAB PINNIKH series XXXII "held at Biomedical Research Institute, AIST, Tsukuba Science City, Japan supported by AIST & JST (Govt. of Japan) and DBT (Govt. of India) from January 20-27, 2019.

2) Attended conference on International Symposium on Biodiversity and Biobanking "Biodiverse 2018 from 27-29 January 2018, organized by IIT, Guwahati, Assam.

3) Participated in one-day workshop on 'Biosafety and Bioethics' held on 22.05.2015 at Conference Hall Vigyan Bhawan, Deorali, Gangtok, organized by Sikkim Biotech Hub, Sikkim State Council of Science and Technology, supported by Dept. of Biotechnology, Govt. of India, Delhi.

4) Participated in one-day workshop on 'Recent Advances in Biotechnology' held on 29th May 2015, organized by Biotech Hub, Sikkim Government College, Tadong, Gangtok.

5) Participated in one-day workshop on 'Food Fortification' held at Conference Hall, Barad Sadan organized by Sikkim University in collaboration with CII-FACE & GAIN on 19.08.2015.

Publications:

1) Shangpliang HNJ, Rai R, Keisam S, Jeyaram K *and* Tamang J P (2018). Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analyzed by high-throughput amplicon sequencing. *Scientific Reports*, DOI 10.1038/s41598-018-19524-6. (Impact Factor: 4.259).

2) Shangpliang HNJ, Sharma S, **Rai R** and Tamang J P (2017). Some technological properties of lactic acid bacteria isolated from *dahi* and *datshi*, naturally fermented milk products of Bhutan. *Frontiers in Microbiology* 8, DOI 10.3389/fmicb.2017.00116. *(Impact Factor: 4.076).*

3) **Rai R**, Shangpliang HNJ and Tamang J P (2016). Naturally fermented milk products of Eastern Himalayas. *Journal of Ethnic Foods* 3: pp 270-275. (Impact Factor: 2.080).

4) **Rai R**, Kharel N and Tamang J P (2016). HACCP model of *Kinema*, a fermented soybean food. *Journal of Scientific and Industrial Research*, 73, pp 588-592. (Impact Factor: 0.735).

LIST OF PUBLICATIONS

SCIENTIFIC REPORTS

OPEN

Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing

Received: 6 June 2017
Accepted: 4 January 2018
Published online: 24 January 2018

H. Nakibapher Jones Shangpliang[1], Ranjita Rai[1], Santosh Keisam[2], Kumaraswamy Jeyaram[2] & Jyoti Prakash Tamang[1]

Naturally fermented milk (NFM) products are popular ethnic fermented foods in Arunachal Pradesh and Sikkim states of India. The present study is the first to have documented the bacterial community in 54 samples of NFM products viz. *chhurpi*, *churkam*, *dahi* and *gheu/mar* by high-throughput Illumina amplicon sequencing. Metagenomic investigation showed that *Firmicutes* (*Streptococcaceae*, *Lactobacillaceae*) and *Proteobacteria* (*Acetobacteraceae*) were the two predominant members of the bacterial communities in these products. *Lactococcus lactis* and *Lactobacillus helveticus* were the predominant lactic acid bacteria while *Acetobacter* spp. and *Gluconobacter* spp. were the predominant acetic acid bacteria present in these products.

Naturally fermented milk (NFM) products are prepared by one of the oldest processes of milk fermentation in the world using raw or boiled milk to ferment spontaneously or by back-sloping method[1]. Some naturally fermented milk products are *chhu, chhurpi, dahi, lassi, misti dahi, mohi, philu, shoyu, somar* and *srikhand* (cow/buffalo/yak milk) of India, Nepal, Pakistan, Bhutan and Bangladesh[2–5], *kurut* of China[6], *aaruul, airag, byasulag, chigee, eezgii, khoormog* and *tarag* of Mongolia[7–9], *ergo* of Ethiopia, *kad, lben, laban, rayeb, zabady, zeer* of Morocco and Northern African and Middle East countries, *rob* (from camel milk), *biruni* (cow/camel milk), *mish* (cow/camel milk) of Sudan, *amasi* (*hodzeko, mukaka wakakora*) of Zimbabwe, *nunu* (from raw cow milk) of Ghana and *kule naoto* of Kenya[10,11], *filmjölk* and *långfil* of Sweden[12], *koumiss* or *kumis* or *kumys* or *kymys* of the Caucasian area[13]. Various cultivation-based studies reported lactic acid bacteria as the predominant microbiota present in the NFM products of the world mostly *Lactococcus lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis*, *Lactobacillus casei/Lb. paracasei, Lb. fermentum, Lb. helveticus*, *Lb. plantarum, Lb. acidophilus, Lb. coryniformis, Lb. curvatus*, *Lb. kefiranofaciens, Lb. kefiri, Lb. buchneri*, *Lb. jensenii, Lb. kitasatonis, Enterococcus faecium, E. faecalis* and *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, and others[11,14–19]. Besides bacteria, yeasts are also present in some NFM products which include *Candida lusitaniae, C. parapsilosis, C. rugosa, C. tropicalis, Kluyveromyces marxianus, Saccharomyces cerevisiae, Galactomyces geotrichum, Issatchenkia orientalis, Kazachstania unispora, Pichia mandshurica*, *P. fermentans, P. kudriavzevii*, and others[8,11,13,16,20–22].

High altitude (upto 4878 m)-naturally fermented milk products of cow (*Bos taurus*) or yak (*Bos grunniens*)-milk prepared by back-sloping are common in the Himalayan states of Arunachal Pradesh and Sikkim in India which include *chhurpi*, *churkam, dahi* and *gheu/mar* (Fig. 1a–f) as a protein-rich food supplement and also as a source of livelihood[5]. *Dahi*, similar to yogurt, is the first product of milk fermentation by back-sloping, and is consumed as savory non-alcoholic beverage. *Gheu/mar* (crude butter) is a fat-rich milk product obtained by a process of milk churning in which the casein-rich soft-variety product called *chhurpi* (cottage cheese-like)

[1]DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, 737102, India. [2]Microbial Resources Division, Institute of Bioresources and Sustainable Development (IBSD), Takyelpat Institutional Area, Imphal, 795 001, Manipur, India. Correspondence and requests for materials should be addressed to J.P.T. (email: jyoti_tamang@hotmail.com)

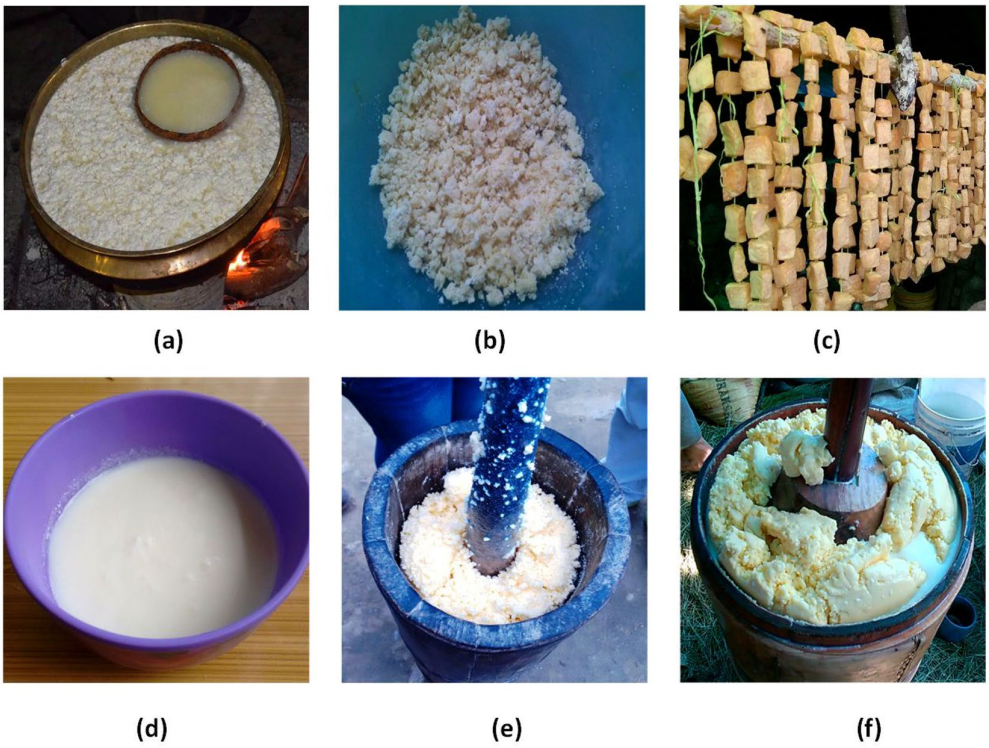


Figure 1. (**a**) *Chhurpi* of Arunachal Pradesh (AP); (**b**) *Chhurpi* of Sikkim; (**c**) *Churkam* of AP; (**d**) *Dahi* of Sikkim; (**e**) *Gheu* of Sikkim; (**f**) *Mar* of AP.

is produced, and is consumed as curry/soup in meals; and *churkam* (hard-variety of *chhurpi*) is the product of dehydrated *chhurpi*, which is used as masticatory as chewing gum in high altitudes. Lactic acid bacteria were predominant with the load of 10^8 cfu/g in the Himalayan fermented milk products[17]. *Lactobacillus bifermentans, Lb. alimentarius, Lb. paracasei* subsp. *pseudoplantarum, Lactococcus (Lc.) lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris*; *Lb. plantarum, Lb. curvatus, Lb. fermentum, Lb. kefir, Lb. hilgardii, Enterococcus faecium* and *Leuconostoc mesenteroides* were reported from *dahi* and *chhurpi* of Sikkim based on phenotypic, biochemical characterization and mol (%) content of G+C of DNA[14,17]. However, no study has been conducted yet on *churkam* and *gheu*/*mar*.

As it is well known that the cultivability of microbiota is still a limiting factor in understanding the natural food fermentation[23,24], application of high throughput metagenomic techniques like Illumina amplicon sequencing may serve to give more insight into microbial ecology of natural food fermentation. Metagenomic studies of various fermented milk products like kefir, buttermilk, cheeses etc have shown a realistic view of the microbial community structure involved in the natural milk fermentation[21,24–28]. In this study we aimed to anlayse the bacterial community structure of fifty-four samples of naturally fermented milk products (*chhurpi, churkam, dahi* and *gheu*/*mar*) of Arunachal Pradesh and Sikkim by Illumina amplicon sequencing. This is the first report on bacterial community in NFM products of the Himalayas using in-depth metagenomic analysis.

Results

Overall microbial community structure. The bacterial composition of the different naturally fermented milk products (*chhurpi, churkam, dahi* and *gheu*/*mar*) was compared at different taxonomic levels (Fig. 2a–c). The bacterial phyla present in four types of NFM products were *Firmicutes* and *Proteobacteria*, respectively (data not shown). Phylum *Firmicutes* was represented by six families belonging to *Streptococcaceae* (24.2%), *Lactobacillaceae* (16.8%), *Leuconostocaceae* (8.0%), *Staphylococcaceae* (6.8%), *Bacillaceae* (1.6%), and *Clostridiaceae* (1.3%); and phylum *Proteobacteria* included *Acetobacteraceae* (26.8%), *Pseudomonadaceae* (3.3%) and *Enterobacteriaceae* (1.2%) (Fig. 1a). The overall bacterial diversity of these NFM products were predominated by species belonging to the lactic acid bacteria: *Lactococcus lactis* (19.7%) and *Lactobacillus helveticus* (9.6%) and *Leuconostoc mesenteroides* (4.5%) (Fig. 2b,c). Additionally, species belonging to the acetic acid bacteria: *Acetobacter lovaniensis* (5.8%), *Acetobacter pasteurianus* (5.7%), *Gluconobacter oxydans* (5.3%), and *Acetobacter syzygii* (4.8%) were also observed (Fig. 2b,c). The percentage of *Enterobacteriaceae* was 1.2% (Fig. 2a), whereas the percentage of genus *Enterococcus* was below 0.5% (data not shown), hence it was not shown at the genus level (Fig. 2b). Percentage of *Streptococcus thermophilus* was below 0.1% (data not shown). The percentage of unclassified bacteria at the taxonomical levels was 7.9% (Fig. 2a–c). Presence of uncultured bacterium was shown in all samples (Fig. 2c).

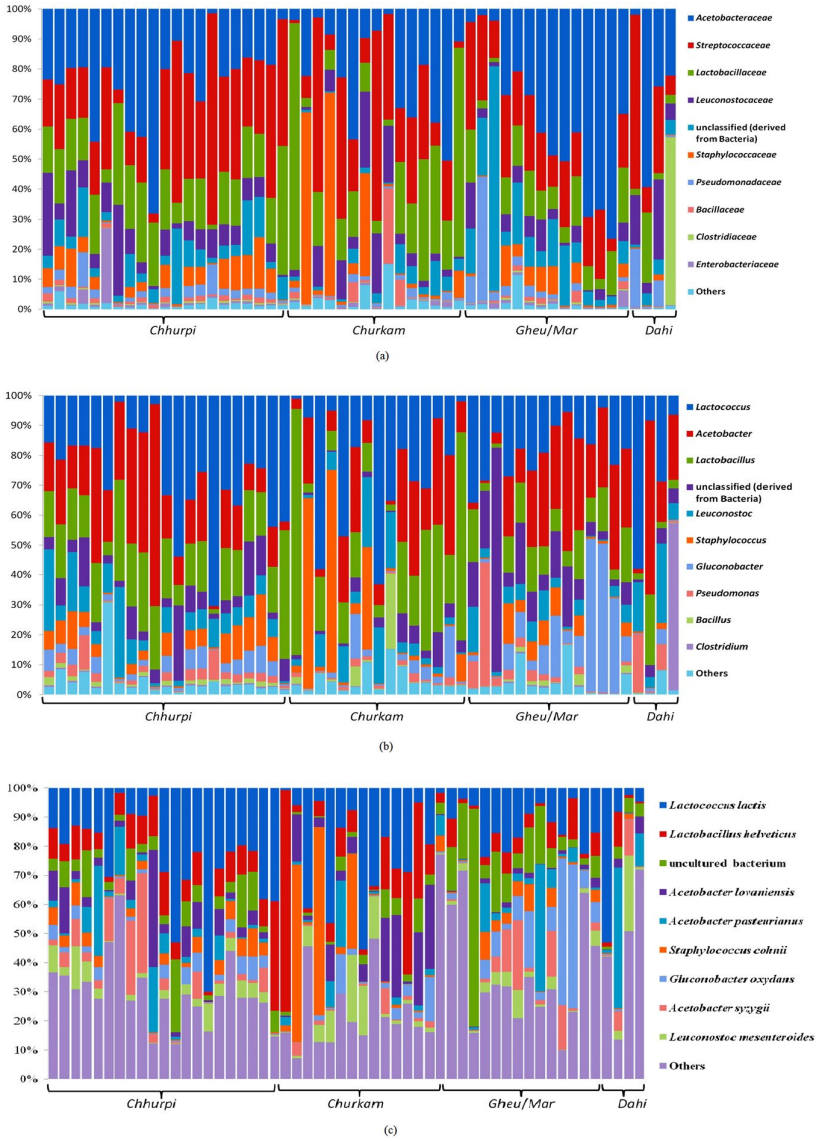


Figure 2. The overall bacterial composition of NFMs: *chhurpi*, *churkam*, *gheu*/*mar* and *dahi* at different taxonomic levels (**a**) Family, (**b**) Genus and (**c**) Species.

Multivariate analysis. PCA using species-level OTUs data showed significant differences among the NFM products studied (Fig. 3). The NFM products collected from two regions (Arunachal Pradesh and Sikkim) showed significant difference in the bacterial community structure (ANOSIM, $p = 0.005$, $R = 0.16$), but however, there was no significant difference between the same products prepared from different sources of milk (cow or yak). This reflects the regional contribution to the bacterial diversity of these products with respect to their location of preparation, but not from the milk source whereby these products are being prepared.

Alpha diversities. Alpha diversities were compared on the basis of states (Sikkim and Arunachal Pradesh)/places of collection of samples, animal's milk source (cow/yak) and product types (Table 1). There was no significant difference between the states/regions and animal's milk source, respectively. However, significance difference ($p = 0.0125$) was observed in terms of product types i.e., *chhurpi* and *churkam* in Chao1 species richness (Fig. 4). *Chhurpi* and *churkam* are two final products of milk fermentation where the latter is produced through a process of dehydration of the former and is usually kept for a longer fermentation. Multivariate analysis of species level OTUs showed a significant difference (ANOSIM $p = 0.002$, $R = 0.16$) between the two products. However, there is no significant difference among the general fermenting bacteria. Also, we observed a significant difference in *Clostridiaceae* ($p = 0.0004$) and *Pseudomonadaceae* ($p = 0.013$) between these two food types (Fig. 5).

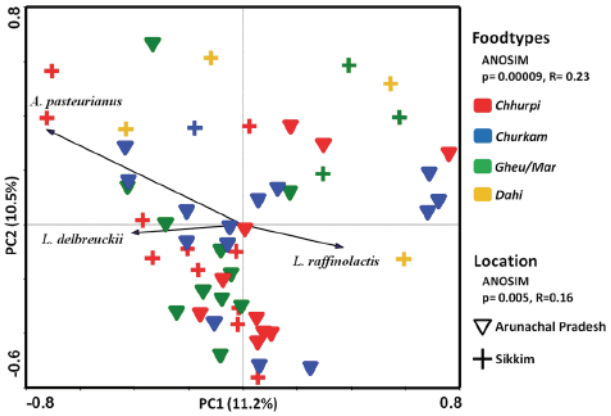


Figure 3. PCA plot shows the difference in bacterial community structure among the NFM products of Arunachal Pradesh and Sikkim. Arrow indicates the species direction. Significant difference is shown by ANOSIM analyzed with 10,000 permutations using Bray-Curtis distances.

Group1	Group 2	Group 1 mean	Group 1 std	Group 2 mean	Group 2 std	t stat	p-value
Chao1							
Chhurpi	*Dahi*	138.6654794	33.93332555	90.56944444	28.79901552	2.549699487	0.0152
Chhurpi	*Churkam*	138.6654794	33.93332555	108.6683546	26.9353883	2.695182315	0.0125
Dahi	*Gheu*	90.56944444	28.79901552	127.6180229	33.10848324	−1.91332029	0.0738
Chhurpi	*Gheu*	138.6654794	33.93332555	127.6180229	33.10848324	0.925146304	0.3583
Churkam	*Gheu*	108.6683546	26.9353883	127.6180229	33.10848324	−1.60079762	0.1171
Dahi	*Churkam*	90.56944444	28.79901552	108.6683546	26.9353883	−1.10004359	0.2864
Shannon							
Chhurpi	*Dahi*	3.639041175	0.736572535	2.657764997	0.378296426	2.493760723	0.0158
Chhurpi	*Churkam*	3.639041175	0.736572535	2.860086707	0.47435654	3.400743965	0.0022
Dahi	*Gheu*	2.657764997	0.378296426	3.339920996	0.823489314	−1.51693208	0.1459
Chhurpi	*Gheu*	3.639041175	0.736572535	3.339920996	0.823489314	1.089687949	0.2738
Churkam	*Gheu*	2.860086707	0.47435654	3.339920996	0.823489314	−1.82046908	0.0789
Dahi	*Churkam*	2.657764997	0.378296426	2.860086707	0.47435654	−0.73983568	0.4743

Table 1. Alpha diversity profiles of NFM products of India.

Discussion

In this study, bacterial diversity was explored by barcoded Illumina MiSeq amplicon sequencing of the 16 S rRNA gene (V4-V5 region). The applied method using high throughput sequencing detected *Lactococcus lactis, Lb. helveticus, Acetobacter lovaniensis, A. pasteurianus, A. syzygii, Gluconobacter oxydans* and *Leuconoctoc mesenteroides* (above 1%) in all 4 samples of NFM products. Reads of OTUs in present study could not detect *Lb. farciminis*, *Lb. biofermentans*, *Lb. hilgardi*, *Lb. paracasei* subsp. *pseudoplantarum*, *Lb. hilgardii*, *Lb. paracasei* subsp. *paracasei* which were reported earlier in *chhurpi* and *dahi* based on limited phenotypic characterization[14,17]. However, *Lb. helveticus* (9.6%) was detected in the present culture-independent method which was not reported in culture dependent method earlier. *Lb. helveticus* is known to be present in dairy products[29]. A major composition of *Lactococcus lactis* (*Streptococcaceae*) and *Lb. helveticus (Lactobacillaceae)* was found to be the most predominant species along with *Leuc. mesenteroides* (*Leuconostocaceae*) in the NFM products of India, which still form what are commonly known as the primary cultures in milk fermentation[1]. Metagenomics-based studies of other milk products around the world like kefir, cheeses, have also reported to harbour species of *Lactobacillus*, *Lactococcus* and *Leuconostoc*[25,26,30,31] as the dominant bacteria in general. Apart from the common known lactic acid bacteria group, a relatively high abundance of *Proteobacteria*-associated *Acetobacteraceae* (acetic acid bacteria) was observed in *gheu*/*mar* products. *Acetobacteraceae* members have also been reported in milk-related products[19,25,32,33], and their dominance in *gheu/mar* (churned before heating) products than the subsequent downstream products (*chhurpi* and *churkam*) may be due to the effect of heating during the processing steps. Even though the *Acetobacteraceae* members were still present in *chhurpi* and *churkam*, the abundance was generally low. During the fermentation of *chhurpi* and *churkam*, we observed an increase in the abundance of *Streptococcaceae* (*Lactococcus*) and subsequently a build-up in the *Lactobacillaceae* (*Lactobacillus*) population in *churkam*.

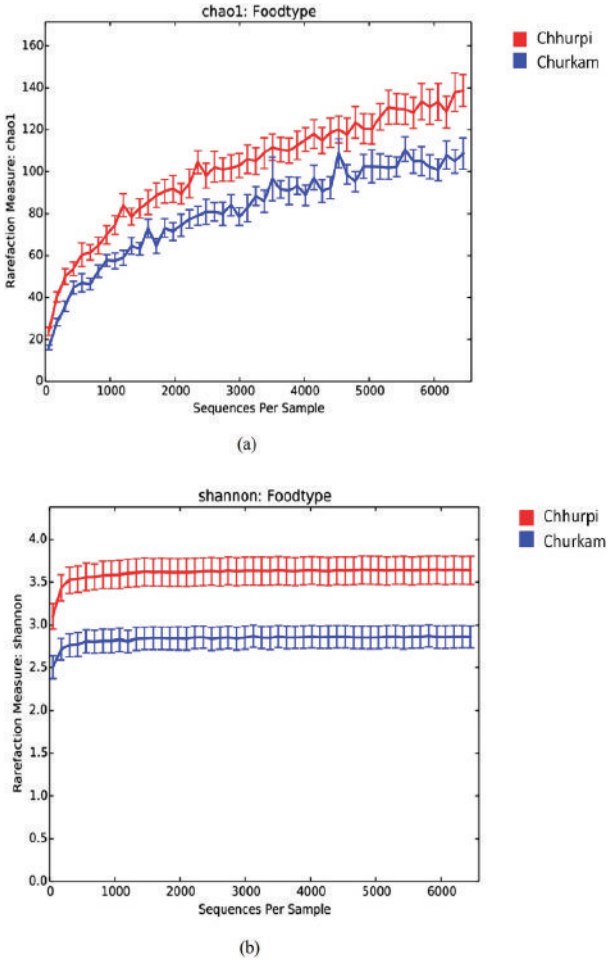


Figure 4. Difference in the bacterial alpha diversity indices of *chhurpi* and *churkam* (**a**) Chao1 species richness and (**b**) Shannon Diversity Index.

Based on OTUs system, the percentage of *Enterobacteriaceae* and genus *Enterococcus* was very low in NFM samples analyzed. *Enterococcus faecalis, Ent. faecium* along with *Lactococcus lactis* subsp. *lactis* were reported from *dahi* of Bhutan based on 16 S rRNA gene sequencing[6]. *Nunu*, African NFM product, is frequently contaminated with pathogenic *Enterobacteriaceae*, demonstrated by short-read-alignment-based bioinformatics tools which may be used for high-throughput food safety testing[34]. *Staphylococcaceae*, *Bacillaceae*, *Clostridiaceae* and *Pseudomonadaceae* were observed at relatively low level in this study probably as contaminants. *Pseudomonadaceae* (*Pseudomonas fluorescens)* is usually present in milk and milk products as sources of contaminants[35] and *Clostridiaceae* (*Clostridium tyrobutyricum*) is another bacterium found in cheese causing late blowing defect[36]. These contaminants were probably associated with the overall handling process, since samples are naturally fermented milk products, and there is no controlled process involved. Contamination of unwanted or rather non-fermenting bacteria are known to have acquired from various sources of production environment[37,38]. Presence of uncultured bacterium was shown in all samples analyzed. Uncultured bacterium group at species level were obtained using OTUs method, as the database could not assign them to any of their closest taxa. OTUs system put sequences into bins based on similarity of sequences within a data set to each other[39]. Moreover, limitations to using OTUs-based method is that the clustering algorithms are computationally intensive, relatively slow, and require significant amounts of memory[40].

However, the predominance of few species were observed in a particular product showing the remarkable diversity of microbiota among 4 analyzed samples of NFM products and subsequently a build-up in the Lactobacillaceae (*Lactobacillus*) population in *churkam*. *Lactococcus lactis* was predominant in *chhurpi*, *dahi* and *churkam*, whereas in *gheu/mar* samples, it was relatively less. *Lb. helveticus* was dominant in *churkam* comparable to other 3 NFM products. However, *Leuc. mesenteroides* was predominant in *dahi* samples. Though we observed a fairly equal distribution between *Lactococcus* and *Acetobacter* species in 4 NFM products, however, at species level *Lactococcus* was represented only by *Lc. lactis* whereas *Acetobacter* was represented by *A. lovaniensis*, *A. pasteurianus*, *A. syzygii* and *Gluconobacter oxydans*. Diversity in bacterial species among the 4 NFM products

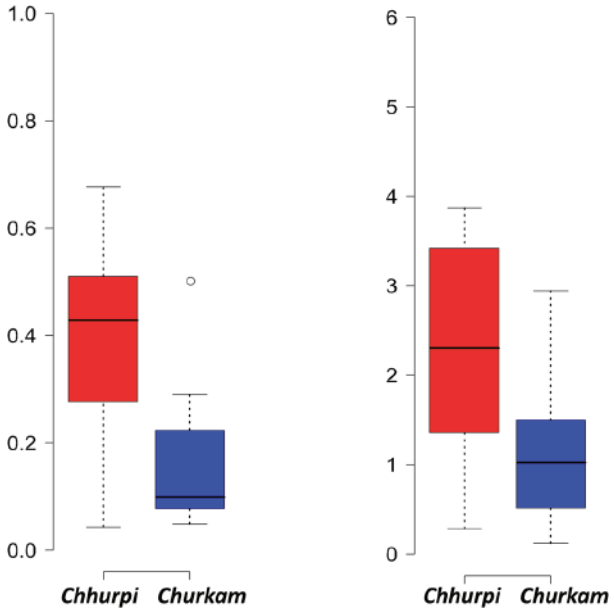


Figure 5. Boxplot showing the difference in the relative abundance of (**a**) *Clostridiaceae* and (**b**) *Pseudomonadaceae* between *chhurpi* and *churkam*.

was observed based on alpha diversity analysis. However, significance difference was observed only in between *chhurpi* and *dahi* ($p = 0.0152$) and *chhurpi* and *churkam* ($p = 0.0125$), respectively.

Conclusion

Earlier reports on *chhurpi* and *dahi* of North East India was based on limited culture-dependent analysis with some species of lactic acid bacteria. However, in the present study the NGS data of *chhurpi*, *churkam, dahi* and *gheu* showed the abundance of *Lactococcus lactis* (*Streptococcaceae*), *Lb. helveticus (Lactobacillaceae)* with *Leuc. mesenteroides* (*Leuconostocaceae*) as one the main bacterial species which may be the reliable information on microbial profile of NFM products. The application of NGS culture-independent methods to study the microbial ecology of fermented foods is of great significance in understanding the products, where Illumina sequencing has been shown to be one of the reliable tools in this study. Further studies on selective culturing of dominant bacteria, development of probiotic starter cultures and standardisation of processing methods may lead to industrialisation of ethnic food products.

Materials and Methods

Sampling. Fifty-four samples of naturally fermented milk products (*chhurpi*, *churkam dahi* and *gheu*/*mar*) were collected from high altitude mountains (1650–2587 meter) in Arunachal Pradesh ($n = 35$) and hills and mountains (381–4878 meter) in Sikkim ($n = 19$) of India (Table 2). The products were aseptically collected from the traditional production centres, transported in an ice-box and stored in the laboratory at −20 °C.

Metagenomic DNA extraction. Metagenomic DNA was extracted by two different methods based on the nature of the samples i.e., lipid-rich sample (*gheu/mar)* and casein-based samples (*dahi, chhurpi* and *churkam*). For the *gheu*/*mar* (lipid-rich) samples, extraction of DNA was performed as per method I as described in[48] with some modifications. This method was chosen on the basis of the product being rich in its fatty content. The usage of a combination of petroleum ether:hexane (1:1) serves the purpose of dissolving the fat content resolving the product into two phases after rigorous vortexing. Briefly, 2 mL of the sample melted in low temperature was homogenized with 2 ml citrate buffer (2%). To this, 4 ml of petroleum ether: hexane (1:1) was added followed by vortexing and 10 min incubation at room temperature. 2 mL of the lower part of the homogenate was transferred to a sterile 2 ml screw-cap tube containing 0.5 g of zirconia/silica beads (0.1 mm) and 4 glass beads (2 mm). The tubes were centrifuged and the pellet resuspended in 150 µl proteinase-K buffer [50 mM Tris-Cl, 10 mM EDTA (pH 8), 0.5% (w/v) SDS]. After overnight incubation at 65 °C with 25 µl proteinase K (25 mg/ml), it was treated with 150 µl of 2X breaking buffer [4% Triton X-100 (v/v), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris (pH 8), 2 mM EDTA (pH 8)]. After addition of phenol (pH 8.0), the samples were treated in a bead beater three times (30 sec beating, 10 sec in ice) and further purified with chloroform: isoamyl alcohol mixture (24:1). Lastly, DNA was precipitated with ethanol and the pellet is dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA).

For the casein-based samples (*dahi*, *chhurpi* and *churkam*), metagenomic DNA was extracted using the method of Keisam *et al.*[41]. This method was shown to recover maximum DNA yield from fermented milks[41], hence it was also applied in this study. Briefly, 10 g or 10 ml of the samples were mixed with 90 mL 2% sodium citrate buffer and homogenized in a stomacher at 200 rpm for 2 min. *Churkam* (hard-cheese) samples were first grinded into powder before the homogenization. 1.5 mL of the homogenate was transferred to a sterile centrifuge

Sample	Sample Code	Animal	State	Region/District	Location	Altitude (meter)	pH
Chhurpi	Ch1Cc	Cow	Arunachal Pradesh	Tawang	Cheghar	1705	5.32 ± 0.01
	Ch1Sc			Tawang	Samchin	1650	5.32 ± 0.02
	Ch1Tc			Tawang	Tawang	2587	5.33 ± 0.02
	Ch2Bc			West Kameng	Dirang	2095	5.35 ± 0.01
	Ch2Tc			Tawang	Tawang	2587	5.32 ± 0.01
	Ch6Bc			West Kameng	Bomdila	2339	5.33 ± 0.01
	SCCD		Sikkim	West Sikkim	Dentam	1500	6.05 ± 0.01
	SCCLG			South Sikkim	Lingee	1370	6.03 ± 0.02
	SCCNT			East Sikkim	Nimtar	619	5.89 ± 0.01
	SCCPK			East Sikkim	Pakyong	1120	6.03 ± 0.01
	SCCS			East Sikkim	Singtam	381	5.89 ± 0.01
	SCCTH			West Sikkim	Thingling	1780	5.89 ± 0.01
	SC1CYG			South Sikkim	Yangang	1370	6.11 ± 0.02
	Ch1By	Yak	Arunachal Pradesh	West Kameng	Dirang	2061	5.42 ± 0.02
	Ch3Ty			Tawang	Tawang	2587	5.35 ± 0.01
	Ch4Ty			Tawang	Tawang	2587	5.41 ± 0.01
	Ch5By			West Kameng	Bomdila	2340	5.42 ± 0.01
	SC1YYS		Sikkim	North Sikkim	Yumesamdong	4878	5.87 ± 0.03
	SC2YYS			North Sikkim	Yumesamdong	4878	5.88 ± 0.02
	SC3YYS			North Sikkim	Yumesamdong	4878	5.89 ± 0.01
	SC4YYS			North Sikkim	Yumesamdong	4878	5.90 ± 0.01
Churkam	Ck1Bc	Cow	Arunachal Pradesh	West Kameng	Bomdila	2339	5.71 ± 0.01
	Ck1Kc			Tawang	Kudung	1695	5.71 ± 0.01
	Ck1Sc			Tawang	Samchin	1650	5.72 ± 0.01
	Ck1Tc			Tawang	Tawang	2587	5.71 ± 0.01
	Ck2Bc			West Kameng	Bomdila	2339	5.72 ± 0.01
	Ck2Kc			Tawang	Kudung	1695	5.73 ± 0.01
	Ck2Sc			Tawang	Samchin	1650	5.72 ± 0.01
	Ck3Kc			Tawang	Kudung	1695	5.72 ± 0.01
	Ck3Sc			Tawang	Samchin	1650	5.72 ± 0.01
	Ck4Bc			West Kameng	Dirang	2095	5.74 ± 0.01
	Ck4Sc			Tawang	Samchin	1650	5.71 ± 0.01
	DCCLA		Sikkim	North Sikkim	Lachung	2700	6.34 ± 0.03
	Ck1Ty	Yak	Arunachal Pradesh	Tawang	Tawang	2587	5.82 ± 0.01
	Ck5By			West Kameng	Bomdila	2340	5.82 ± 0.01
	Ck6By			West Kameng	Bomdila	2340	5.87 ± 0.02
Gheu/Mar	Gh1Bc	Cow	Arunachal Pradesh	West Kameng	Dirang	2088	6.53 ± 0.02
	Gh3Kc			Tawang	Kudung	1695	6.52 ± 0.01
	Gh3Sc			Tawang	Samchin	1650	6.52 ± 0.01
	Gh4Cc			Tawang	Cheghar	1705	6.55 ± 0.01
	Gh5Bc			West Kameng	Dirang	2095	6.53 ± 0.01
	Gh5Tc			Tawang	Tawang	2587	6.55 ± 0.02
	Gh7Bc			West Kameng	Bomdila	2339	6.53 ± 0.01
	Gh2By	Yak		West Kameng	Bomdila	2339	6.62 ± 0.01
	Gh2Ty			Tawang	Tawang	2587	6.62 ± 0.01
	Gh4By			West Kameng	Dirang	2102	6.56 ± 0.02
	Gh6Ty			Tawang	Tawang	2587	6.61 ± 0.01
	GH1YYS		Sikkim	North Sikkim	Yumesamdong	4878	6.62 ± 0.01
	GH2YYS			North Sikkim	Yumesamdong	4878	6.63 ± 0.01
	GH3YYS			North Sikkim	Yumesamdong	4878	6.63 ± 0.01
Dahi	DHCLA	Cow	Sikkim	North Sikkim	Lachung	2700	4.14 ± 0.02
	DHCT			East Sikkim	Tadong	1649	4.23 ± 0.02
	DHCTH			West Sikkim	Thingling	1780	4.12 ± 0.02
	DHYYS	Yak		North Sikkim	Yumesamdong	4878	4.33 ± 0.02

Table 2. Sample details of the NFM products of India.

tube and centrifuge for 10 min at 18000 × g. To the pellet, 400 µl TES buffer [50 mM Tris, 1 mM EDTA, 8.7% sucrose] 50 KU lysozyme, 25 U mutanolysin and 20 U lyticase were added and incubated at 37 °C for 1 h. After incubation, proteinase-K (25 mg/mL) was added to the mixture and further incubated at 65 °C for 1 h, followed by addition of GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% sarkosyl). The sample was treated with 7.5 M ammonium acetate followed by purification with choloroform: isoamyl alcohol (24:1). Finally, DNA was precipitated with ethanol and the pellet dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA). In all cases, absence of contaminating DNA in the laboratory prepared reagents was confirmed by extracting DNA from sterile water and observing negative PCR amplification with universal bacterial primers. The quality ($A_{260/280}$) and quantity of the extracted DNA was checked using a spectrophotometer (NanoDrop ND-1000, USA). DNA was stored at −20 °C until required.

Barcoded Illumina MiSeq Sequencing. For in-depth bacterial community analysis, barcoded Illumina MiSeq amplicon sequencing targeting the V4-V5 region of the 16 S rRNA gene was conducted as described earlier[49]. The forward primer F563–577 (5′-AYTGGGYDTAAAGNG-3′) and barcoded reverse primers R924–907 (5′-CCGTCAATTCMTTTRAGT-3′) with an 8 bp barcode in its 5′-end was used for sample multiplexing[42]. Each PCR reaction was performed in a total volume of 25 µl with a template-free reaction that acts as a control. The following PCR conditions were used for amplification- initial denaturation (98 °C for 5 min); denaturation (98 °C for 15 sec), annealing (55 °C for 30 sec) and elongation (72 °C for 30 sec). The PCR reaction was run for 28 cycles with a final extension process of 72 °C for 5 min. The 430 bp sized products were separated in a 1.5% agarose gel (w/v) and the target bands were carefully excised from the gel with a sterile scalpel blade and then purified using QIAquick gel extraction kit (Qiagen, New Delhi, India) as per the manufacturer's instructions. The purified DNA was quantified with Qubit dsDNA BR Assay Kit (Invitrogen) in a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) and the individual were samples pooled in equimolar proportions. The final DNA pool was sent to the NGS facility in Xcelris Genomics (Ahmedabad, India) for paired-end MiSeq sequencing (2 × 300 bp). The raw sequence reads obtained was analysed using the default settings in MG-RAST[43] and an open-source bioinformatics pipeline QIIME v1.8.0[44]. A total of 7,614,683 post-quality filtered sequences originating from 54 samples belonging to 4 food types of NFM samples were uploaded to MG-RAST server with the MG-RAST ID number 4732361 to 4732414. The reads were subjected to secondary quality filtering to remove non-rRNA sequences before clustering into operational taxonomic units (OTUs) and subsequent generation of OTU tables at four different taxonomic levels (phylum, family, genus and species) using the SILVA SSU database in MG-RAST. Eukaryota-specific and unassigned OTUs were removed before performing further analysis.

Statistical Analysis. Normalisation of the OTUs relative abundance data was performed by log transformation $\log_{10}(x_i + 1)$. To understand the variation in the microbial community structure of different food types, PCA was plotted using Canoco software v4.52 (Wageningen University, The Netherlands). Significant difference in the bacterial community structure amongst the four food type was evaluated by ANOSIM with 10,000 permutations using Bray-Curtis similarity index in PAST v2.17. Any significant difference in the abundance of individual taxa at four different taxonomic levels between the four food types was tested by p-value calculation using Student's twov-tailed paired t-test and ANOVA. p-value < 0.05 was considered statistically significant and the differences in taxon abundance were represented as boxplots using BoxPlotR[45,46]. Species level-OTUs table was rarefied at a depth of 50 to 6482 sequences using the multiple_rarefactions.py script in QIIME for generation of alpha diversities rarefaction curves. Rarefaction plots were generated for Chao1 richness, diversity indices (Fisher alpha, Shannon), Shannon's equitability and Good's coverage using the make_rarefaction_plots.py script[44]. Significant differences in the alpha indices amongst the food types were calculated using the script compare_alpha_diversity.py in QIIME.

Data availability. Sequence data associated with this present work have been uploaded to MG-RAST server with the MG-RAST ID number 4732361 to 4732414.

References

1. Tamang, J. P., Watanabe, K. & Holzapfel, W. H. Review: Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* **7**, 377, https://doi.org/10.3389/fmicb.2016.00377 (2016).
2. Harun-ur-Rashid, M., Togo, K., Useda, M. & Miyamoto, T. Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk "Dahi" in Bangladesh. *Pakistan J. Nutr.* **6**, 647–652 (2007).
3. Sarkar, S. Innovations in Indian fermented milk products-a review. *Food Biotechnol.* **22**, 78–97 (2008).
4. Tamang, J. P. *Himalayan fermented foods: microbiology, nutrition and ethnic value. CRC Press*. (Taylor and Francis Group, New York, 2010).
5. Rai, R., Shangpliang, H. N. J. & Tamang, J. P. Naturally fermented milk products of the Eastern Himalayas. *J. Ethn. Foods.* **3**, 270–5 (2016).
6. Shangpliang, H. N. J., Sharma, S., Rai, R., Tamang J. P. Some technological properties of lactic acid bacteria isolated from Dahi and Datshi, naturally fermented milk products of Bhutan. *Front. Microbiol.* **8**, https://doi.org/10.3389/fmicb.2017.00116 (2017).
7. Sun, Z. *et al*. Identification and characterization of the dominant lactic acid bacteria from *kurut*: the naturally fermented yak milk in Qinghai, China. *J. Gen. Appl. Microbiol.* **56**, 1–10 (2010).
8. Watanabe, K. *et al*. Diversity of lactic acid bacteria and yeasts in airag and tarag, traditional fermented milk products from Mongolia. *World J. Microbiol. Biotechnol.* **24**, 1313–1325 (2008).
9. Takeda, S. *et al*. The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. *Animal Sci. J.* **82**, 571–579 (2011).
10. Mathara, J. M. *et al*. Isolation, identification and characterization of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *Int. J. Food Microbiol.* **94**, 269–278 (2004).
11. Akabanda, F. *et al*. Taxonomic and molecular characterization of lactic acid bacteria and yeasts in *nunu*, a Ghanaian fermented milk product. *Food Microbiol.* **34**, 277–283 (2013).
12. Mayo, B., Ammor, M. S., Delgado, S., Alegría, A. Fermented milk products. In: Tamang, J. P. & Kailasapathy, K. (Eds) *Fermented foods and beverages of the World*, CRC Press, Taylor & Francis Group, New York. pp. 263–288 (2010).

13. Wu, R. *et al*. Isolation and preliminary probiotic selection of lactobacilli from Koumiss in Inner Mongolia. *J. Basic Microbiol.* **49**, 318–326 (2009).
14. Tamang, J. P. *et al*. Identification and enzymatic profiles of the predominant lactic acid bacteria isolated from soft-variety Chhurpi, a traditional cheese typical of the Sikkim Himalayas. *Food Biotechnol.* **14**, 99–112 (2000).
15. Mathara, J. M. *et al*. Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. *Int. J. Food Microbiol.* **126**, 57–64 (2008).
16. Dewan, S. & Tamang, J. P. Microbial and analytical characterization of Chhu - A traditional fermented milk product of the Sikkim Himalayas. *J. Sci. Ind. Res.* **65**, 747–52 (2006).
17. Dewan, S. & Tamang, J. P. Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. *Antonie Van Leeuwenhoek.* **92**, 343–52 (2007).
18. Yu, J. *et al*. Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. *J. Dairy Sci.* **94**, 3229–3241 (2011).
19. Oki, K., Dugersuren, J., Demberel, S. & Watanabe, K. Pyrosequencing analysis on the microbial diversity in Airag, Khoormog and Tarag, traditional fermented dairy products of Mongolia. *Bio. Microbiota Food Health* **33**, 53–64 (2014).
20. Gadaga, T. H., Mutukumira, A. N. & Narvhus, J. A. Enumeration and identification of yeast isolates from Zimbabwean traditional fermented milk. *Int. Dairy J.* **10**, 459–466 (2000).
21. Liu, W. *et al*. High-throughput sequencing for the detection of the bacterial and fungal diversity in Mongolian naturally fermented cow's milk in Russia. *BMC Microbiol.* **15**, 45 (2015).
22. Qviris, L. A. *et al*. Isolation, identification and characterization of yeasts from fermented goat milk of the Yaghnob valley in Tajikistan. *Front. Microbiol.* **7**, https://doi.org/10.3389/fmicb.2016.01690 (2016).
23. Oliver, J. D. The viable but nonculturable state in bacteria. *J Microbiol* **43**(1), 93–100 (2005).
24. Delbès, C., Ali-Mandjee, L. & Montel, M. C. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. *Appl. Environ. Microbiol.* **73**, 1882–91 (2007).
25. Dobson, A., O'Sullivan, O., Cotter, P. D., Ross, P. & Hill, C. High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiol. Lett.* **320**, 56–62 (2011).
26. Alegría, Á., Szczesny, P., Mayo, B., Bardowski, J. & Kowalczyk, M. Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and -independent approaches. *Appl. Environ. Microbiol.* **78**, 1890–8 (2012).
27. Quigley, L. *et al*. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Appl. Environ. Microbiol.* **78**, 5717–23 (2012).
28. Jayashree, S., Pushpanathan, M., Rajendhran, J. & Gunasekaran, P. Microbial diversity and phylogeny analysis of buttermilk, a fermented milk product, employing 16S rRNA-based pyrosequencing. *Food Biotechnol.* **27**, 213–21 (2013).
29. Moser, A., Berthoud, H., Eugster, E., Meile, L. & Irmler, S. Detection and enumeration of *Lactobacillus helveticus* in dairy products. *Int. Dairy J.* **68**, 52–9 (2016).
30. Marsh, A. J., O'Sullivan, O., Hill, C., Ross, R. P. & Cotter, P. D. Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. *PLoS One.* **8**, 7 (2013).
31. Planý, M. *et al*. Metagenomic analysis of Slocak Bryndza cheese using next-generation 16S rDNA amplicon sequencing. *Nova Biotechnol. Chimica.* **15**(1), 23–34 (2016).
32. Liu, W. J. *et al*. A survey of the bacterial composition of kurut from Tibet using a culture-independent approach. *J. Dairy Sci.* **95**, 1064–72 (2012).
33. Leite, A. M. O. *et al*. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiol.* **31**, 215–21 (2012).
34. Walsh, A. M. *et al*. Strain-level metagenomic analysis of the fermented dairy beverage nunu highlights potential food safety risks. *Appl. Environ. Microbiol*. https://doi.org/10.1128/AEM.01144-17 (2017).
35. Wiedmann, M., Weilmeier, D., Dineen, S. S., Ralyea, R. & Boor, K. J. Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Appl. Environ. Microbiol.* **66**(5), 2085–2095 (2000).
36. Panelli, S., Brambati, E., Bonacina, C., Feligini, M. Detection of *Clostridium tyrobutyricum* in milk to prevent late blowing in cheese by automated ribosomal intergenic spacer analysis. *J. Food Sci.* **78** (2013).
37. Doyle, C. J., Gleeson, D., O'Toole, P. W., Cotter, P. D. High-throughput sequencing highlights the significant influence of seasonal housing and teat preparation on the raw milk microbiota. *Appl. Environ. Microbiol*. https://doi.org/10.1128/AEM.02694-16 (2016).
38. Ssajjakambwe, P. *et al*. Milk hygiene in rural southwestern Uganda: prevalence of mastitis and antimicrobial resistance profiles of bacterial contaminants of milk and milk products. *Vet. Med. Int*. 6, https://doi.org/10.1155/2017/8710758 (2017).
39. Schloss, P. D. & Westcott, S. L. Assessing and improving methods used in Operational Taxonomic Unit-based approaches for 16S rRNA gene sequence analysis. *Appl. Environ. Microbiol.* **77**(10), 3219–3226 (2011).
40. Schloss, P. D. *et al*. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537–7541 (2009).
41. Keisam, S., Romi, W., Ahmed, G. & Jeyaram, K. Quantifying the biases in metagenome mining for realistic assessment of microbial ecology of naturally fermented foods. *Sci. Rep.* **6**, 34155 (2016).
42. Romi, W., Ahmed, G. & Jeyaram, K. Three-phase succession of autochthonous lactic acid bacteria to reach a stable ecosystem within 7 days of natural bamboo shoot fermentation as revealed by different molecular approaches. *Mol. Ecol.* **24**, 3372–89 (2015).
43. Meyer, F. *et al*. The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics.* **9**, 386 (2008).
44. Caporaso, J. G. *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* **7**, 335–6 (2010).
45. Spitzer, M., Wildenhain, J., Rappsilber, J. & Tyers, M. BoxPlotR: a web tool for generation of box plots. *Nat. Methods.* **11**, 121–2 (2014).
46. Ruggirello, M., Dolci, P. & Cocolin, L. Detection and viability of *Lactococcus lactis* throughout cheese ripening. *PLoS One.* **9**, 1–14 (2014).

Acknowledgements

Authors are grateful to Science and Engineering Research Board-Department of Science and Technology, New Delhi for research project (Sanction order: SB/EMEQ-367/2014, dated: 21.07.2014), DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre of DBT and Microbial Resources Division, Institute of Bioresources and Sustainable Development (IBSD), Imphal, Manipur.

Author Contributions

H.N.J.S. and R.R. contributed to this present work equally as first co-authors which is a part of their research work. S.K. helps and assists in all the molecular work and N.G.S. (Bioinformatics and statistical) analyses. K.J. and J.P.T. have framed this research paper along with all the authors involved. All authors critically revised, read and approved the final manuscript with final check by J.P.T.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 February 2017
doi: 10.3389/fmicb.2017.00116

Some Technological Properties of Lactic Acid Bacteria Isolated from *Dahi* and *Datshi*, Naturally Fermented Milk Products of Bhutan

*H. N. J. Shangpliang, Sharmila Sharma, Ranjita Rai and Jyoti P. Tamang**

Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, India

Dahi and *datshi* are common naturally fermented milk (NFM) products of Bhutan. Population of lactic acid bacteria (LAB) in *dahi* (pH 3.7) and *datshi* (pH 5.2) was 1.4×10^7 and 3.9×10^8 cfu/ml, respectively. Based on 16S rRNA gene sequencing isolates of LAB from *dahi* and *datshi* were identified as *Enterococcus faecalis, E. faecium, Lactococcus lactis* subsp. *lactis.* LAB strains were tested for some technological properties. All LAB strains except *E. faecalis* CH2:17 caused coagulation of milk at both 30°C for 48 h. Only *E. faecium* DH4:05 strain was resistant to pH 3. No significant difference ($P > 0.05$) of viable counts was observed in MRS broth with and without lysozyme. All LAB strains grew well in 0.3% bile showing their ability to tolerate bile salt. None of the LAB strains showed >70% hydrophobicity. This study, being the first of its microbiological analysis of the NFM of Bhutan, has opened up to an extent of research work that gives a new insight to the products.

Keywords: technological properties, lactic acid bacteria, *dahi, datshi*, naturally fermented milk products

OPEN ACCESS

Edited by:
Andrea Gomez-Zavaglia, Center for Research and Development in Food Cryotechnology (CONICET), Argentina

Reviewed by:
Bernadette Dora Gombossy De Melo Franco, University of São Paulo, Brazil
Prakash M. Halami, Central Food Technological Research Institute (CSIR), India

***Correspondence:**
Jyoti P. Tamang
jyoti_tamang@hotmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *29 June 2016*
Accepted: *17 January 2017*
Published: *01 February 2017*

Citation:
Shangpliang HNJ, Sharma S, Rai R and Tamang JP (2017) Some Technological Properties of Lactic Acid Bacteria Isolated from Dahi and Datshi, Naturally Fermented Milk Products of Bhutan. Front. Microbiol. 8:116. doi: 10.3389/fmicb.2017.00116

INTRODUCTION

Naturally fermented milk (NFM) products are prepared by the practice of one of the oldest techniques of milk fermentation known as the 'back-sloping' method in which a previous batch of a fermented product is used to inoculate the new batch (Josephsen and Jespersen, 2004; Tamang et al., 2016b). NFM products are prepared and consumed daily in Bhutan. Some NFM products of Bhutan are *dahi*, *datshi*, *mohi*, *gheu*, hard-*chhurpi* (*chugo*/*churkam*) and *hitpa*. *Dahi* (**Figure 1A**) is a yogurt-like NFM product of Bhutan, which is traditionally prepared by allowing the boiled milk to undergo spontaneous fermentation at room temperature for 2–3 days with the inoculation of the previous *dahi* sample. *Dahi* is drunk as a refreshing non-alcoholic beverage in Bhutan. *Datshi* (**Figure 1B**) is a cottage cheese like product, which is prepared by churning *dahi* for 10–15 min until a clumping product; butter (locally called *gheu*) is extracted. The butter is collected in another vessel and the buttermilk, locally called *mohi* is then heated for 15–20 min for the curdling of the product, called *datshi,* which is made into round small balls. It is consumed as curry in main meals in Bhutan. Most of these NFM products are occasionally used for religious ceremonies in Bhutan. Some people are economically dependent upon these NFM products where they sell at local markets. Some NFM products of other countries were well studied such as *dahi, misti dahi, shrikhand, chhu, chhurpi, philu* and *somar* of India, Nepal, Pakistan, and Bangladesh (Tamang et al., 2000; Dewan and Tamang, 2006, 2007; Harun-ur-Rashid et al., 2007; Sarkar, 2008; Patil et al., 2010; Tamang, 2010), *kurut* of China (Sun et al., 2010), *aaruul, airag, byasulag, chigee, tarag,* and *khoormog* of Mongolia (Watanabe et al., 2008; Takeda et al., 2011; Oki et al., 2014), *ergo* of Ethiopia, *lben, rayeb, zabady,* and *zeer* of Morocco and Northern African and Middle East

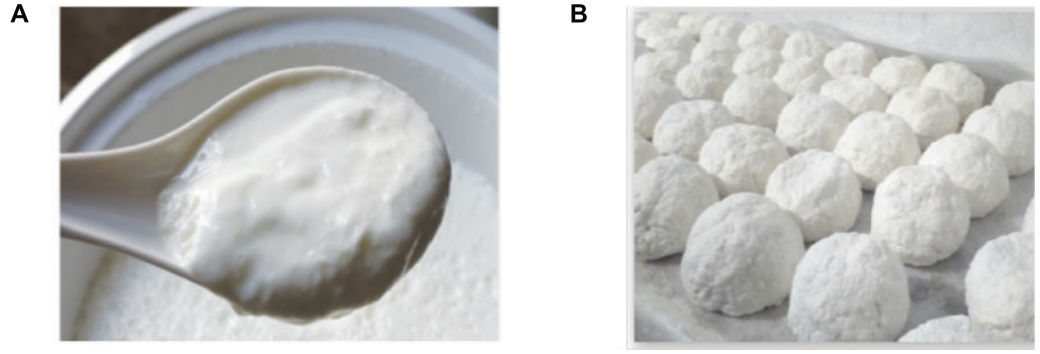


FIGURE 1 | (A) *Dahi* and **(B)** *datshi*.

countries, *rob* (from camel milk), *biruni*, *mish* (cow/camel milk) of Sudan, *amasi* (*hodzeko, mukaka wakakora*) of Zimbabwe, *nunu* of Ghana (Akabanda et al., 2013), *filmjölk* and *långfil* of Sweden (Mayo et al., 2010), and *koumiss* or *kumis* or *kumys* or *kymys* of the Caucasian area (Wu et al., 2009). Among species of lactic acid bacteria (LAB), *Lactococcus lactis* subsp. *cremoris,* and *Lc. lactis* subsp. *lactis* are the dominant microbiota along with other mesophilic lactobacilli (*Lactobacillus casei/Lb. paracasei, Lb. fermentum, Lb. helveticus*, *Lb. plantarum,* and/or *Lb. acidophilus*), *Enterococcus faecium,* species of *Leuconostoc* and *Pediococcus* in NFMs (Tamang et al., 2000, 2016b; Mathara et al., 2004; Dewan and Tamang, 2006, 2007; Patrignani et al., 2006; Watanabe et al., 2008; Wu et al., 2009; Hao et al., 2010; Yu et al., 2011; Akabanda et al., 2013; Oki et al., 2014). Technological properties including probiotics characters have been extensively studied in some NFM products of the world (Patrignani et al., 2006; Dewan and Tamang, 2007; Harun-ur-Rashid et al., 2007; Wu et al., 2009; Tamang et al., 2016a). Till date, there has been no report on the microbiological analysis and technological properties of the NFM from Bhutan, making this research the first of this kind. This paper is aimed to determine some technological properties of the LAB isolates from two popular NFM products of Bhutan- *dahi* and *datshi* such as acidification and coagulation, resistance to low pH, tolerance against bile, lysozyme tolerance and hydrophobicity assay, and also to isolate and identify LAB species by 16S rRNA sequencing.

MATERIALS AND METHODS

Samples

A total number of eight fresh samples of *dahi* (4) and *datshi* (4) were collected from Tabthangbu village, Bhutan in pre-sterilized sampling bags and were transported to the laboratory in an icebox carrier, stored at 4°C and analyzed within a week.

Microbiological Analysis

Samples (10 ml) were homogenized with sterile physiological saline (90 ml) in a stomacher lab-blender (400, Seward, London, UK) for 1 min, and were serially diluted in the same diluent. LAB were enumerated on MRS agar (M641, HiMedia, Mumbai, India) plates under anaerobic conditions in an anaerobic gas-pack system (LE002, HiMedia, Mumbai, India) and incubated at 30°C for 48–72 h (Dewan and Tamang, 2007). Colonies were selected randomly from the plates which contained less than 10 colonies, according to Leisner et al. (1997). Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media, followed by microscopic examinations. LAB isolates were preserved at −20°C in MRS broth (M369, HiMedia, Mumbai, India) mixed with 20% (v/v) glycerol.

Determination of pH

The pH of samples was determined using a pH meter (Crison basic 20, Barcelona, Spain) calibrated with standard buffers.

Phenotypic Characterization

Cell morphology of all isolates and their motility was determined using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties including sugar fermentations, following the methods of Schillinger and Lücke (1987) and Dykes et al. (1994).

Molecular Identification

DNA Extraction

Based on similar sugar fermentation and other phenotypic characteristics criteria, six representative strains of LAB were randomly selected from 44 strains of LAB. Total genomic DNA of six representative strains of LAB was extracted from 2-ml samples of overnight cultures grown in MRS broth at 30°C according to the methods of Martín-Platero et al. (2007). DNA was quantified using fluorometer (Qubitol® 3.0, Fisher Scientific, USA).

16S rRNA Gene Sequencing

The 16S rRNA gene was amplified by PCR mixtures (25 μL) contained approximately 30–50 ng template DNA, 1 μM forward primer 27F and 1 μM reverse primer 1492R (Lane, 1991)

TABLE 1 | Phenotypic characteristics of the lactic acid bacteria (LAB) isolated from *dahi* and *datshi* of Bhutan.

Representative Isolates (no. of grouped strains)	Growth at 45°C	Sugar fermentation										Tentative genera
		Arabinose	Fructose	Galactose	Melibiose	Ribose	Xylose	Raffinose	Aesculin	Melezitose	Salicin	Rhammnose	
*DH4:05 (12)	10/2	7/5	+	+	+	9/3	–	+	+	–	–	–	*Enterococcus*
**CH1:14 (3)	+	+	+	+	+	2/1	2/1	+	+	+	+	–	*Enterococcus*
CH2:02 (10)	9/1	+	+	+	–	–	–	+	6/4	5/5	+	–	*Enterococcus*
CH2:17 (4)	2/2	+	–	+	3/1	–	+	2/2	+	+	+	–	*Enterococcus*
CH3:03 (7)	+	+	+	+	3/4	+	+	6/1	+	–	+	+	*Enterococcus*
CH4:01 (8)	6/2	+	–	+	4/4	–	–	–	+	–	–	+	*Lactococcus*

**DH, denotes isolates from dahi samples; **CH, denotes isolates from datshi samples. All strains were Gram-positive, catalase negative, coccoids, non-motile and non-sporing; +, all strains positive; -, all strains negative; (../..), number of positive/negative strains. All strains grew at 10 and 15°C. All strains fermented cellobiose, mannose and maltose.*

using a PCR Master Mix (Promega, Canada) performed under the standard PCR amplification procedure in a SimpliAmp^{TM} Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR amplicons were checked for their purity on 1% agarose gel electrophoresis in the presence of ethidium bromide (10 mg/mL), which was later analyzed by the Gel Doc System (Ultra-Violet Products Ltd, UK). Sequencing service was outsourced.

Phylogenetic Analysis

The BLAST (Basic Phylogenetic Local Alignment Search Tool) program was used for comparing DNA databases for sequence similarities available in the NCBI database. Five different strains/species from each BLAST results were chosen for phylogenetic analysis using Molecular Evolutionary genetics Analysis software (MEGA version 6).

Technological Properties

Activation of LAB Strains

Enterococcus faecalis CH1:14, *E. faecalis* CH2:02, *E. faecalis* CH2:17, *E. durans* CH3:03, *Lactococcus lactis* subsp. *cremoris* CH4:01 and *E. faecium* DH4:05, isolated from *dahi* and *datshi*, were grown in MRS broth for 16-24 h at 30°C, and were used for determinations of acidification and coagulation, tolerance against bile, and lysozyme tolerance. Activation of LAB strains for resistance to pH 3 and hydrophobicity were mentioned below.

Acidification and Coagulation

Acidification and coagulation ability of LAB strains were assayed by inoculating 10% skim milk (RM1254, HiMedia, Mumbai, India) at 1% level and incubated at 30°C for 72 h. Observation was made for commencement of clotting, followed by pH measurement (Olasupo et al., 2001).

Tolerance against Bile

MRS broth containing 0.3% bile was inoculated with active cultures for 4 h (Prasad et al., 1998) and viable cells were enumerated in MRS agar plates after 24 h incubation and growth was recorded.

Lysozyme Tolerance

10 mL of MRS broth with lysozyme (MB098-1G, HiMedia, India) and without lysozyme, respectively, was inoculated with 1 mL of both culture suspensions of 10^8 cfu/ml cell concentration and incubated at 30°C for 24 h and viable cells were enumerated in MRS agar plates after 24 h incubation (Brennan et al., 1986).

Resistance to Low pH

Active cultures were harvested by centrifugation and pellets were washed once in phosphate-saline buffer (PBS, pH 7.2), re-suspended in PBS (pH 3) and incubated in MRS agar plates at 30°C for 24 h, and growth was recorded (Prasad et al., 1998).

Hydrophobicity Assay

Bacterial affinity to hydrocarbons was determined and results were expressed according to Perez et al. (1998), modified by Tamang et al. (2009) as follows. Fresh cultures were grown in MRS broth at 30°C for 24 h and centrifuged at 8,000 *g* for

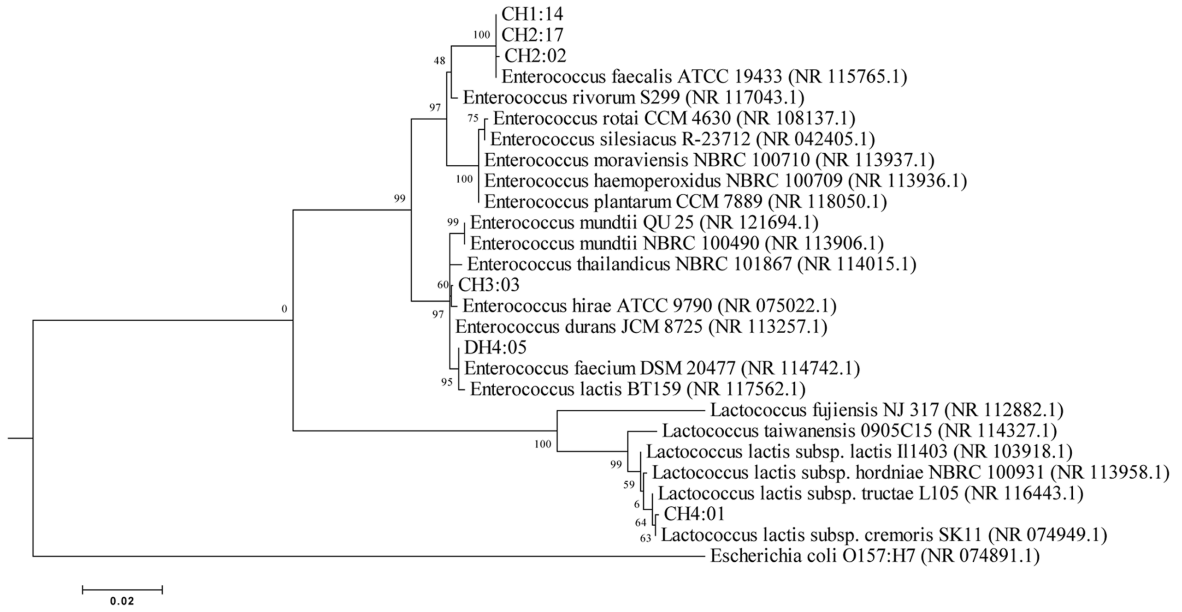


FIGURE 2 | Phylogenetic tree based upon the Neighbor-Joining of 16S rDNA sequences (*E. coli* 84 to 1437) derived by PCR with the primer 27F and 1492R.

TABLE 2 | Identification table based on NCBI-BLAST.

Isolates	Length (bp)	Max Score	Query coverage (%)	*E*-value	% Identification	Closest Known Relative (Strain No., GenBank Accession No.)
CH1:14	1406	2591	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH2:02	1370	2525	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH2:17	1386	2556	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH3:03	1384	2536	99	0.0	99	*Enterococcus durans* (JCM 8725, NR 113257.1)
CH4:01	1361	2508	100	0.0	99	*Lactococcus lactis* subsp. *cremoris* (SK11, NR 074949.1)
DH4:05	1378	2542	100	0.0	99	*Enterococcus faecium* (DSM 20477, NR 114742.1)

TABLE 3 | Technological properties of the LAB isolates from *dahi* and *datshi* of Bhutan.

Isolates	pH at Commencement of clotting	Coagulation (hours)		Resistance to pH 3	[a]Lysozyme tolerance	[b]Bile tolerance	(%) Hydrophobicity
		24	48				
E. faecium DH4:05	5.54	-	+	+	+	+	17.53
E. faecium CH1:14	5.24	-	+	-	+	+	56.58
E. faecalis CH2:02	5.52	-	+	-	+	+	8.91
E. faecalis CH2:17	5.50	-	-	-	+	+	5.99
E. faecium CH3:03	5.00	+	+	-	+	+	1.3
Lc. lactis subsp. *lactis* CH4:01	4.70	+	+	-	+	+	3.02

Data represent an average of three sets of experiments. +, indicates growth (>10^6 cfu/ml) of LAB strains; [a]no significant difference ($P > 0.05$) of viable LAB counts in MRS broth with and without lysozyme after incubation (30°C/24 h) was considered as a strain resistant to lysozyme.; [b]MRS broth with 0.3% bile.

5 min. The pellet was washed with 9 ml of Ringer solution (Merck, Germany) and thoroughly mixed. Suspension (1 ml) was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai, India) in duplicates and mixed thoroughly. Phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully transferred to a new tube and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows:

$$\text{hydrophobicity } \% = \left[A_0 - A/A\right] \times 100,$$

where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane.

RESULTS AND DISCUSSION

Dahi and *datshi* are acidic fermented milk products showing an average pH of 3.7 ± 0.17 and 5.2 ± 0.12, respectively. Isolation of LAB was performed on the classical media i.e., *Lactobacillus* MRS Agar media under anaerobic conditions at 30°C incubation for 48 h. The microbial load of LAB in *dahi* was 1.4×10^7 cfu/ml and in *datshi* was 3.9×10^8 cfu/mL, respectively. A total of 44 LAB isolates were isolated from *dahi* and *datshi* and phenotypically characterized and were randomly grouped into six representative strains based on similar sugar fermentation and other phenotypic characteristics (**Table 1**). These isolates were tentatively identified as *Enterococcus* and *Lactococcus* (**Table 1**).

Total genomic DNA of 6 representative strains of LAB was extracted and amplified and were identified by partial 16S rRNA gene sequencing which were compared to the NCBI database for their phylogenetic relationship by using the software MEGA 6 (**Figure 2**). On the basis of molecular identification, the following species of LAB were identified from *dahi* and *datshi* of Bhutan with percentage similarity of LAB: *E. faecalis* CH1:14 (99%), *E. faecalis* CH2:02 (99%), *E. faecalis* CH2:17 (99%), *E. durans* CH3:03 (99%), *Lactococcus lactis* subsp. *cremoris* CH4:01 (99%), and *E. faecium* DH4:05 (99%; **Table 2**).

Lactococcus lactis subsp. *lactis, Lc. lactis* subsp. *cremoris, E. faecium, E. faecalis, Leuconostoc mesenteroides* and *Pediococcus* and lactobacilli (*Lactobacillus casei, Lb. fermentum, Lb. helveticus*, *Lb. plantarum,* and/or *Lb. acidophilus*), were reported from many NFM products of different countries (Tamang et al., 2000; Mathara et al., 2004; Dewan and Tamang, 2006, 2007; Patrignani et al., 2006; Watanabe et al., 2008; Wu et al., 2009; Hao et al., 2010; Yu et al., 2011; Akabanda et al., 2013).

Lactic acid bacteria strains were tested for some technological properties (**Table 3**). All LAB strains except *E. faecalis* CH2:17 caused coagulation of milk at both 30°C for 48 h with a significant drop in pH (**Table 3**). Coagulation of milk by LAB strains reveals their potential as starters or adjunct cultures in the production of NFM of Bhutan. Only *E. faecium* DH4:05 strain showed positive result indicating its resistance to pH 3 in applied method (**Table 3**). Resistance to pH 3 is often used *in vitro* assays to determine the resistance to stomach pH (Prasad et al., 1998). Resistances to the lysozyme by all six strains of LAB were evaluated in MRS broth with and without lysosome at 30°C for 24 h (**Table 3**). Lysozyme is capable of lysing bacteria, but it doesn't impair activities of LAB (Saran et al., 2012). Tolerance against bile was also tested and found that all LAB strains grew well in 0.3% bile showing their ability to tolerate bile salt. The mean intestinal bile concentration is 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h (Prasad et al., 1998). The probiotic bacteria survival in the gastrointestinal transit is primordial, and implies in the ability of microorganisms to survive at the stomach acidity and bile, so that they can exert their beneficial effects on the host (Pozza et al., 2011).

Bacterial affinity to hydrocarbons, such as hexadecane, proved to be a simple method to determine cell surface hydrophobicity (van Loosdrecht et al., 1987). None of the LAB strains showed >70% hydrophobicity (**Table 3**). A percent hydrophobic index greater than 70% was classified as hydrophobic (Nostro et al., 2004). Hence, LAB strains from *dahi* and *datshi* do not show hydrophobic character in the applied method. However, these limited technological properties are not enough to validate the potential probiotic uses of these isolates.

CONCLUSION

Based on 16S rRNA gene sequencing isolates of LAB, isolated from *dahi* and *datshi* of Bhutan, were identified as *E. faecalis, E. faecium, Lactococcus lactis* subsp. *lactis* and some strains showed promising technological properties. This is the first report on NFM of Bhutan, which may be used as baseline data for further research on NFM products.

AUTHOR CONTRIBUTIONS

HS: Molecular analysis of LAB isolates. SS: Isolation and phenotypic characterization. RR: Determination of technological properties of isolates. JT: Compilation of data and preparation of manuscript.

FUNDING

DST vide its sanction order: SB/EMEQ-367/2014, dated: 21.07.2014.

ACKNOWLEDGMENTS

Authors are grateful to Department of Science and Technology, New Delhi for research project. Authors are also thankful to Dr. K. Jeyaram and Dr. W. Romi of Institute of Bioresource and Sustainable Development, Imphal, India for analysis of the sequences in this paper.

REFERENCES

Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Glover, R. L. K., Nielsen, D. S., and Jespersen, L. (2013). Taxonomic and molecular characterization of lactic acid bacteria and yeasts in nunu, a Ghanaian fermented milk product. *Food Microbiol.* 34, 277–283. doi: 10.1016/j.fm.2012.09.025

Brennan, M., Wanismail, B., Johnson, M. C., and Ray, B. (1986). Cellular damage in Dried *Lactobacillus acidophilus*. *J. Food Prot.* 49, 47–53. doi: 10.4315/0362-028X-49.1.47

Dewan, S., and Tamang, J. P. (2006). Microbial and analytical characterization of Chhu, a traditional fermented milk product of the Sikkim Himalayas. *J. Sci. Indus. Res.* 65, 747–752.

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

Dykes, G. A., Britz, T. J., and von Holy, A. (1994). Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum packaged Vienna sausages. *J. Appl. Bacteriol.* 76, 246–252. doi: 10.1111/j.1365-2672.1994.tb01623.x

Hao, Y., Zhao, L., Zhang, H., Zhai, Z., Huang, Y., Liu, X., et al. (2010). Identification of the bacterial biodiversity in koumiss by denaturing gradient gel electrophoresis and species-specific polymerase chain reaction. *J. Dairy Sci.* 93, 1926–1933. doi: 10.3168/jds.2009-2822

Harun-ur-Rashid, M., Togo, K., Useda, M., and Miyamoto, T. (2007). Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk "Dahi" in Bangladesh. *Pakistan J. Nutr.* 6, 647–652. doi: 10.3923/pjn.2007.\break647.652

Josephsen, J., and Jespersen, L. (2004). "Handbook of food and beverage fermentation technology," in *Starter Cultures and Fermented Products*, Vol. 3, eds Y. H. Hui, L. Meunier-Goddik, ÅS. Hansen, J. Josephsen, W. K. Nip, P. S. Stanfield, et al. (New York, NY: Marcel Dekker, Inc), 23–49.

Lane, D. J. (1991). "16S/23S rRNA Sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackenbrandt and M. Goodfellow (New York, NY: John Wiley & sons), 115–147.

Leisner, J. J., Rusul, G., Wee, B. W., Boo, H. C., and Mohammad, K. (1997). Microbiology of Chili Bo, a popular Malaysian food ingredient. *J. Food Prot.* 60, 1235–1240. doi: 10.4315/0362-028X-60.10.1235

Martín-Platero, A. M., Valdivia, E., Maqueda, M., and Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Anal. Biochem.* 366, 102–104. doi: 10.1016/j.ab.2007.03.010

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

Mayo, B., Ammor, M. S., Delgado, S., and Alegría, A. (2010). "Fermented milk products," in *Fermented Foods and Beverages of the World*, eds J. P. Tamang and K. Kailasapathy (Boca Raton, FL: CRC Press), 263–288.

Nostro, A., Cannatelli, M. A., Crisafi, G., Musolino, A. D., Procopio, F., and Alonzon, V. (2004). Modifications of hydrophobicity, in vitro adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Lett. Appl. Microbiol.* 38, 423–427. doi: 10.1111/j.1472-765X.2004.01509.x

Oki, K., Dugersuren, J., Demberel, S., and Watanabe, K. (2014). Pyrosequencing analysis of the microbial diversity of airag, khoormog and tarag, traditional fermented dairy products of Mongolia. *Biosci. Microbiol. Food Health* 33, 53–64. doi: 10.12938/bmfh.33.53

Olasupo, N. A., Schillinger, U., and Holzpafel, W. H. (2001). Studies on some technological properties of predominant lactic acid bacteria isolated from Nigerian fermented foods. *Food Biotechnol.* 15, 157–167. doi: 10.1081/FBT-100107627

Patil, M. M., Pal, A., Anand, T., and Ramana, K. V. (2010). Isolation and characterization of lactic acid bacteria from curd and cucumber. *Indian J. Biotechnol.* 9, 166–172.

Patrignani, F., Lanciotti, R., Mathara, J. M., Guerzoni, M. E., and Holzapfel, W. H. (2006). Potential of functional strains, isolated from traditional Maasai milk, as starters for the production of fermented milks. *Int. J. Food Microbiol.* 107, 1–11. doi: 10.1016/j.ijfoodmicro.2005.08.004

Perez, P. F., Minnaard, Y., Disalvo, E. A., and De Antoni, G. L. (1998). Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microbiol.* 64, 21–26.

Pozza, M. S. S., Miglioranza, L. H. S., Garcia, J. E., Garcia, S., and Pozza, P. C. (2011). Human gastrointestinal tract resistance of *Lactobacillus* strains isolated from infant faeces. *Ciec. Agrairias* 32, 1021–1032. doi: 10.5433/1679-0359.2011v32n3p1021

Prasad, J., Gill, H., Smart, J., and Gopal, P. K. (1998). Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. *Int. Dairy J.* 8, 993–1002. doi: 10.1016/S0958-6946(99)00024-2

Saran, S., Bisht, M. S., Singh, K., and Toetia, U. S. (2012). Analyzing probiotic attributes to assess comparatively two isolates of *Lactobacillus acidophilus* in prebiotics, honey and inulin. *DHR Int. J. Biomed. Life Sci.* 2, 26–34.

Sarkar, S. (2008). Innovations in Indian fermented milk products-a review. *Food Biotechnol.* 22, 78–97. doi: 10.1080/08905430701864025

Schillinger, U., and Lücke, F. K. (1987). Identification of lactobacilli from meat and meat products. *Food Microbiol.* 4, 199–208. doi: 10.1016/0740-0020(87)90002-5

Sun, Z., Liu, W., Gao, W., Yang, M., Zhang, J., Wang, J., et al. (2010). Identification and characterization of the dominant lactic acid bacteria from kurut: the naturally fermented yak milk in Qinghai. *China. J. Gen. Appl. Microbiol.* 56, 1–10. doi: 10.2323/jgam.56.1

Takeda, S., Yamasaki, K., Takeshita, M., Kikuchi, Y., Tsend-Ayush, C., Dashnyam, B., et al. (2011). The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. *Animal Sci. J.* 82, 571–579. doi: 10.1111/j.1740-0929.2011.00874.x

Tamang, J. P. (2010). *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic Values*. New York, NY: CRC Press.

Tamang, J. P., Dewan, S., Thapa, S., Olasupo, N. A., Schillinger, U., and Holzapfel, W. H. (2000). Identification and enzymatic profiles of predominant lactic acid bacteria isolated from soft variety chhurpi, a traditional cheese typical of the Sikkim Himalayas. *Food Biotechnol.* 14, 99–112. doi: 10.1080/08905430009549982

Tamang, J. P., Shin, D. H., Jung, S. J., and Chae, S. W. (2016a). Functional properties of microorganisms in fermented foods. *Front. Microbiol.* 7:578. doi: 10.3389/fmicb.2016.00578

Tamang, J. P., Tamang, B., Schillinger, U., Guigas, C., and Holzapfel, W. H. (2009). Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. *Int. J. Food Microbiol.* 135, 28–33. doi: 10.1016/j.ijfoodmicro.2009.07.016

Tamang, J. P., Watanabe, K., and Holzapfel, W. H. (2016b). Review: diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* 7:377. doi: 10.3389/fmicb.2016.00377

van Loosdrecht, M. C. M., Lyklema, J., Norde, W., Schraa, G., and Zehnder, A. J. B. (1987). The role of bacterial cell wall hydrophobicity in adhesion. *Appl. Environ. Microbiol.* 53, 1893–1897.

Watanabe, K., Fujimoto, J., Sasamoto, M., Dugersuren, J., Tumursuh, T., and Demberel, S. (2008). Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional fermented milk products of Mongolia. *World J. Microbiol. Biotechnol.* 24, 1313–1325. doi: 10.1007/s11274-007-9604-3

Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., et al. (2009). Isolation and preliminary probiotic selection of lactobacilli from Koumiss in Inner Mongolia. *J. Basic Microbiol.* 49, 318–326. doi: 10.1002/jobm.200800047

Yu, J., Wang, W. H., Menghe, B. L. G., Jiri, M. T., Wang, H. M., Liu, W. J., et al. (2011). Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. *J. Dairy Sci.* 94, 3229–3241. doi: 10.3168/jds.2010-3727

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

J Ethn Foods 3 (2016) 270–275

Contents lists available at ScienceDirect

Journal of Ethnic Foods

journal homepage: http://journalofethnicfoods.net

Original article

Naturally fermented milk products of the Eastern Himalayas

Ranjita Rai, H. Nakibapher Jones Shangpliang, Jyoti P. Tamang*

Department of Microbiology, School of Life Sciences, Sikkim University, Tadong, Sikkim, India

ARTICLE INFO

Article history:
Received 30 September 2016
Received in revised form
1 November 2016
Accepted 16 November 2016
Available online 3 December 2016

Keywords:
chhurpi
dahi
Eastern Himalayas
mohi
naturally fermented milk products

ABSTRACT

Background: Pastoralists comprising different ethnic groups of people dominate the Eastern Himalayas. Traditional knowledge in the Eastern Himalayas reflects the common linkage of origin and settlement of the ethnic groups in the regions. The practice of milk fermentation along the Eastern Himalayan regions shows similar types of ethnic naturally fermented milk (NFM) products that are regularly prepared by different ethnic groups of people.

Methods: A survey of various types of NFM products of Eastern Nepal, Darjeeling Hills, Sikkim, and Arunachal Pradesh in India, and Bhutan and their methods of preparation, mode of consumption, and ethnic values was documented as per the standard method.

Results: Sikkim and Nepal have several varieties of NFM products, which include *dahi*, *mohi*, *gheu*, soft *chhurpi*, hard *chhurpi*, *dudh-chhurpi*, *chhu*, *somar*, *maa*, *philu*, and *shyow*. The main products, which are daily prepared in Arunachal Pradesh, are *mar*, *chhurpi/churapi*, *churkam*, and *churtang/chhurpupu*. NFM products of Bhutan are *dahi*, *datshi*, *mohi*, *gheu*, *chugo*, and *hitpa*.

Conclusion: Unique types of NFM products have been reported from the Eastern Himalayas. Although these are minor products, they are of high biological importance.

1. Introduction

The Eastern Himalayan region lies between the latitudes 26°40′–29°30′ North and longitudes 88°5′–97°5′ East and covers a total area of 93,988 km^2, comprising the Eastern part of Nepal; Darjeeling Hills, Sikkim, and Arunachal Pradesh in India; and Bhutan. Agriculture and livestock are the major livelihoods of the ethnic people in the Eastern Himalayas [1]. Domestic livestock includes cows, oxen, goats, pigs, sheep, yaks, "joe/churru" (hybrid of cow and yak), buffalo, and poultry, which are mainly used for meat, hair, milk and milk products, and eggs. Naturally fermented milk (NFM) products are popular only in a few regions of the Eastern Himalayas of Nepal, Darjeeling Hills, Sikkim, Bhutan, and some parts of Arunachal Pradesh. In the other states of Northeast India, except for Assam and Tripura, milk and milk products are not a part of the traditional foods because no fermented milk products have been reported from Meghalaya, Nagaland, Mizoram, and Manipur, where pastoral systems are rare. Pastoralism is the major livestock practice of the ethnic people of the Eastern Himalayas where only certain tribes are associated with it, namely, Sherpa, Bhutia, and Nepali (Sikkim), Bjobs (Western Bhutan), Brokpas (Eastern Bhutan), and Brokpas (Arunachal Pradesh) [1–3]. NFM products are mostly prepared only from cows and yaks, which usually thrive at high altitudes. In Arunachal Pradesh, yaks are reared only in two districts, West Kameng and Tawang, and NFM products are only found in these regions. Amongst the different tribes of Arunachal Pradesh, yak raisers, locally known as Brokpas, a pastoral community belonging to the Monpa tribe, are associated with preparation of NFM products. NFM products are prepared from both cows' and yaks' milk, however, only a few surveys have been reported from yak products [3–6]. In Sikkim, yaks are found at high altitude in North Sikkim and the border area between Sikkim and Nepal at West Sikkim. In Bhutan, yaks are mostly found in the eastern and western part of the country [2]. The present study aimed to document the ethnic NFM products of the Eastern Himalayas including some eastern parts of Nepal, Darjeeling Hills, Sikkim, Arunachal Pradesh, and Bhutan.

* Corresponding author. Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Tadong 737102, Sikkim, India.
E-mail address: jyoti_tamang@hotmail.com (J.P. Tamang).

http://dx.doi.org/10.1016/j.jef.2016.11.006

2. Materials and methods

A field survey was conducted in randomly selected villages in Eastern Nepal (Dharan, Dhankuta, Hiley, and Damak), Darjeeling Hills (Darjeeling and Kalimpong), Sikkim (Namchi, Rhenock, Lachung, Lachen, Uttare, Sorang, and Chakung), Bhutan (Punakha, Paru, and Thimpu), and Arunachal Pradesh (Tawang and West Kameng), representing the various ethnic communities. Information was documented on types of major and minor ethnic NFM products, their traditional methods of preparation, mode of consumption, and culinary, socioeconomic, and ethnic values.

3. Results

3.1. Ethnic NFM products of the Eastern Himalayas

The production of NFM products is similar in various regions of the Eastern Himalayas. The main products that are daily prepared in Arunachal Pradesh include *mar*, *chhurpi/churapi*, *churkam*, and *churtang/chhurpuppu* [3–6]. Sikkim has several varieties of NFM products prepared on a daily basis by the ethnic people, which includes *dahi*, *mohi*, *gheu*, soft *chhurpi*, hard *chhurpi*, *dudh-chhurpi*, *chhu*, *somar*, *maa*, *philu*, and *shyow*. *Dahi* (curd), *mohi* (buttermilk), and *gheu* (butter) are familiar in all regions of the Himalayas whereas *chhurpi*, *chhu*, and *philu* are confined mostly to the Bhutia community. *Somar* is exclusively prepared and consumed by the Sherpa of Nepal and Sikkim living at high altitudes. NFM products of Bhutan include *dahi*, *datshi*, *mohi*, *gheu*, *chugo*, and *hitpa*. Table 1 shows the list of major and minir ethnic NFM products of the Eastern Himalayas.

3.2. Traditional method of preparation of NFM products in the Eastern Himalayas

In Arunachal Pradesh, raw milk is churned in a specially made wooden vessel, locally known as a sop/shoptu/zopu (Figs. 1, 2). In colder seasons, raw milk is either warmed up in a fireplace before churning, or warm water is poured into the vessel during the churning process for better separation of the butter from the milk. NFM products can be further categorized into two types based on the time duration of fermentation of the processed milk. Short period fermented products include *mar*, *chhurpi*, and *churkam* (Figs. 1, 2). *Mar* (artisanal butter) is a fat-rich product that is separated from the whole milk by a churning process in a specially made wooden vessel locally known as a sop, leaving behind *dhara* (buttermilk). *Dhara* is further boiled for 25–30 minutes until a clumping solid (*chhurpi*) is formed, which is collected leaving the liquid residue (*churku*) behind. *Chhurpi* is spontaneously fermented at room temperature for only a few days and is also the main source of the production of two other products, *churkam* and *churtang*. For the preparation of *churkam*, *chhurpi* is immediately collected in a cloth after boiling and is hanged for a few minutes, which is later placed in between two stones for drying the remaining liquid up to 4–5 hours. The covering cloth is then carefully unwrapped and the semidried product is cut into small cubes of variable length (2–4 cm) and breadth (1–1.2 cm), which are then boiled along with *churku* until it is almost dried. The pieces are then sewn together in a thread with 20 pieces each making a roll. The dried products (*churkam*) are then hung for 3–4 days at room temperature inside the tent and are supplied to the local market for selling. Besides *churkam*, *chhurpi* can also be used to prepare *churtang/chhurpupu* (longer-period fermentation). However, in this process, *chhurpi*, after collection, is packed in an animal skin (calf skin by Zhorchut tribes, and Yak skin by *Mongnang*) and fermentation is for a duration of 6 months to > 1 year and some would even keep it longer for 3–20 years. This practice is also a form of preservation of *chhurpi* for a longer time.

The traditional method of preparation can be briefly summarized as follows: *dahi* is the main NFM product of Nepal, Darjeeling Hills, and Sikkim, and it also used for the preparation of several other milk products: *gheu*, *mohi*, soft *chhurpi*, and *chhu*. For the preparation of *dahi/shyow*, fresh or boiled milk (after cooling to

Table 1
NFM products of the Eastern Himalayas.

NFM	Milk source	Product characterization & mode of consumption	Region
Chhu/sheden	Cow or yak milk	Soft, strong flavored; curry	Darjeeling Hills, Sikkim
Chhur chirpen	Yak milk & crab apple	Pressed, light yellowish brown, side dish	Arunachal Pradesh
Chhur singba/chhur mingba	Yak milk	Pressed, light yellowish brown, side dish	Arunachal Pradesh
Chhurpi (soft variety)/*churapi*	Cow or yak milk	Soft, cheese-like; curry, pickle	Sikkim, Darjeeling Hills, Arunachal Pradesh
Chhurpi (hard variety)	Cow or yak milk	Hard mass, masticator	Sikkim, Darjeeling Hills, Arunachal Pradesh, Bhutan
Chungo	Cow or yak milk	Hard mass, masticator	Bhutan
Churtang/chhurpupu	Yak/cow milk	4–5 y old *chhurpi,* strong-flavored, curry	Arunachal Pradesh
Churkham	Fresh and old *chhurpi*	Soft cheese packed in yak skin & sun dried, eaten as masticator, mouth freshener	Arunachal Pradesh
Dahi	Cow/buffalo/yak milk	Curd; savory	All
Datshi	Cow or yak milk	Soft, cheese-like; curry, pickle	Bhutan
Dudh chhurpi	Cow milk	Hard mass, masticator	Darjeeling Hills, Sikkim
Gheu/ghee	Cow/buffalo milk	Butter	All
Hitpa	Cow or yak milk	*Datshi* packed in yak's skin, 1–2 y fermentation; strong-flavored, curry	Bhutan
Lassi	Cow/buffalo milk	Buttermilk; refreshing beverage	All
Maa/mar	Yak milk	Butter	Sikkim
Marchang	Yak ghee & barley flour *kongpu*	Side dish	Arunachal Pradesh
Mohi	Yak milk	Butter milk; refreshment	All
Philu	Yak milk	Cream; fried curry with butter	Sikkim
Phrung	Yak milk	Hard mass, masticator	Arunachal Pradesh
Shyow	Yak milk	Curd, savory	Sikkim
Somar	Cow or Yak Milk	Paste, flavored; condiment	Nepal, Darjeeling Hills, Sikkim

NFM, naturally fermented milk.

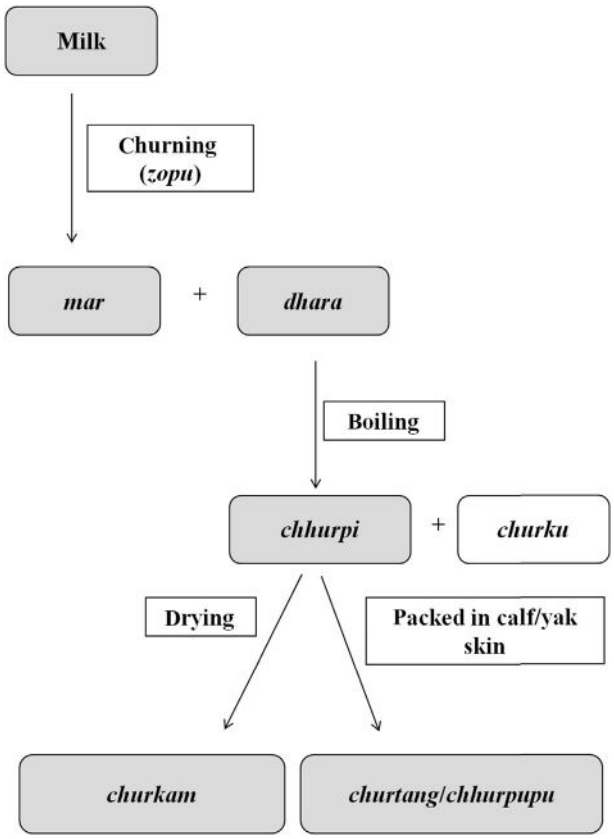


Fig. 1. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Arunachal Pradesh.

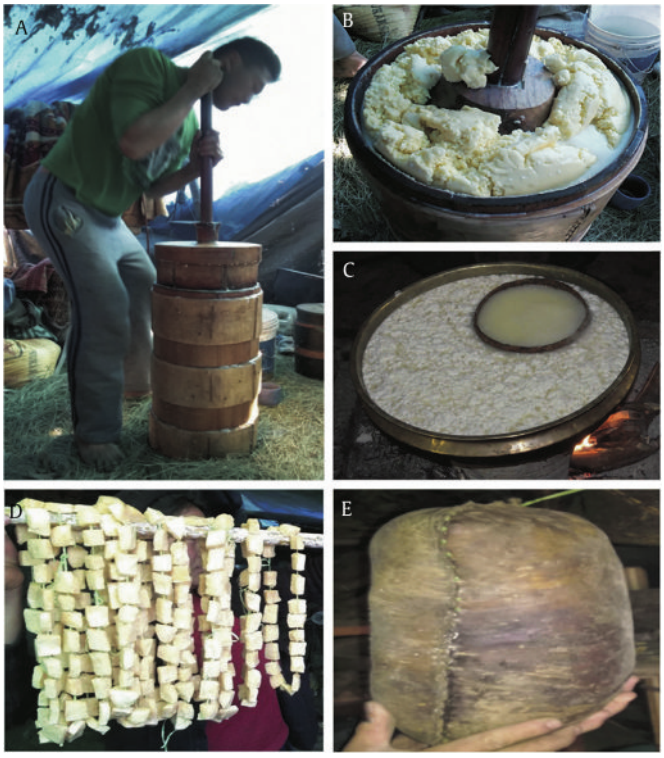


Fig. 2. Naturally fermented milk products of Arunachal Pradesh. (A) Brokpa churning milk in a wooden vessel (*sop*/*shoptu*/*zopu*); (B) *mar*; (C) *chhurpi*; (D) *churkam*; and (E) *churtang*.

room temperature) is fermented for 1–2 days by the addition of an old culture (*dahi*); a process known as back-sloping technique (Figs. 3, 4). Milk (fresh/boiled) is churned in a hollow wooden vessel container (theki), leaving behind *gheu* at the top of the container and a liquid byproduct, *mohi* (buttermilk). In Bhutia and Lepcha dialect, *mohi* is known as *kachhu*, whereas in the Western Himalayas, buttermilk is called *lassi*. *Philu* is a cream-like fermented product that is prepared by pouring fresh milk into a wooden vessel, where a thick mesh of dried creeper or sticks are kept inside that holds the milk. For two or three times a day, the milk is poured into the vessel, which is kept for 6–7 days, and some would even keep it for up to 15 days of fermentation. *Gheu* is an artisanal butter in Nepali, which is also known as *ghee* or *makhan* in Hindi, *maa* in Tibetan, and *mor* in Lepcha. *Mohi* can be further processed into soft *chhurpi*, hard *chhurpi*, and *dudh-chhurpi*. Soft *chhurpi*/*chhu*/*sheden* is formed when the buttermilk is boiled for about 15 minutes and is collected by sieving out using a cloth, which is hung by a string to drain out the remaining whey. When a fresh *chhurpi* is kept in a tight container for 10–15 days, the final product is known as *somar*. Soft *chhurpi* is further processed to form hard *chhurpi*, which is prepared by overpressing the highly stringy mass that is wrapped in a cloth over stones, and is usually fermented under pressure at room temperature for about 2 days. However, the hard variety, which is prepared from yak's milk, is called *dudh-chhurpi*.

The preparation of NFM products in Bhutan is similar to that in Sikkim and Arunachal Pradesh. *Dahi* is prepared from boiled or raw milk that is fermented at room temperature for about 15 days (Fig. 5). It is used for the preparation of several other ethnic milk products such as *gheu* (*mar*), *mohi*, *datshi*, and *chugo* (Fig. 6). *Dahi* is further processed into *mar*/*gheu* by churning in a special wooden

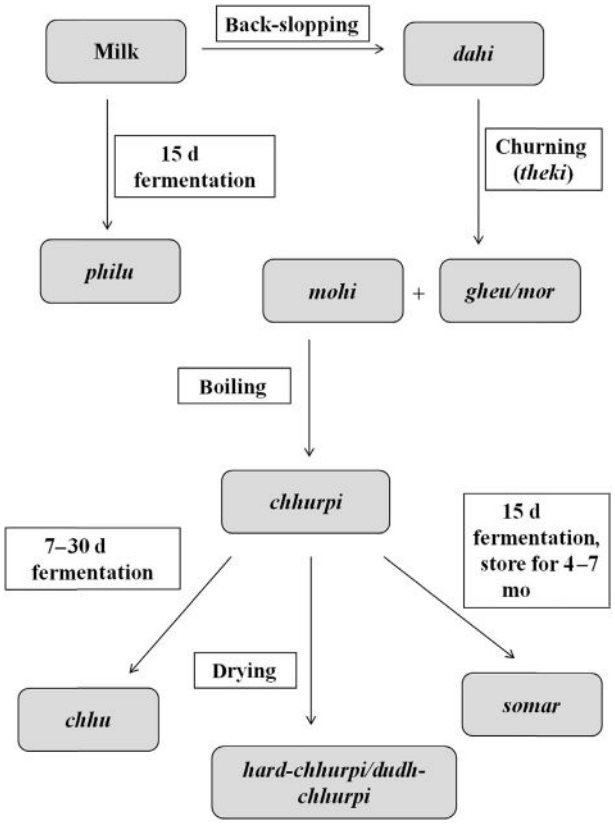


Fig. 3. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Sikkim and Darjeeling Hills in India, and Nepal.

Fig. 4. Naturally fermented milk products of Sikkim and Darjeeling Hills in India, and Nepal. (A) *Gheu*, (B) hard *chhurpi*, (C) *philu*, (D) soft *chhurpi*, (E) *dudh-chhurpi*.

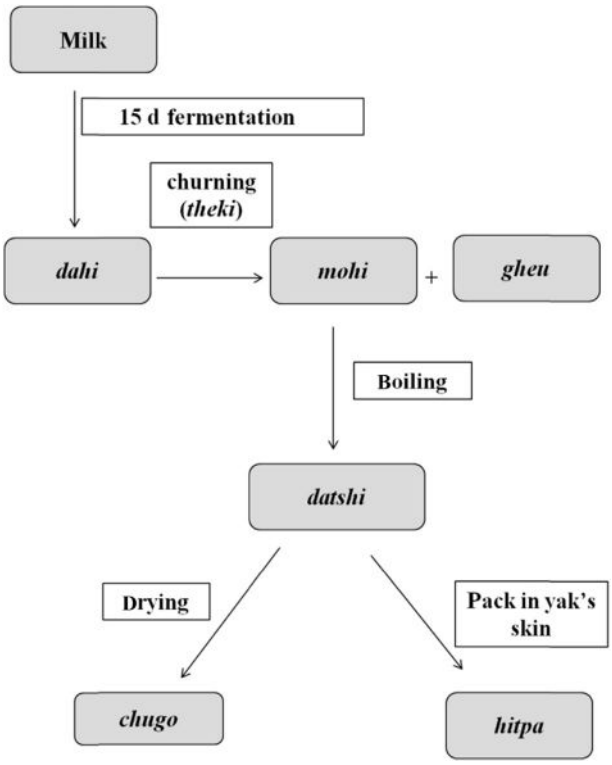


Fig. 5. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Bhutan.

container, locally called theki. After this process, *mar* is collected, leaving the liquid residue behind, which is called *mohi*. *Mohi* is further processed to yield *datshi*, by boiling until clumping, a process similar to the preparation of soft *chhurpi* of Sikkim, India. *Datshi* is collected in a cloth, dried where the remaining liquid residue is almost drained out and then *chugo* is formed, which is also similar to *dudh-chhurpi* and *churkam* of Sikkim and Arunachal Pradesh, India. *Hitpa* is formed by fermentation of *datshi* for a longer period of time (~1 year), which is usually packed in yak's skin; another product that shares similarity to *churtang* of Arunachal Pradesh.

3.3. Mode of consumption and ethnic values

Dahi is consumed directly as a nonalcoholic beverage in Nepal, Darjeeling Hills, Sikkim, and Bhutan, but is uncommon in Arunachal Pradesh. It is also consumed after mixing it with rice or *chuira* (beaten rice). *Mohi*/*kachhu* is consumed as a cooling beverage during hot days and also to overcome tiredness. *Gheu*/*mar* is also consumed freshly as it is. In Sikkim, *gheu* is further purified by boiling until the oily liquid separates from the unwanted dark-brown precipitate, locally called *khar*, which is consumed along with steamed rice or mixed in dal and curry. *Mar* (butter) is the main ingredient of a beverage made of tea and salt, locally known as *shui zha*/*maar zha* or commonly as *namak tea* (butter tea). It is also mixed in the preparation of dishes or consumed raw by just mixing with rice. *Gheu* is also used to prepare traditional cereal-based snacks and varieties of sweets; *Maa* is used for cooking and frying edible items. *Gheu*/*mar* is a highly prized milk product and serves as a major source of income for farmers in the Eastern Himalayas and is sold all the year round in the local markets. *Gheu* costs about Indian Rupees (Rs) 250–350/kg. *Mar* is priced for Rs 250/kg. Soft-variety *chhurpi* is prepared as a curry, cooked in edible oil or *gheu* along with onions, tomato, and chillies or wild edible ferns (*Diplazium esculentum*) and is eaten with boiled rice. It is also used to prepare *aachar* or pickle by mixing it with chopped cucumber, radish, and chillies, and as soup. One kilogram of soft *chhurpi* costs about Rs 120–150, which is usually packed in the leaves of the fig (*Ficus* sp.). In Arunachal Pradesh, *chhurpi* can be prepared in a variety of ways and is also consumed raw and is available in markets at the rate of Rs 400/kg. Tastier versions of *chhurpi* can be prepared as *chur chirpen* (milk boiled with crab apple), soybean (*libi*) *chhurpi* (with soybean), and *chhurpi* chutney (paste with tomato, *Allium* spp.). *Chhu* is prepared as soup and as curry by cooking in *maa* (butter) along with onions, tomato, and chillies, and mixed with salt. It has a sour taste with a strong aroma and is used as an appetizer. In Bhutan, *datshi* is usually made as round small balls. *Emadatshi* is a popular delicious food in Bhutan, which is creamy white gravy comprising mainly cheese (*datshi*), potatoes, and thin sliced chillies. *Dudh-chhurpi*, hard *chhurpi*, and *churkam* are available as cube-shaped solids of variable sizes and are mostly eaten as a nutritious masticator or as a mouth freshener, and *chhurpi* chewing gives extra energy at high altitudes. The hard-variety *chhurpi* costs about Rs 500/kg. In Arunachal Pradesh, local people use *churkam* as greetings for friends and loved ones, and it is usually sold in cubes of 20 pieces a roll at the rate of Rs 120–150/roll. *Somar* is prepared as a soup-based curry and is consumed mostly by the older generation of the *Sherpa*, which is believed to cure digestive problems and control diarrhea. However, *somar* is not sold in the market and is only prepared in the household. *Philu* is also cooked as a curry and it is eaten as a side dish along with

Fig. 6. Naturally fermented milk products of Bhutan. (A) A man churning milk using theki, (B) theki, (C) *dahi*, (D) *ghee*, (E) *chhurpi*, and (F) *chugo*.

boiled rice, and sometimes, it is mixed with meat and vegetables. *Philu* is an expensive ethnic milk product sold in local markets in Sikkim costing Rs 200/kg. *Churtang/chhurpupu* is also prepared in the same way as that of *chhurpi*. It is also used to cure stomach pain where a small amount is mixed with a beverage made of indigenous barley or finger millet and is given to people suffering from stomach ache; it is also used to prepare marchang, which is known to cure body ache. *Churtang* is also of high value to the people and costs about Rs 1600/kg and more, depending upon the size and duration of fermentation. The longer the fermentation, the more the value it possesses.

4. Discussion

The yaks are considered as an important domesticated animal in the Eastern Himalayan [2]. Yaks are usually found in the colder regions near the snow-capped mountains of the Himalayas, whereas cows are mostly found in the lower regions. Apart from yaks, cows are also the main livestock of the Himalayan pastoralism. In Arunachal Pradesh, NFM products are prepared from both cows' and yaks' milk, however, only a few surveys have been reported from yak products [3–6]. Additionally, cows' milk is just as important as yaks' milk, especially in the Tawang Regions where most herders (*brokpas*) rear cows as well as yaks. In Sikkim, NFM products are prepared from both cows and yaks, where yaks are mostly found in the northern and western regions.

There are many similarities among the ethnic people of the Eastern Himalayas, and their traditional knowledge of preparation of NFM products also reflects their common culture and tradition, and most importantly, religion [1]. As discussed earlier, most of the NFM products of the Eastern Himalayas are similar in their production and most differ only in the use of different dialects that the different tribe speak. This implies that the ethnic people of the Eastern Himalayas share traditional knowledge that leads back to Tibetan origin. The NFM products of the Eastern Himalayas are also similar to those prepared in the Western Himalayas [7].

In the high mountains of Tawang in Arunachal Pradesh, cattle rearing seems to be one of the most challenging occupation for the brokpas tribes, as most of them have to stay in jungles and move around almost every 3–4 months from one place to another in search of a suitable place for their cattle. Yak herders usually stay in the higher altitudes of these regions as their cattle are more suitable to the cold regions that the snow-capped mountains provide, whereas the cow herders usually stay in the lower regions in warmer places. However, the difficulty of the practice of cattle rearing seems to be almost of equal measure as these herders need to move and follow their cattle from time to time whenever there is shortage of food. In some cases, herders do use this product as an exchange for grasses with people who would bring to them as in the form of a barter system. The nomad pastoralists face a lot of challenges in the mountains, and it seems to be difficult for them to carry out their tradition and livelihood in the future [2,8], where not only the stocks of cattle are decreasing, but also the market for these products is also narrow and localized. Livestock such as cows are known to have a social impact in many societies and dairying played an important role in early religious practice [9]. In Hinduism, cows are considered sacred, and their milk and milk products are used in every religious and cultural ceremony. The importance of cow and milk products have been mentioned in the Rig Veda, the oldest sacred book of the Hindus, where it is known in ancient Indian history that *dahi*, *buttermilk*, and *ghee* were widely consumed during the time of Lord Krishna time about 3000 BC [10].

Major and minor ethnic NFM products in the Eastern Himalayas are unknown to the outside world. The knowledge of the ethnic people of this region about production of NFM products with high biological importance, as well as ethnic values, has been documented for the first time.

Conflict of interest

There is no conflict of interest.

Acknowledgments

We are grateful to the local people of the Eastern Himalayas for their indigenous knowledge of milk fermentation, as well as to the Department of Science and Technology, New Delhi for the research project.

References

[1] Tamang JP. Fermented milks. In: Tamang JP, editor. Himalayan fermented foods: microbiology, nutrition, and ethnic values. Boca Raton: CRC Press, Taylor and Francis; 2010. p. 95–109.
[2] Wangchuk D, Dhammasaccakarn W, Tepsing P and Sakolnakarn TPN. The yaks: heart and soul of the Himalayan tribes of Bhutan. E3 J Environ Res Manage 2013:3189–96.
[3] Bora L, Bam J, Paul V and Maiti S. Traditional milk, meat processing and preservation techniques of the Yak pastoralists of Arunachal Pradesh. Indian J Traditional Know 2014;13:216–21.
[4] Singh RK, Singh A and Sureja AK. Traditional foods of Monpa tribe of West Kameng, Arunachal Pradesh. Indian J Traditional Knowl 2007;6:25–36.

[5] Tiwari SC and Mahanta D. Ethnological observation on fermented food products of certain tribes of Arunachal Pradesh. Indian J Traditional Knowl 2007;6:106–10.
[6] Tomar SK, Singh R, Gupta SC, Arora DK, Joshi BK and Kumar D. Phenotypic and genotypic characterization of lactobacilli from Churpi cheese. Dairy Sci Technol 2009;89:531–40.
[7] Raj A and Sharma P. Fermented milk products of Ladakh. Indian J Traditional Knowl 2015;1:132–8.
[8] Singh RK. Indigenous knowledge of yak breeding and management by Brokpa community in Eastern Himalaya, Arunachal Pradesh. Indian J Traditional Knowl 2009;8:495–501.
[9] McCormick F. Cows, milk and religion: the use of dairy produce in early societies. Anthropozool 2012;47:101–13.
[10] Prajapati JB and Nair BM. The history of fermented foods. In: Farnworth R, editor. Handbook of fermented functional foods. New York: CRC Press; 2003. p. 1–25.

Journal of Scientific & Industrial Research
Vol 73, September 2014, pp. 588-592

HACCP model of *kinema*, a fermented soybean food

R Rai, N Kharel and J P Tamang*
Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Tadong 737102, Sikkim, India

Received 10 February 2014; revised 30 June 2014; accepted 28 July 2014

The Hazard Analysis Critical Control Points (HACCP) system basically applies on food processing to identify specific hazards and measures for their control to ensure the safety of foods. *Kinema* is a naturally fermented soybean food of the Eastern Himalayas. The present study on HACCP of *kinema* revealed that marketed *kinema* has higher microbial load as compared to the one prepared under laboratory condition and home-made *kinema*. Furthermore, the Critical Control Point (CCP) was checked during *kinema* preparation in both traditionally prepared method and *kinema* prepared under laboratory condition. HACCP model for optimised production of *kinema* has been proposed.

Keywords: Fermented foods, *Kinema*, HACCP

Introduction

Kinema is a sticky, ammonia-flavoured, naturally fermented soybean food produced by the Himalayan women of Sikkim, Darjeeling hills, Eastern Nepal and Bhutan and is eaten as curry[1]. *Bacillus subtilis* is the dominant and functional bacterium in *kinema* along with *Enterococcus faecium, Candida parapsilosis* and *Geotrichum candidum*[2,3]. The Hazard Analysis and Critical Control Points (HACCP) system is the science based and systematic which identifies specific hazards and measures for their control to ensure the safety of foods[4,5,6]. HACCP is used in the food industry to identify potential food safety hazards so that key actions called Critical Control Points (CCPs) can be taken to reduce or eliminate the risk of the hazards being realized, and the system is used at all stages of food production and preparation processes including packaging, distribution, etc[4]. HACCP was introduced to foodservice by Bill Vomvoris in 1987[5]. In 1993, the Codex Alimentarius Commission endorsed the HACCP system as the most cost-effective approach for ensuring the safety of food[7]. The aim of this study is to focus on assessment of microbiological safety and to formulate possible application of HACCP system in both traditionally prepared and laboratory-prepared *kinema*.

*Author for correspondence
E-mail: jyoti_tamang@hotmail.com

Materials and Methods

Survey

Aao village, near Pakyong in East Sikkim was selected for the study. A survey was conducted in fifteen households who practices *kinema* preparation by traditional method. Most of them belong to Limboo community. They prepare *kinema* weekly and sell to the nearby market.

Sample Collection

The sample was collected from in and around Gangtok. For determination of CCP samples were obtained from different stages of *kinema* processing. Collected samples were analyzed for microbial counts. Approximately 100 g of unfermented soybean cotyledon, soaked soybean cotyledons and freshly fermented condiments were collected in sterile poly-bags and transported to laboratory for analysis. Tap water was collected from laboratory for analysis. *Kinema* was prepared under laboratory condition following the guidelines mentioned elsewhere Tamang[8].

Microbiological analysis

10 g of sample was homogenized with 90 ml of 0.85 % (w/v) sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution (10^{-1} to 10^{-8}) in the same diluents was made. Spore-forming bacilli were isolated on nutrient agar (MM012, HiMedia), after inactivation of vegetable cells by heating at 100 ^{0}C for 2 min[9] and then incubated at 37 ^{0}C for 24 h. Enumeration of pathogenic bacteria from the food samples was done in selective media such as *Bacillus cereus* agar base

(M833, HiMedia) for *Bacillus cereus*, Violet Red Bile Glucose agar w/o lactose (M581, HiMedia) for enterobacteriaceae[10].

Selective enumeration of *Staphylococcus aureus* was carried out on spread plates of Baird-Parker Agar Media (MM043, HiMedia), with appropriate addition of Egg Yolk Tellurite Emulsion (FD046, HiMedia)[11]. *Salmonella-Shigella* Agar (M108, HiMedia) was used for the detection of *Salmonella* and *Shigella* and *Listeria* identification agar base (M1064, HiMedia) with *Listeria* selective supplement (FD 061, HiMedia) for *Listeria* in the samples following the standard method of Metaxopolous *et al*.[11] Most Probable Number (MPN) counts of coliforms was determined as described by Harrigan[12]. The water samples were tested for MPN which included presumptive, confirmatory and completed. Microbiological data obtained were transformed into logarithms of the numbers of colony forming unit (cfu) per g of sample.

Preparation of *kinema* under laboratory condition

Kinema was prepared in laboratory using monoculture of *Bacillus subtilis* (KK-2B10), previously isolated and identified[8]. Strain of *B. subtilis* (KK-2B10) was cultured on a nutrient broth at 37°C for 24 h. Yellow varieties of soybeans (*Glycine max*) were purchased from Gangtok. A 100 g of soybean was washed and soaked overnight (~12 h) at the ratio of 1:10 (w/v). The soaked soybean was autoclaved for 45 min to soften the seeds. A 0.1ml of fresh inoculum was inoculated on autoclaved soybeans, and then slowly stirred with a sterile glass rod and incubated at 50°C for 24 h.

Preparation of *kinema* by traditional method

100 g of soybean was washed and soaked overnight (~12 h) with clean water at the ratio of 1:10 (w/v). The soaked soybean was boiled for 45 min to soften the seeds. Cooked soybeans was cracked by using pestle and added 1% of wood ash then packed in *nevara (Ficus hookeriana)* leaves and kept in warm place for 2-3 days for fermentation[1].

Identification of bacterial isolates

Initial characterization of bacterial isolates included colony and cell morphology, Gram staining and other standard biochemical tests[3]. Rapid biochemical identification test kits (HiMedia) were used to identify the bacterial isolates of *kinema*. Biolog (Biolog Inc., USA) was also used for identification.

HACCP

HACCP (Hazard Analysis and Critical Control Points) was determined following the method of Gupta *et al*.[4] with slight modification. For the purpose of this study, HACCP team was constituted which comprised individuals having adequate knowledge and work experience viz., food microbiologist, research scholars and local personnel regarding *kinema*.

Result and Discussion

Microbial load in laboratory-made and home-made *kinema* was shown in Table 1. In market sample of *kinema* the bacterial load of *E.coli* ranged from 5.2×10^3 cfu/g to 7.5×10^8cfu/g. *Enterobacteriaceae* was found with 10.1×10^5cfu/g in *kofta,* 5×10^4 cfu/g in sausage and 37.8×10^4 cfu/g in *shawerma*[13,14]. Microbial population of *Shigella* in market sample was found to be 7×10^5cfu/g to 5.2×10^7cfu/g. It was reported that *Shigella* can easily be transmitted by person to person contact, food and water to create adequate exposure for infection[15]. The microbial count of *Salmonella* in market kinema was 6×10^4 cfu/g to 7.3×10^4 cfu/. It was reported that

Table 1 - Microbial count of laboratory-made and home-made *kinema*

Sample	Lab-made *Kinema* (Monoculture)			Home-made *Kinema* (Traditionally)		
	cfu/g (x 10^4)					
	E. coli	*Staphylococcus*	*Bacillus*	*E. coli*	*Staphylococcus*	*Bacillus*
Raw soybeans	3.7	1.6	0.05	3.7	1.6	0.05
Tap Water collected from Tadong	0.01	0.02	ND	0.5	0.02	ND
Autoclaved Soybean	ND	ND	ND	ND	ND	0.02
Fresh *kinema*	0.04	300	ND	0.01	120	15

ND = not detected.
Enterobacteriaceae, Shigella, Salmonella, Listeria and *Vibrio* were not detected in any sample.
Data shows a mean average of 3 sets of experiment

60.8% of *Salmonella* spp. are the contaminating agent in retail raw foods[16]. About 10^7 -10^8 cfu/g of population of *Staphylococcus* spp was determined in market *kinema* samples. Species of *Staphylococci* survive in a wide variety of food especially those require manipulation during processing including fermented food products like cheeses[17]. The growth of *Bacillus* spp. ranged 6.0×10^4 cfu/g to 9×10^7cfu/g in market sample. *Doenjang* , one of the most common soybean fermented food of Korea was found contaminated with *B. cereus*[18]. It was mentioned that ingestation of more than 10^5 cfu of *B.cereus* per gram of food may cause food poisioning[19]. The load of *Vibrio* was 10^2 - 10^4cfu/g in market *kinema*. The present study highlighted the prevalence of higher bacterial population in market *kinema* as compared to the one prepared under laboratory condition and homemade (Fig 1). Preliminary identification of isolates was done by colony morphology, simple staining and Gram staining. The bacteria isolates were identified based on standard microbiological methods[20]. For further differentiation of isolates a rapid test was performed by using biochemical test kits. The results of test kits were compared with the given biochemical test kit chart, the isolates were 50-80% positive similarity and identified as *E.coli, Salmonella enteritidis, Shigella sonnei*, *Staphylococcus aureus*, *Staphy. chromogens, Vibrio parahaemolyticus, V. orientalis* and *V. fluvialis.* For taxonomic identification of *Bacillus* spp. from *kinema*, the methods described by Cowan[21] were followed and isolated strains were identified as *Bacillus cereus.* Unidentified isolates were inoculated at Biolog, and were identified as *Serratia liquefaciens/grimesii, Proteus penneri/vulgaris*, *Providencia rettgeri.*Multiple tube fermentation (MTF) technique was performed to detect coliforms. For presumptive test MPN index of coliforms from tap water of Tadong contained 13/100 ml of coliform with reference to standard MPN index chart which ranged from 4-35 colonies. Similarly, the water collected from Entel area of Gangtok contained 1600/100ml of coliform with respect to standard chart which had confidence limit ranging from 400-4600 colonies. Biochemical tests were performed to confirm *E. coli*[22]. It was reported that the characteristic colouration of *E.coli* colonies on EMB agar was green metallic sheen and production of gas bubbles on lactose broth indicated positive test[23]. Most of the isolates from water sample showed positive result to indole and methyl red (data not shown).

Implementation of HACCP in this investigation identified the point of contamination during processing of *kinema* and tries to reduce the contamination load in it. Only the microbiological hazards i.e. bacterial contaminations were taken for current study. During implementation of HACCP, CCP was applied at four processing steps during preparation; they are (i) raw cotyledons (ii) water (iii) during cooking (iv) freshly prepared *kinema*. Fig 2 and 3 red box indicate the possible site of contamination during *kinema* preparation at

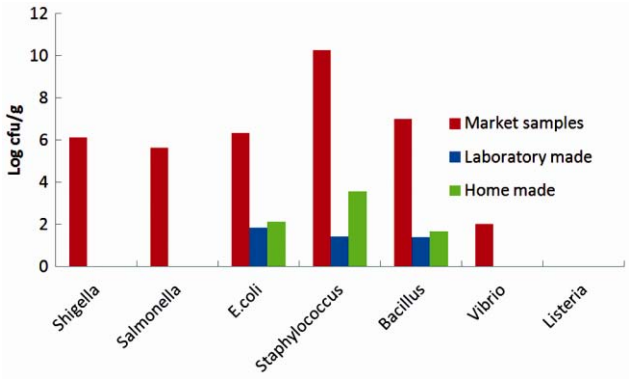


Fig 1 Graphical representation of comparison of microbial load of market, laboratory-made and home-made *kinema*.

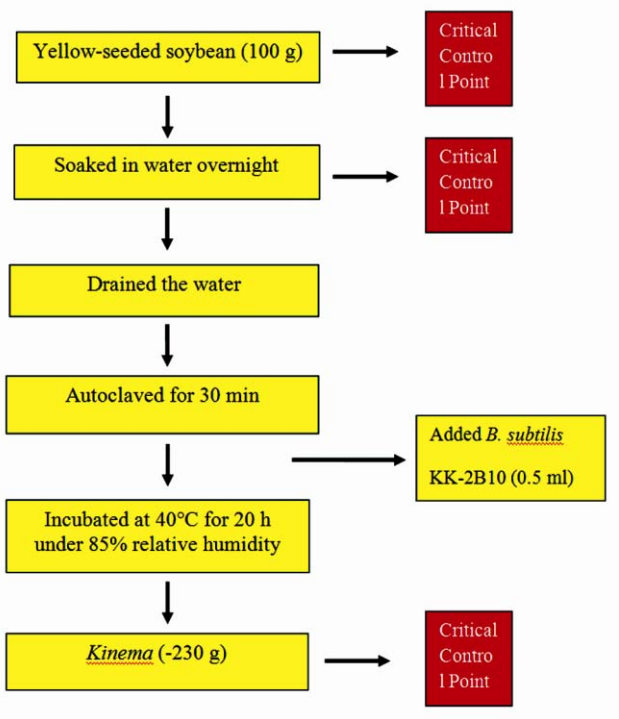


Fig 2 Determination of CCP in monoculture used *kinema* at laboratory.

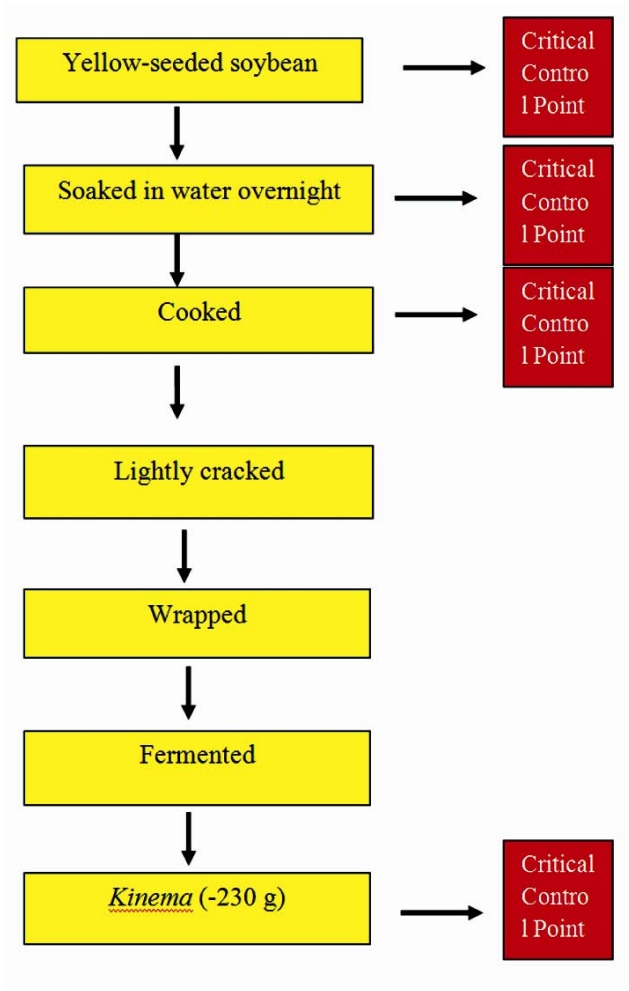


Fig 3 Determination of CCP in traditionally processed *kinema*

laboratory and at home. During analysis raw soybean was found contaminated with *Bacillus*, probably contaminated from soil. To eliminate or reduce the hazards to an acceptable level, proper cooking of soybean is required as in this study cooked cotyledons (autoclaved) did not contain any microorganism (Table 1). Similarly, tap water was found contaminated with coliform and to reduce this hazard, boiled water should be used instead of tap water. Freshly prepared *kinema* was found contaminated with *Enterobacteriaceae, Staphylococcus* and *Bacillus* (Table 1). Handling during *kinema* processing may add the microorganisms to the *kinema* sample. It was stated that personal hygiene was imperative because humans are the largest sources of contamination in food[24]. If proper handling and self hygiene is maintained throughout the processing it may help in reduction of microbial hazards to an acceptable level.

Conclusion

Implementation of HACCP during *kinema* processing may help to reduce the pathogenic load to an acceptable level. This current study was able to find four critical control points during processing and also found existence of preventive control measures. General awareness of self hygiene during preparation should provide to the handlers.

References

1 Tamang J P, Chettri R & Sharma R M, Indigenous knowledge on North-East women on production of ethnic fermented soybean foods. *Indian J Traditional Knowl,* **8** (1) (2009) 122-126.

2 Sarkar P K, Tamang J P, Cook P E & Owens J D, *Kinema*- a traditional soybean fermented food: Proximate composition and microflora. *Food Microbiol,* **11** (1994) 47-55.

3 Tamang J P, Native Microorganisms in the fermentation of *Kinema*. *Indian J Microbiol,* **43** (2) (2003) 127-130.

4 Gupta A, Sharma P C & Verma A K, Application of food safety management system (HACCP) in food industry. *Indian Food Industry*, **29** (2) (2010) 39-46.

5 King P, Implementing a HACCP Program. *Food Manage*, **27** (12) (1992) 54-58.

6 FAO, Guidelines for the application of the hazard analysis critical control point (HACCP) system in Rome. *Food and Agricultural Organization*, (Codex Alimentarius Commission (1993).

7 Panisello P J & Quantick P C, Technical barriers to hazard analysis critical control point (HACCP). *Food Control,* **12** (2001) 165–173.

8 Tamang J P, Development of Pulverised Starter for *Kinema* Production. *J Food Sci Technol,* **36** (5) (1999) 475-478.

9 Tamang J P & Nikkuni, S, Selection of starter culture for production of kinema, fermented soybean food of the Himalaya. *World J Microbiol Biotechnol,* **12** (6) (1996) 629-635.

10 Han B Z, Beumer R R, Rombouts F M & Nout M J R, Microbiological safety and quality of commercial sufu- a Chinese fermented soybean food. *Food Control*, **12** (2001) 541-547.

11 Metaxopoulos J, Samelis J & Papadelli M, Technological and microbiological evaluation of traditional process as modified for the industrial manufacturing of dry fermented sausages in Greece. *Italian J Food Sci,* **13** (2001) 3-18.

12 Harrigan W F, *Laboratory Methods in Food Microbiology.* **3rd Edition**. Academic Press, London. (1998).

13 Arthur T M, Bosilevac J M, Nou X, Shackelford S, Wheeler T , Kent M , Jaroni D, Pauling B, Allen D & Koohmaraie M, *Escherichia coli* O157 Prevalence and Enumeration of Aerobic bacteria, *Enterobacteriaceae,* and *Escherichia coli* O157 at various steps in commercial beef processing plants. *J Food Protec*, **67** (4) (2004) 658–665.

14 Mutairi, M F, The Incidence of *Enterobacteriaceae* causing food poisoning in some meat products. *Advance J Food Sci Technol,* **3**(2) (2011) 116-121.

15 Bennett J V, Holmberg S D, Rogers M F & Solomon S L. Infectious and parasitic diseases. *American J Preventive Medicine,* **3** (1987) 102- 114.

16 Van T T H, Moutafis G, Istivan T, Tran L T & Coloe P J, Detection of *Salmonella* spp. in Retail Raw Food Samples from

Vietnam and Characterization of Their Antibiotic Resistance. *Appl Environ Microbiol*, **73** (21) (2007) 6885–6890.

17 Loir Y, Baron F & Gautier M, Staphylococcus *aureus* and food poisoning. *Genetics Mol Res,* **2** (1) (2003) 63-76.

18 Lim J S, Kim M R, Kim W & Hong K W, Detection and differentiation of non-emetic and Emetic *Bacillus cereus* strains in food by Real-Time PCR. *J Korean Soc Appl Biol Chem*, **54** (1) (2011) 105-111.

19 Kotiranta A, Lounatmaa K & Haapasalo M, Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infection,* **2** (2000) 189-198.

20 Oranusi S U, Oguoma O I & Agusi E, Microbiological quality assessment of foods sold in student's cafeterias. *Global Res J Microbiol,* **3**(1) 2013)1 -7.

21 Cowan S T & Steel K J, Manual for the Identification of Medical Bacteria. Cambridge University Press, London, UK (1965).

22 Singh P & Prakash A, Isolation of *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* from Milk products sold under market conditions at Agra region. *Acta Agriculturae Slovenica*, **92**(1) (2008) 83–88.

23 Reddy U B, Chandrakanth N, Indu P S, Nagalakshmi R V & Usha K B, Isolation and Characterization of faecal *coliforms* in street vended fruit juices and its safety evaluation: A Case Study of Bellary City, India. *Int J Food Safety*, **11** (2009) 35-43.

24 Marriot N, Principles of Food Sanitation. Van Nostrand Reinhold Company, New York (1985).