Phenotypic and Genotypic Identification of Microorganisms from Some Naturally Fermented Milk Products of Arunachal Pradesh

A Thesis Submitted

To

Sikkim University

For the Degree of Doctor of Philosophy

By

H. Nakibapher Jones Shangpliang

Supervisor: Professor Dr. Jyoti Prakash Tamang

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

DECEMBER 2021

Dedicated to my mother (mei)

Mrs. Grazilian Shangpliang

DECLARATION

I declare that the present Ph.D thesis entitled **"Phenotypic and Genotypic Identification of Microorganisms from Some Naturally Fermented Milk Products of Arunachal Pradesh"** submitted by me for the award of the degree **of Doctor of Philosophy in Microbiology** of Sikkim University (central university) under the supervision of **Professor Dr. Jyoti Prakash Tamang**, Professor, Department of Microbiology, School of Life Sciences, 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.

H. Nakibapher Jones Shangpliang

.....

H. Nakibapher Jones Shangpliang
Registration No.: 14/Ph.D/MCB/02
Research Scholar
Department of Microbiology
School of Life Sciences
Sikkim University
(https://cus.ac.in)

Date: 29th December 2021
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

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This is to certify that the PhD thesis entitled **"Phenotypic and Genotypic Identification of Microorganisms from Some Naturally Fermented Milk Products of Arunachal Pradesh"** submitted to **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Shri H. Nakibapher Jones Shangpliang** 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 him under my supervision and guidance. He 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.

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SUPERVISOR and HEAD

प्रो. ज्योति प्रकाश तामांग
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

विभागाध्यक्ष/Head
प्रो. ज्योतिप्रकाश तामांग
Professor Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
माइक्रोबायोलॉजीविभाग/Department of Microbiology
सिक्किमविश्वविद्यालय/SIKKIM UNIVERSITY
Science Building, Tadong-737102, Sikkim

Place: Tadong, Gangtok
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6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
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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

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SIKKIM UNIVERSITY

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"Phenotypic and Genotypic Identification of Microorganisms from Some Naturally Fermented Milk Products of Arunachal Pradesh"

Submitted by **Shri H Nakibapher Jones Shangpliang** under the supervision of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Sikkim University, Tadong 737102, Sikkim, India.

H Nakibapher Jones Shangpliang
.....

H Nakibapher Jones Shangpliang
Signature of the candidate

.....

प्रो. ज्योति प्रकाश तामांग
Professor Dr. Jyoti Prakash Tamang
Supervisor

विभागाध्यक्ष/Head
प्रो. ज्योतिप्रकाश तामांग
Professor Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
माइक्रोबायोलॉजीविभाग/Department of Microbiology
सिक्किमविश्वविद्यालय/SIKKIM UNIVERSITY
Science Building, Tadong-737102, Sikkim

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प्रो. ज्योतिप्रकाश तामांग
Professor Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
माइक्रोबायोलॉजीविभाग/Department of Microbiology
सिक्किमविश्वविद्यालय/SIKKIM UNIVERSITY
Science Building, Tadong-737102, Sikkim

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H. Nakibapher Jones Shangpliang

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INTRODUCTION

Animal milk is highly nutritive with several health promoting benefits (Poppitt, 2020) and has been consuming for last 10,000 years (Curry, 2013). Among mammalians' milk, cow milk is most popular and is widely consumed across the world, however, milk from other mammals including buffalo, sheep, goat, mare, camel and yak may have been historically and culturally more important in certain regions/countries in the world (Tamang et al., 2020). However, the milk highly perishable, thus, fermentation is evolved as the major way to preserve milk and its nutrients, either by spontaneous or natural fermentation (Groenenboom et al., 2020) or back-slopping (Tamime and Robinson, 2007, Demirci et al., 2022). Naturally fermented milk products are one of the oldest fermented foods consumed by different ethnic communities in the word since 6000 BCE (Parker et al., 2018; Tamang et al., 2020). Wide varieties of traditional and commercial fermented milk products with more than 400 generic names are produced worldwide (Robinson and Tamime 2006; Tamime 2002; Khorshidian et al. 2020). Types of fermented milk products are based on several factors, including mode of coagulation (enzyme or acid or acid plus heat) (Tamime and Robinson 2007); the means by which whey separation occurs (Jørgensen et al. 2019); or by the nature of fermentation (lactic acid bacteria alone or lactic acid bacteria with fungal or other adjunct cultures) (Akabanda et al., 2014; Groenenboom et al., 2020; Albayrak and Duran, 2021). Common as well as lesser-known artisan, unique and exotic fermented dairy products are produced and consumed in different parts of the world which include *airag* (Mongolia), *amasi* (South Africa, Zimbabwe), ayran (Turkey, Kazakhstan and Russia), *bongo* (Uganda), dadih/dadiah (Indonesia), *dhanaan* (Somalia), *kefir* (Russia, Europe, and South America), *garris* (Somalia), *koumiss* (Russia), *kurut* (Tibet in China), *laban rayeb* (Egypt), *leben*/*lben* (North, East Central Africa), *lait caillé* (Senegal and Burkina Faso), *långfil* (Sweden), *nono* (Nigeria), *nunu* (Ghana), *tarag* (Mongolia), *viili*

(Finland), etc. (Sulieman et al., 2005; Watanabe et al., 2008; Liu et al., 2012; Akabanda et al., 2013; Berhe et al., 2017; Parker et al., 2018; Bayili et al., 2019; Bengoa et al., 2019; Mukisa et al., 2020; Tamang et al., 2020; Arnold et al., 2021; Fagbemigun et al., 2021; Kaledina et al., 2021; Widyastuti et al., 2021; Zhadyra et al., 2021).

Fermented foods, in particular, fermented milk products, are a good source of many beneficial microorganisms (probiotics), prebiotics and bioactive compounds which are also considered as good nutraceutical agents and functional foods (Tamang et al., 2016; Rezac et al., 2018; Ghosh et al., 2019; García-Burgos et al., 2020; Marco et al., 2021). It has also been described that the consumption of fermented milk products does improve gastrointestinal and cardiovascular health, cancer risk, weight management, diabetes, and metabolic health (Savaiano and Hutkins, 2021). Additionally, the significance of fermented milk products is their abundance of potential probiotic microorganisms which, apart from confer health benefits to host, are also of great importance in food industries (Rezac et al., 2018; Gao et al., 2021). Probiotics are defined "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (Hill et al., 2014; Martín and Langella, 2019). There are several microorganisms which are considered as probiotics or claimed to be having probiotic properties which include lactobacilli viz. *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lactobacillus acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. gallinarum*, *Lb. gasseri*, *Lb. johnsonii*, and *Limosilactobacillus reuteri*; *Bifidobacterium* species- *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, and *B. longum*; other LAB- *Enterococcus faecalis*, *E. faecium*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Sporolactobacillus inulinus* and *Streptococcus thermophilus*; and non-LAB- *Bacillus*

cereus var. *toyoi*, *Escherichia coli* strain *nissle*, *Propionibacterium freudenreichii*, *Saccharomyces cerevisiae* var. *boulardii* (Holzapfel et al., 2001; Fijan, 2014; Lazo-Vélez et al., 2018; García-Burgos et al., 2020; Gao et al., 2021). Among the well-known probiotic, *Lacticaseibacillus rhamnosus* GG is the most studied in the whole world (Hussain et al., 2021). Though there are several new strains to be claimed as probiotics, however, there are rigid criteria for a new strain to qualify as such, for food applications and dietary supplements, which briefly should include its adequate characterization, safe usage, clinical trial evidence, and long shelf life (Binda et al., 2020).

Consumption of naturally fermented milk (NFM) products are also common in different regions of India which are mostly community-specific and regions-based (Tamang, 2021), which include *dahi, lassi, misti dahi, srikhand, chhu, chhurpi, mohi, philu, shoyu, somar* (cow/yak milk) (Dewan and Tamang, 2006, 2007; Rai et al. 2016; Tamang et al., 2000, 2021). Arunachal Pradesh is one of North-eastern states of India with an area of 83,743 km², and a population of about 1.255 million as of 2012 **(**https://www.arunachalpradesh.gov.in/**).** Arunachal Pradesh is geographically located in the Eastern Himalayan region having an altitude range of 160 ft - 23,160 ft and borders internationally with like Tibet in China, Bhutan, and Myanmar. and nationally with Assam and Nagaland, two other North-eastern Indian states (Fig. A). Arunachal Pradesh is considered as one of the richest north-eastern regions of India in the sense of traditional foods and beverages. Traditional foods are mainly based on yak milk, soybean (*Glycine maxx* Merrill), buckwheat (*Fagopyrum esculentum* Moench), Amaranthus, maize, barley, chilli, and various indigenous fruits and vegetables (Singh et al., 2007). Milk is processed into different types of products by the *Brokpas* (*Monpas*) in Arunachal Pradesh, and the common products are butter (*Mar*) and cheese (*Chhurpy*) (Bora et al., 2014). Major animal resources of Arunachal Pradesh are cow, ox, mithun,

goat, pig, sheep, buffalo, poultry, and yak), and fishes from hill rivers, streams, and lakes (Tamang, 2010; Pandey et al. 2020). Different ethnic groups of people in Arunachal Pradesh are mostly pastoral and prepare fermented products which includes naturally fermented milk (NFM) products (Shrivastava et al., 2020; Tamang et al., 2012, 2021). *Monpas*, sub ethnic group of *Brokpa* community, are the indigenous people of India residing in Tawang and West Kameng districts of Arunachal Pradesh, and traditional practice of milk fermentation is mostly associated with these groups as they are cattle herders (cow/yak). Till date, there is very limited work that has been carried out on the study of the NFM products till date, hence the selection of AP as study site is justifiable.

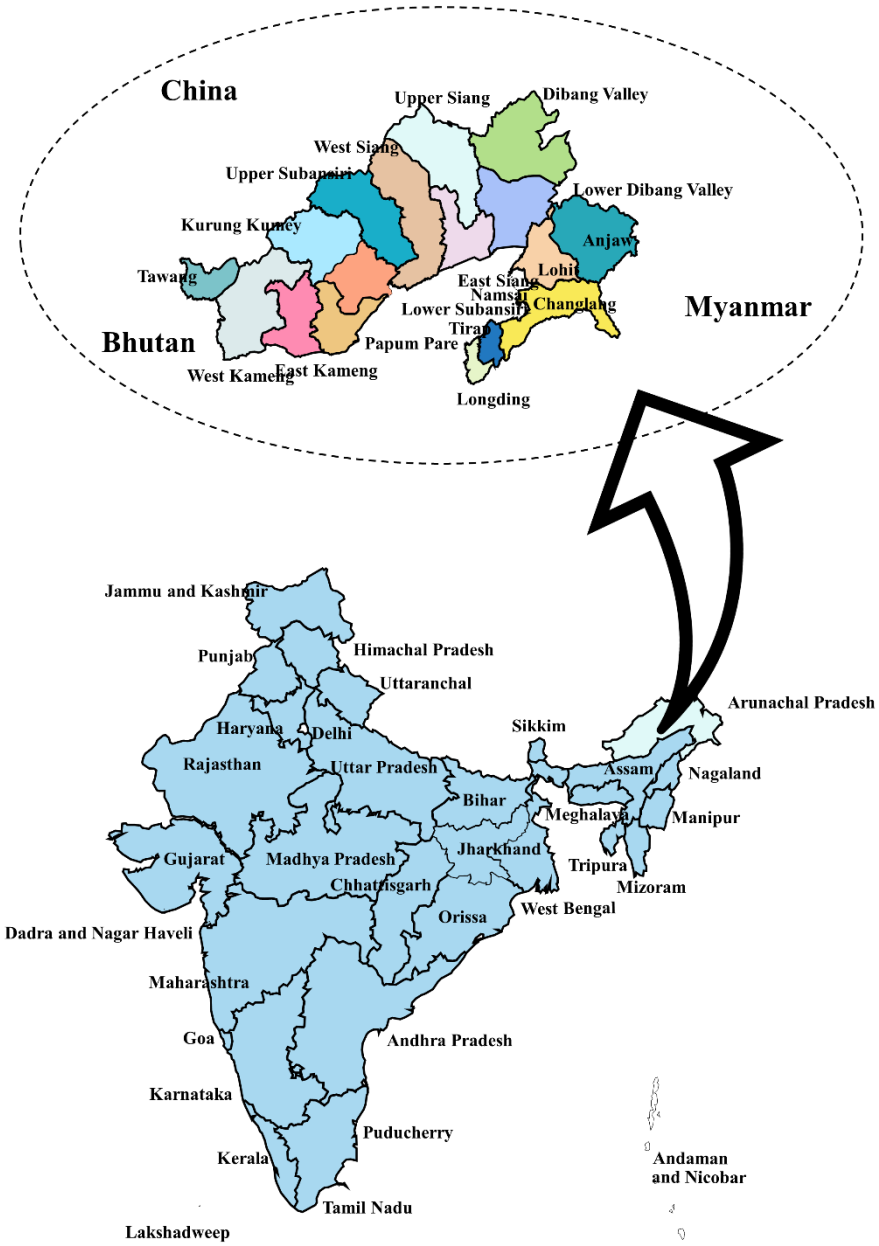


Figure A: Map of Arunachal Pradesh

Previous report on the NFM of Arunachal Pradesh

The NFM products of Arunachal Pradesh are considered rare, exotic, and unexplored. Till date, *Lactobacillus paracasei* and *Lactobacillus plantarum* of *chhurpi* of Arunachal Pradesh has been reported (Tomar et al., 2009).

Research gap

Extensive exploration of the NFM products have not been applied and moreover, the application of high-throughput sequencing technology has not been used to profile the microbial diversity in larger volumes. Additionally, probiotic evaluation of the isolates has also not been studied. Hence, this present Thesis aims to emphasize on the bacterial diversity of these exotic products and probiotic evaluation which could lead into the development of starter cultures with health beneficial properties.

Experimental design to study the objectives

In this present Thesis, before sample collection, survey, and documentation of the available NFM products was carried out in the first year. Following this, six rare/exotic NFM products which were available in Arunachal Pradesh were considered for this present study. These includes cow-milk *mar*, yak-milk *mar*; cow-milk *chhurpi*, yak-milk *chhurpi*, cow-milk *churkam* and yak-milk *churkam*. Based on the above-mentioned research gap, experiments were designed accordingly to fulfill the following objectives:

- Survey and documentation of the available NFM products of Arunachal Pradesh
- Isolation and characterization by phenotypic and genotypic (16S rRNA gene sequencing) methods
- Illumina-based next generation sequencing analysis for direct profiling of bacteria from samples

- Probiotic screening and evaluation using standard experimental tests which includes probiotic marker gene PCR amplification

Objectives

- To document the naturally fermented milk (NFM) products of Arunachal Pradesh.
- To identify the native microorganisms using phenotypic characterization
- To study the microbial diversity of NFM of Arunachal Pradesh using culture-dependent techniques.
- To study the microbial community in NFM by culture-independent technique.
- To determine some probiotics characters of isolates.

REVIEW OF LITERATURE

Naturally fermented milk (NFM) products are one of the popular and widely available ethnic fermented foods which are associated with different cultures all around the world (Tamang et al., 2020). These products are usually fermented from different animal milk source including buffalo, camel, donkey, ewe, goat, mare, yak, and cow (Faccia et al., 2020). Most NFM products are very similar to each other as they do share their similarity in the method of production and product characteristics (Zhong et al., 2016), thereby, leading to similar products with different local vernacular languages/dialects. The practice of milk fermentation is usually associated with cattle herders who depends their livelihood on cattle rearing (Tamang, 2010).

Fermented milk products of the world

Fermented milk/dairy products can be present almost all around the world. Yoghurt and cheese are probably the most common products where industrialized versions are available in the market. This is usually observed in the European countries, where single strain or a consortium of known beneficial microorganisms are used under optimum conditions. However, there are many NFM products which are still being prepared traditionally/indigenously in different regions of the world that are associated with different communities/races. Most NFM products are commonly available in continents like Asia, Africa, South America, and Europe; however, in North America and Australia, most fermented foods are only produced with defined starter cultures (Tamang et al., 2020). Traditional/Naturally fermented milk products are prepared from various domesticated animals which includes buffalo, camel, donkey, ewe, goat, mare, yak, and cow (Faccia et al., 2020) and they are usually available as yoghurt-like/fermented drink, fermented cream, artisanal-butter, buttermilk, and cheese-like (Fig. B). Some of the popular/common traditional fermented milk products of the world are as follows:

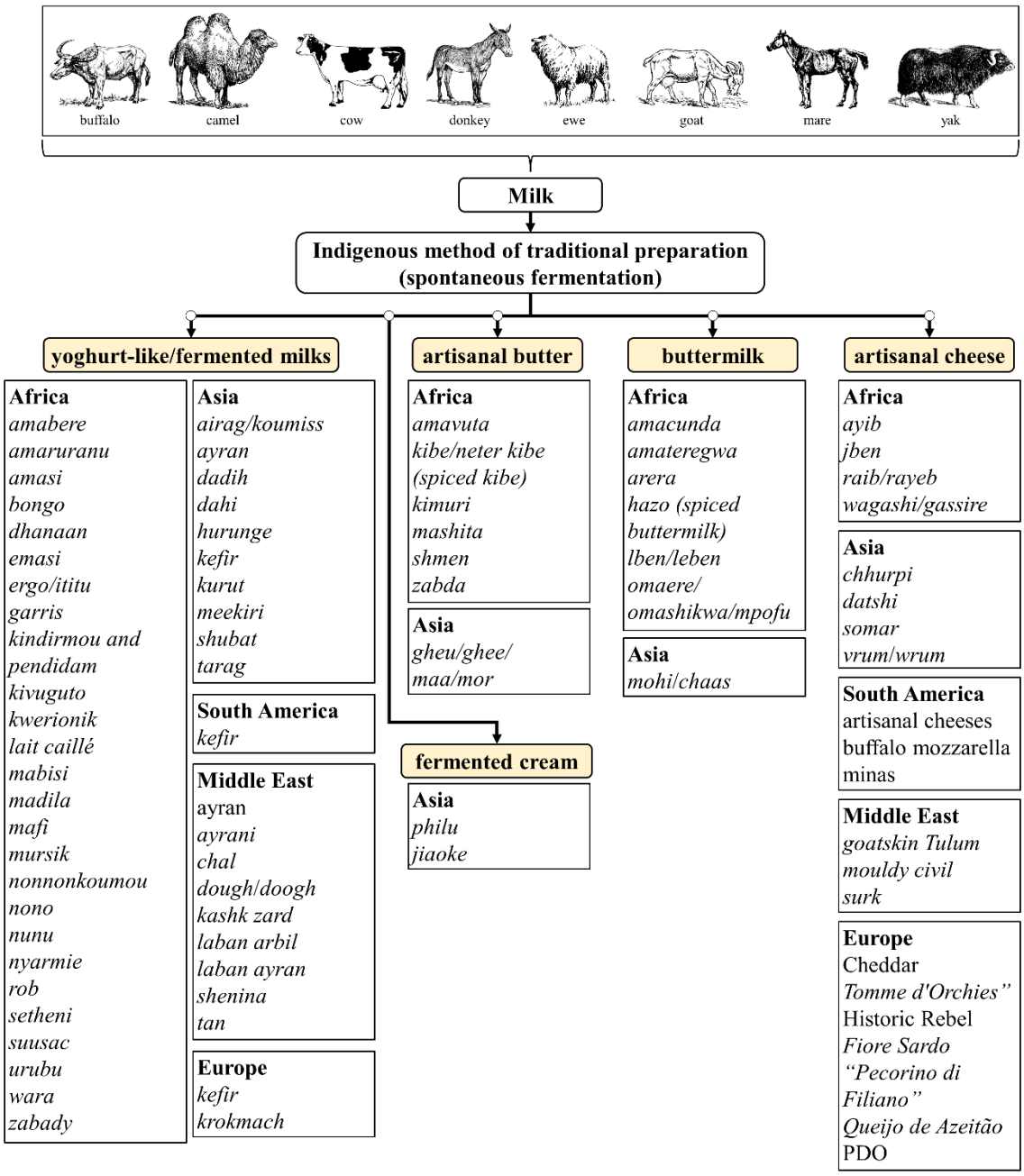


Figure B: Types of naturally/traditional fermented milk products of the world. Milk (raw/boiled) is usually fermented into four different types of products as a form of preservation and prolongation of milk which includes- yoghurt-like or fermented milks alike, fermented cream, artisanal butter, buttermilks, and artisanal cheeses.

NFM products of Africa and their microbial diversity

Yoghurt-like/fermented milk products

1) ***Amabere amaruranu***- It is a fermented milk usually prepared by the neolithic agro-pastoralist inhabitants of present-day Kenya, known as the *abagusii* (Nyambane et al., 2014). It is usually prepared from cow's milk and can be found in the Kisii regions located on the South-western part of the country. Using culture-dependent based study, lactic acid bacteria and yeasts have been identified as the fermenters with the help of bioMérieux analytical profile index (API) identification system (Nyambane et al., 2014). Recently, *Lactiplantibacillus plantarum* subsp. *plantarum* has been identified as the main LAB strain with potential probiotic attributes (Sichangi et al., 2020). Furthermore, *Epicoccum* spp. (fungal) and *Staphylococcus warneri* (non-fermenter) have also been identified (Sichangi et al., 2020).
2) ***Amasi***- It is a home-made yoghurt-like product that can be found in the eastern coastal regions of South Africa (KwaZulu-Natal) and Zimbabwe (Gwanda), which is commonly prepared from cow's milk (Todorov et al., 2007; Osvik et al., 2013). In Zimbabwe, it is also known as *mukaka wakakora*/*zifa* by the ethnic people known as *Shona* (*Bantu*) and the ethnic people, *Ndebele*, called it *amasi* (Gadaga et al., 1999). Using Denaturing gradient gel electrophoresis (DGGE, culture-independent method), the major LAB species associated with this product was *Lactococcus lactis*, where other minor LAB species observed, belonged to *Lactobacillus*, *Leuconostoc* and *Enterococcus* (Osvik et al., 2013). Predominant yeast identified includes- *Saccharomyces cerevisiae*, *Candida lusitaniae*, C. *colliculosa* and *S. dairenensis*; and minor species including *Dekera bruxillensis*, *C. lipolytica* and *C. tropicalis*, and *C. kefyr* (Gadaga et al., 2000).

3) ***Bongo***- Another popular milk product, named *bongo*, can be found in the western and central part of Uganda. It is also yoghurt-like product that is usually prepared from cow's milk (Mukisa et al., 2020). Mukisa et al. (2020) assessed the microbiological content of the product and reported the predominance of lactic acid bacteria and yeasts, but however, no further identification of the specific strains was studied.

4) ***Dhanaan***- It is popular fermented camel milk which is usually prepared in the Somali Regional State (Ethiopia) by spontaneous fermentation (and back slopping) at room temperature (Berhe et al., 2017). Next generation sequencing analysis using Ion Personal Genome Machine (PGM) revealed the predominance of LAB genera which comprises of *Streptococcus*, *Lactococcus*, and *Weissella*; other bacterial genera include *Acinetobacter*, *Brenneria*, *Buttiauxella*, *Clostridium*, *Cronobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Lelliottia*, *Obesumbacterium*, *Pectobacterium*, *Salmonella*, *Shigella*, *Shimwellia*, and *Tatumella* (Berhe et al., 2019).

5) ***Emasi***- It is another rarely available yoghurt-like NFM product usually prepared in the Hhohho region of the Kingdom of Eswatini (formerly Swaziland) (Simatende et al., 2019). It has been also reported to be predominated with LAB species which includes *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, and *Levilactobacillus brevis* (Simatende et al., 2019).

6) ***Ergo/ititu***- It is a fermented yoghurt-like product commonly prepared in Ethiopia, which is prepared from raw milk of cattle (Gonfa et al., 2001; Berhe et al., 2017). It has a thick smooth and semi-solid in appearance with pleasant odour and taste usually prepared by the smallholder farmers, particularly married women (Gonfa et

al., 2001). It is also known to be prepared from either goat or camel's milk in the lowland regions of Ethiopia (Gonfa et al., 2001). Identified LAB strain from this product have reported the presence of *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Streptococcus thermophilus*, *S. acidominimus*, *Enterococcus faecalis* var. *liquefaciens*, *S. bovis*, *S. mitis*, *S. agalactiae*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leu. lactis*, and *Lactococcus lactis* subsp. *lactis* (Gonfa et al., 2001; Mulaw et al., 2019). No yeast species have been reported from this product.

7) ***Garris***- *Garris* is another traditionally fermented milk products prepared from camel's milk via semi-continuous fed-batch fermentation (Sulieman et al., 2005), which is usually found prepared in countries like Sudan and Somalia. In Sudan, it is also known as *hameedh* or *humadah*- which can be translated as "sour" (Shori, 2012). It is a LAB-predominated product where several species reported includes *Enterococcus faecium*, *Lactobacillus acidophilus*, *Companilactobacillus alimentarius*, *Ligilactobacillus animalis*, *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *Carnobacterium divergens*, *Limosilactobacillus fermentum*, *Lb. gasseri*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *lactis*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lactococcus raffinolactis*, *Lacticaseibacillus rhamnosus*, *Streptococcus infantarius* subsp. *infantarius*, *S. lactis*, *S. lactis* subsp. *diactylactis* (Hassan et al., 2008; Abdelgadir et al., 2008; Ashmaig et al., 2009). Two yeast species have also been identified *viz*., *Kluyveromyces marxianus* and *Issatchenkia orientalis* (Abdelgadir et al., 2008).

8) ***Kindirmou*** **and** ***pendidam***- It is another fermented milk product which is yoghurt-like in nature and is usually prepared in Cameroon (Maroua, Garoua and Ngaoundere) (Maïworé et al., 2019; Sohanang et al., 2021). Another similar product

is also prepared in Cameroon, named *pendidam*. The difference between the two products is that *kindirmou* is usually prepared using fresh boiled cow milk which is incubated for 12 h (back-slopping process) and is consumed after addition of sugar; whereas *pendidam* serves as acidifying porridges that contains less milk fat and usually fermented for 48 to 72 h (back-slopping process) (Maïworé et al., 2019). Both culture-dependent and -independent studies have been used to study the microbial diversity of these products which revealed the presence of both lactic acid bacteria- *Pediococcus acidilactici*, *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*; and yeast species- *Galactomyces candidum, Candida parapsilosis, Torulaspora delbrueckii*, *Saccharomyces cerevisiae*/*paradoxus* and *Kluyveromyces marxianus* (Maïworé et al., 2019; Sohanang et al., 2021).

9) ***Kivuguto***- It is a fermented cow yoghurt-like product popularly prepared in Rwanda (Karenzi et al., 2013). LAB species identified includes- *Lactococcus lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc pseudomesenteroides* (Karenzi et al., 2012).

10) ***Kwerionik***- *Kwerionik* is a traditional cultured milk produced in Eastern Uganda (Nakavuma et al., 2011). Several LAB species have been identified using culture-dependent analysis which includes *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lacticaseibacillus casei* (Basonym: *Lactobacillus casei, Lactococcus lactis* subsp. *lactis, Enterococcus faecalis*, *Enterococcus faecium* and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Pinto et al., 2006; Nakavuma et al., 2012). Similarly, yeast species reported includes *Candida krusei, Candida kefyr, Kodamaea* (*Pichia*) *ohmeri*, *Candida intermedia*, *Candida lusitaniae*, *Candida pelliculosa*, *Candida lambica*, *Candida guilliermondii* and *Candida holmii* (Nakavuma et al., 2011).

11) ***Lait caillé***- It is a spontaneously fermented milk product prepared unpasteurized raw milk usually found in Northern Senegal (Parker et al., 2018) and Burkina Faso (Bayili et al., 2019). Predominant microorganisms reported includes LAB- *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Weissella paramesenteroides*, *Lactococcus lactis*, *Enterococcus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Acetobacter* spp., *Lactobacillus* spp., and yeasts- *Candida parapsilosis*, and *Saccharomyces cerevisiae* (Parker et al., 2018; Bayili et al., 2019).

12) ***Mabisi***- *Mabisi* is a traditional Zambian fermented milk which is usually prepared from cow's milk. Traditionally, it is spontaneously fermented using calabash/gourd as fermentation container, but however, plastic and metal containers have also been used for fermentation (Groenenboom et al., 2020). Two versions of the product are available depending on the cattle herders- *tonga* and *barotse* (or *lozi*) *mabisi*, and the cattle they reared are also commonly known by the same names (Moonga et al., 2021). Microbial profiles of *mabisi* revealed the predominance of lactic acid bacteria- *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*; with minor acetic acid bacteria- *Acetobacter*, and non-fermenters- *Acinetobacter*, *Aeromonas*, *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Kluyvera* (Schoustra et al., 2013; Moonga et al., 2020; Groenenboom et al., 2020; Moonga et al., 2021).

13) ***Madila***- It is a popular yoghurt-like fermented milk that is prepared in most South African countries particularly in Botswana (Tswana communities); and after 3-4 days of fermentation, it is collected whereby the whey is removed using a cloth (by hanging) (Ohenhen et al., 2013). Predominant bacterial species isolated and identified from this product includes- *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Levilactobacillus brevis*, *Limosilactobacillus fermentum*, *Lactococcus lactis*, and few other minor non-

fermenters/contaminants *viz*., *Staphylococcus aureus, Bacillus cereus,* and *Escherichia coli* (Ohenhen et al., 2013).

14) ***Mafi***- It is a spontaneous fermented milk product which is generally prepared in Lesotho (Gadaga et al., 2021). No microbial reports have been documented till date on the predominant fermenters.

15) ***Mursik***- It is another ethnic fermented milk product of Kenya which is associated with the Kalenjin community, which is prepared from cow and goat's milk and spontaneously fermented using calabash/gourd, locally known as *sotet* (Nduko et al., 2017). The most common microorganisms involved in the fermentation of *mursik* includes lactic acid bacteria- *Lentilactobacillus kefiri*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* (Basonym: *Lactobacillus fermentum*), *Levilactobacillus brevis* (Nieminen et al., 2013; Digo et al., 2017). Among the yeast species- *Candida krusei*, *Candida kefyr*, *Candida sphaerica*, and *Saccharomyces fermentati* were detected (Nieminen et al., 2013).

16) ***Nonnonkoumou***- This is a fermented curdled milk product found in Côte d'Ivoire or simply known as Ivory Coast (Christelle et al., 2021). This product has not been thoroughly studied for its microbiological content. Till date, only physiological properties and microbiological enumeration of the product have been explored (Christelle et al., 2020; Christelle et al., 2021). Though lactic acid bacteria were reported to be predominant, however, yeasts and fungal have also been enumerated with other pathogenic contaminants (Christelle et al., 2020). No taxonomic identification of the microorganism reported has also been studied.

17) ***Nono***- It is fermented yoghurt-like product usually prepared by the nomadic cattle-rearing ethnic groups of Nigeria, known as *fulani* (Bankole and Okagbue, 1992). It

is usually prepared from cow's milk but occasionally it is also prepared from goat's milk (Obadina et al., 2013). Several predominant species of lactic acid bacteria have been reported from this product which includes *Lactobacillus helveticus*, *Limosilactobacillus fermentum*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, and *Weissella* spp. and yeast genera including *Saccharomyces*, *Candida*, *Cyberlindnera*, *Meyerozyma*, *Trichosporon* and *Galactomyces*, where the most frequently species are *Saccharomyces cerevisiae* followed by *Candida glabrata* (Diaz et al., 2019; Ayanniran et al., 2020; Fagbemigun et al., 2021).

18) ***Nunu***- Another similar product, called *nunu*, is a popular fermented yoghurt-like product usually prepared in Ghana and other western part of Africa (Akabanda et al., 2010). The predominant LAB species reported includes *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, and *Leuconostoc mesenteroides*, whereas minor LAB species includes *Lactobacillus helveticus*, *Enterococcus faecium*, *Enterococcus italicus*, *Weissella confusa*, and *Lactococcus* spp. (Akabanda et al., 2013). On the other hand, predominant yeast species reported were *Pichia kudriavzevii*, and *Saccharomyces cerevisiae*, with other minor species includes- *Candida kefyr*, *Candida parapsilosis*, *Candida rugosa*, *Candida stellata*, *Candida tropicalis*, *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *Saccharomyces pastorianus*, *Yarrowia lipolytica*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces rouxii* (Akabanda et al., 2013).

19) ***Nyarmie***- It is another version of yoghurt-like fermented product in Ghana like *nunu*. For preparation of *nyarmie*, milk is first pasteurized at 65-75 °C for 30-45 min before fermentation unlike the preparation of *nunu* (Obodai and Dodd, 2006). Predominant isolated and identified LAB species reported includes *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp.

bulgaricus, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactococcus lactis* and one yeast species- *Saccharomyces cerevisiae* (Obodai and Dodd, 2006).

20) ***Rob***- It is a fermented yoghurt-like product which is prepared in Sudan (Abdelgadir et al., 2001). Isolated microbial species identified from rob includes LAB- *Limosilactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, and *Streptococcus salivarius*; and yeasts- *Saccharomyces cerevisiae*, and *Candida kefyr* (Abdelgadir et al., 2001; Abdullah and Osman, 2010).

21) ***Sethemi***- It is a traditional South African fermented yoghurt-like product which is fermented using gourds or clay pots (Kebede et al., 2007a). The predominance of LAB was reported from this product, however not identified, and yeast species viz., *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Cryptococcus curvatus*, *Cryptococcus humicola*, *Kluyveromyces marxianus*, and *Candida albicans* were identified (Kebede et al., 2007a; Kebede et al., 2007b).

22) ***Suusac***- It is fermented camel milk product that is prepared in Kenya and Somalia (Lore et al., 2005). It is reported to be predominated with LAB species and few yeast species. Identified LAB species includes- *Latilactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Ligilactobacillus salivarius*, *Lactococcus raffinolactis*, *Lactococcus lactis* subsp. *lactis*, *Streptococcus infantarius* subsp. *infantarius*, *Streptococcus thermophilus* and *Leuconostoc mesenteroides* subsp. *mesenteroides*; while identified yeast species includes *Saccharomyces cerevisiae*, *Candida famata*, *Candida guilliermondii*, *Candida inconspicua*, *Candida krusei*, *Candida lusitaniae*, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Geotrichum*

penicillatum, *Rhodotorula mucilaginosa*, *Trichosporon cutaneum*, and *Trichosporon mucoides* (Lore et al., 2005; Njage et al., 2011; Jans et al., 2012).

23) ***Urubu***- It is a yoghurt-like product of Burundi that is spontaneously fermented from raw cow's milk at room temperature using earthen pot or suitable container (Aloys and Angeline, 2009; Mattiello et al., 2018). Till date, no report on the microbial composition of this product has been documented.

24) ***Wara***- *Wara* is another ethnic Nigerian yoghurt-like product which may be eaten raw or fired in oil and is also known to be traditionally prepared by the *fulani* herdsmen (Adesulu-Dahunsi et al., 2020). Several LAB species have been identified which includes- *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Enterococcus faecium* and *Enterococcus faecalis* (Olasupo et al., 1994; Oguntoyinbo and Okueso, 2013; Abdulkarim et al., 2020; Olajugbagbe et al., 2020). No yeast communities/species have been reported from this product.

25) ***Zabady***- It is regarded as the oldest form of fermented yoghurt-like product in the world, especially in the Middle Eastern countries (particularly found in Egypt) (El-Baradei et al., 2008). Major LAB species identified from this product includes- *Streptococcus thermophilus*, *Lactococcus raffinolactis*, *Lactococcus garvieae*, *Lactococcus lactis*, *Leuconostoc citreum*, *Lactococcus garvieae*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus johnsonii* (El-Baradei et al., 2008).

Artisanal butter products

1) ***Amavuta***- It is another naturally fermented milk (artisanal butter) product that is locally found in Burundi, which is basically an artisanal butter formed after churning

of cow's milk (Aloys and Angeline, 2009; Mattiello et al., 2018). No microbiological study has been reported from this product so far.

2) ***Kibe***- It is basically an artisanal/traditional butter, commonly prepared in Ethiopia from cow, goat, or sheep milk with a white to light yellowish in appearance (Gonfa et al., 2001; Berhe et al., 2017). When boiling with spices, it is then called as *neter kibe* (traditional spiced butter) (Gonfa et al., 2001). Till date, no microbiological record is available of this product.

3) ***Mashita***- It is an artisanal butter which is prepared in western Uganda (Ongol and Asano, 2009). Till date, not much microbial diversity has been studied in this product only that of Ongol and Asano, where bacterial and yeast diversity have been profiled using culture-dependent and DGGE-based culture-independent study (Ongol and Asano, 2009). The predominance LAB species present in *mashita* includes Lacticaseibacillus *paracasei*, *Lactobacillus helveticus*, *Lactiplantibacillus plantarum* and *Schleiferilactobacillus perolens*, *Bifidobacterium* sp., *Enterococcus faecium*, *Levilactobacillus brevis*, *Lactobacillus acetotolerans, Lactococcus raffinolactis, Lactococcus lactis* subsp. *lactis* and *Streptococcus salivarius* (Ongol and Asano, 2009). Among the acetic acid bacteria (AAB) species, *Acetobacter aceti*, *Acetobacter lovaniensis*, *Acetobacter orientalis* and *Acetobacter pasteurianus* were detected (Ongol and Asano, 2009). *Brettanomyces custersianus*, *Candida silvae*, *Geotrichum* sp., *Issatchenkia occidentalis*, *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Trichosporon asahii* were main yeast species detected (Ongol and Asano, 2009).

4) ***Kimuri*** **and** ***amacunda***- It is a Rwandese artisanal butter produced after churning *kivuguto* and removal of *amacunda* (buttermilk) (Karenzi et al., 2013). No microbiological study has been reported from these products.

5) ***Shmen***- In the Saharan regions of North Africa, camel milk is also used for preparation of fermented products. *Shmen* is a traditional/artisanal butter that is found in Algeria, which is usually associated with the nomadic ethnic people known as *Tuareg* (or *Touareg*) (Mourad and Nour-Eddine, 2006). This product can be considered as rare as no other microbiological study exist apart from that reported by Mourad and Nour-Eddine, (2006). Using bioMérieux analytical profile index (API) identification system, few species of lactic acid bacteria and yeasts have been identified which includes *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus lactis* ssp. *cremoris*, *Lacticaseibacillus paracasei* subsp. *paracasei* (Basonym: *Lactobacillus paracasei* ssp. *paracasei*), *Leuconostoc pseudomesenteroides*, *Leuconostoc gelidum*, and *Enterococcus faecium*; with only one yeast identified- *Saccharomyces cerevisiae* (Mourad and Nour-Eddine, 2006).
6) ***Zabda***- *Zabda* is an artisanal butter, commonly prepared in Morocco, which resulted from churning of raw/fermented milk (Hamama, 1992). This product is also salted and allow to ferment anaerobic conditions for about 3-6 months with optional addition of aromatic plants to become more rancid, locally known as *smen* (Benkerroum and Tamime, 2004). *Lactococcus lactis* (*Lac. lactis* subsp. *lactis*, and *Lac*. *lactis* subsp. *lactis* biovar. *diacetylactis*) (Hamama, 1992).

Buttermilks

1) ***Amateregwa***- In Burundi, buttermilk is locally known as *amateregwa* (Aloys and Angeline, 2009; Mattiello et al., 2018). No microbiological study has been reported from this product.

2) ***Lben*/*leben***- It is a Moroccan fermented buttermilk which is prepared by simply allow milk to ferment under room temperature and is collected after the removal of the coagulated milk (Hamama, 1992). In Egypt, a similar product exists by the name *laban rayeb* (El-Gendy, 1983). *Lben* is mostly predominated with species of *Lactococcus*, *Leuconostoc* and *Enterococcus*, whereas *Lactobacillus* species were recorded in low number. Bacterial species includes- *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gilvus*, *Enterococcus hirae*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactococcus garvieae*, *Lactococcus lactis* (*Lac. lactis* subsp. *lactis*, and *Lac. lactis* subsp. *lactis* biovar. *diacetylactis*), *Leuconostoc citreum*, *Leuconostoc kimchii*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Pediococcus pentosaceus*, *Weissella cibaria*, *Weissella confusa*, *Weissella paramesenteroides*, and *Weissella viridescens*; and yeast species belong to *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* var *marxianus* (Hamama, 1992; Benkerroum and Tamime, 2004; Ouadghiri et al., 2009; Mangia et al., 2014).

3) ***Arera***- It is fermented milk products usually available in Ethiopia, which is basically a defatted sour milk/buttermilk (Berhe et al., 2017). No microbial composition has been reported.

4) ***Hazo***- It is another Ethiopian buttermilk which is usually mixed with pulses and grains with several herbs/spices- *Alium sativum* (Garlic), *Lepidium sativum* (garden cress), *Ruta chalepensis* (rue) *Ocimum basilicum* (basil), *Cuminum cyminum* (cumin), *Trachyspermum ammi* (adjwain seed), *Trigonella foenum-graecum* (fenugreek), *Piper igrum* (black pepper), *Nigella sativa* (nigella), *Zingiber officinale* (ginger), *Aframomum corrorima* (Ethiopian cardamom), *Curcuma domestica*

(turmeric) (Berhe et al., 2017). The addition of spices is to basically to maintain the quality and improve its nutritional values (Gebreselassie et al., 2012). No microbial composition has been reported.

5) ***Jben***- It is a cheese-like NFM product which resulted from a longer period of fermentation after draining off the whey from *raib* and is sometimes mixed with salt (Hamama, 1992). It is prepared from either goat or ewe milk and is usually found in the Maghreb countries which includes Algeria, Libya, Mauritania, Morocco, and Tunisia (Leksir et al., 2019). Several LAB species have been reported which includes- *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis Lentilactobacillus buchneri*, *Lactococcus lactis subsp. lactis*, *Lactococcus garvieae*, *Lactococcus raffinolactis*, *Leuconostoc lactis*, *Leuconostoc pseudomesenteroides*, *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus saccharominimus* and *Streptococcus* sp. (Hamama, 1992; Ouadghiri et al., 2006).

6) ***Omaere*/*omashikwa*/*mpofu***- In Namibia, buttermilk is one of the fermented milk products that usually available in Oshana, Ohangwena, Oshikoto, Omusati, and Omaheke regions (Misihairabgwi and Cheikhyoussef, 2017). It is usually prepared by collecting fresh milk into calabash, followed by the addition of *omunkuzi* (*Boscia albitrunca*) or *omukwa* (*Adansonia digitata*) roots and back-slopping process using old starter (Misihairabgwi and Cheikhyoussef, 2017). *Omaere* is also prepared in Angola and by the *Herero* (*Bantu*) ethnic groups of Botswana (Plaatjie, 2018). *Omashikwa* is characterized by bitter and rancid flavour, a high acidity, low pH, root-like taste, and slimy consistency (Bille et al., 2007). Through culture-dependent study, LAB species that have been reported includes- *Lactiplantibacillus plantarum*,

Lacticaseibacillus paracasei subsp. *paracasei*, *Lentilactobacillus kefiri*, *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, *Lactococcus lactis* and yeast species includes *Kazachstania unispora*, *Saccharomyces cerevisiae* and *Candida pararugosa* (Heita, 2014; Kutaa, 2017).

Artisanal cheese-like products

1) ***Ayib***- *Ayib* is another well-known fermented milk product usually prepared in Ethiopia which is basically an artisanal/cottage cheese, formed after heated in a clay pot for about 40-70 ^{o}C (Berhe et al., 2017); when added with spice, it is then called *metata ayib* (Geremew et al., 2015). LAB species reported from this product includes *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, and *Lactococcus lactis* (Girma and Aemiro, 2021). In Ethiopia, after the preparation of *ayib*, the removed byproduct acid whey is known as *aguat* (Berhe et al., 2017). No microbiological record is available of this product.
2) ***Raib***- In Morocco, raw milk is allowed to spontaneously ferment at room temperature for 1-3 days depending on the season. After fermentation, the coagulated milk is collected, which is locally called *raib*, which is consumed as such (Hamama, 1992). LAB associated with this product includes- *Enterococcus faecium*, *E. faecalis*, *E. hirae*, *E. durans*, *Lactococcus lactis* (*Lac. lactis* subsp. *lactis*, and *Lac. lactis* subsp. *lactis* biovar. *diacetylactis*), *Lactococcus garvieae*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leu. mesenteroides* subsp. *dextranicum*, *Lactiplantibacillus plantarum* (Hamama, 1992; Elotmani et al., 2002; Bendimerad et al., 2012; Moumene et al., 2016).

3) ***Rayeb***- It is a traditional fermented milk product prepared from raw buffalo's milk in rural areas of Egypt (Abd El Gawad et al., 2010). Using tentative identification, LAB species isolated includes- *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactococcus acidophilus*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Enterococcus faecium*, *Enterococcus durans*, *Streptococcus acidominimus* and *Aerococcus viridans* (Abd El Gawad et al., 2010).

4) ***Wagashi* (or *gassire*)**- In countries like Benin and Niger, milk is usually fermented into an artisanal cheese-like product locally known as *Wagashi* (or *gassire*) (Sessou et al., 2013). Using high-throughput Illumina MiSeq amplicon sequencing, only yeast communities have been studied, where the predominant yeast species reported are *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Candida parapsilosis*, and *Sagenomella keratitidis* (Sessou et al., 2019).

Rare traditional milk products of Africa and Middle East

Some of the rare and unexplored traditional fermented milk products in Africa includes- *mabobo* (Madagascar), *chambiko* (Malawi), *mame* (Tanzania), *umlaza*/*mutivi* (Zimbabwe), *mashoronga* (Zimbabwe), *Lee Naga a Agbora* (Uganda) (Kebede, 2005; Akaichi and Revoredo-Giha, 2014). The distribution of the NFM products documented in Africa is depicted in the Fig. C.

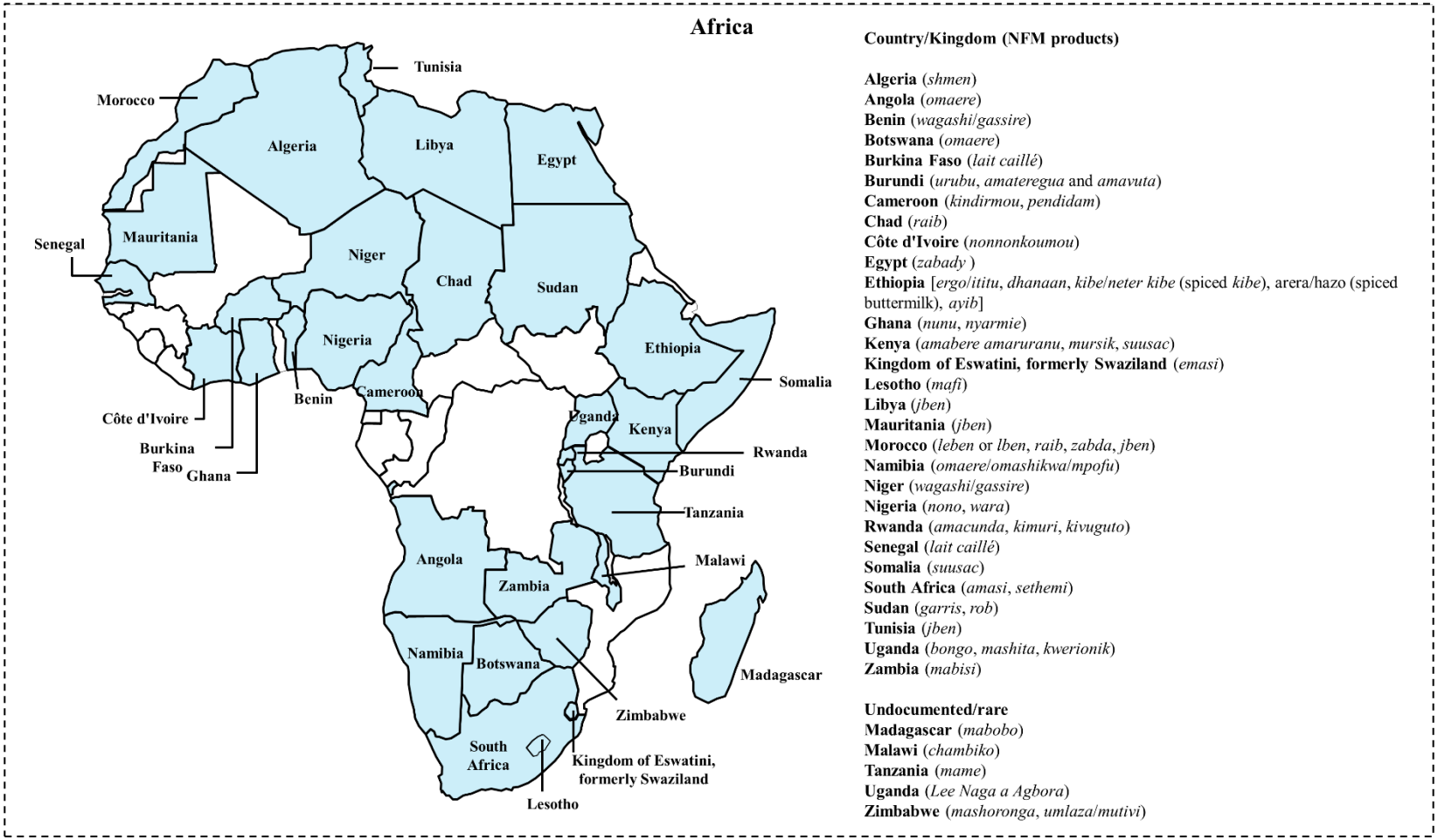


Figure C: Naturally/traditional fermented milk products prepared in the African countries

NFM products of Asia (including Russia, Middle East), South America, Europe, and their microbial diversity

Yoghurt-like/fermented milk products

1) ***Airag***- It (or *koumiss*) is a traditionally fermented milk beverage that is prepared in Mongolia, Kazakhstan, Kyrgyzstan, and some Central Asian regions of Russia, which is prepared form mare's milk (Watanabe et al., 2008). On the other hand, when it is prepared from cow, yak, ewe, goat, and camel's milk, it is termed as *tarag* or *isgelen tarag* (when fermented for a longer time) (Uchida et al., 2007) (Watanabe et al., 2008). Microorganisms involved in the fermentation of airag includes LAB species- *Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lentilactobacillus kefiri, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum,* and *Companilactobacillus farciminis,* whereas yeast species includes- *Saccharomyces dairensis, S. cerevisiae, Issachenkia orientalis, Kluyveromyces marxianus,* and *Kluyveromyces wickerhamii* (Uchida et al., 2007; Watanabe et al., 2008). In tarag, reported LAB species includes- *Lactobacillus helveticus, Lentilactobacillus kefiri, Lactobacillus fermentum, Lacticaseibacillus paracasei* and *Lactobacillus acetotolerans,* and *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus*; and yeast species- *Saccharomyces cerevisiae, Issatchenkia orientalis*, and *Kazachstania unispora* were reported (Uchida et al., 2007; Watanabe et al., 2008). Using 16S rRNA clone library and denaturing gradient gel electrophoresis (DGGE) major bacterial diversity revealed the presence of *Lentilactobacillus hilgardii, Lentilactobacillus kefiri, Lentilactobacillus parakefiri, Lacticaseibacillus paracasei, Lactiplantibacillus pentosus, Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lactococcus lactis* subsp. *lactis, Streptococcus thermophilus, Enterococcus hirae, Bacillus pumilus, Bacillus safensis, Paenibacillus* sp.,

Exiguobacterium profundum, *Kocuria rhizophila*, *Acinetobacter calcoaceticus*, *Gluconobacter cerinus*, *Psychrobacter* sp., and *Acetobacter pasteurianus* (Ringø et al., 2014).

2) ***Ayran***- It is a yoghurt-like fermented product commonly prepared in Turkey (Baruzzi et al., 2016), Kazakhstan, (Zhadyra et al., 2021) and in Russia (Kaledina et al., 2021). It is also known by other names in countries- *ayrani* (Cyprus), *dough*/*doogh* (Iran), *laban arbil* (Iraq), *laban ayran* (Lebanon), *laban ayran* (Syria), *shenina* (Jordan), and *tan* (Armenia) (Baruzzi et al., 2016). LAB species reported from culture-dependent and -independent includes- *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Limosilactobacillus fermentum*, and *Lacticaseibacillus paracasei* (Baruzzi et al., 2016; Zhadyra et al., 2021).

3) ***Dadih***/***dadiah***- It is a fermented buffalo milk which is prepared by the ethnic group, Minangkabau (a.k.a. Minang) of Indonesia (Arnold et al., 2021). Culture-independent analysis have revealed the predominance of LAB genera- *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Acetobacter*, and *Bifidobacterium*; with the detection of unwanted or contaminant genera viz., *Klebsiella*, Chryseobacterium, *Acinetobacter*, *Raoultella*, *Serratia*, *Corynebacterium*, *Staphylococcus*, *Stenotrophomonas*, and *Frateuria* (Sukma et al., 2018; Fatdillah et al., 2021). Culture-dependent study have also reported the presence of potential probiotic bacteria viz., *Lactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Enterococcus faecium* (Collado et al., 2007; Amelia et al., 2020).

4) ***Dahi***- One of the most popular fermented yoghurt-like product available in countries like Pakistan, Bangladesh, Nepal, Bhutan, and India (Tamang et al., 2020).

a. ***Dahi* (Bangladesh)**- Culture-dependent analysis of dahi have revealed the predominance of LAB species including- *Streptococcus bovis*, *Limosilactobacillus fermentum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Enterococcus faecium*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus raffinolactis* and *Pediococcus pentosaceus* (Harun-ur-Rashid et al., 2007). Culture-independent analysis have also shown the predominance of LAB species with the predominance of Firmicutes-associated *Lactobacillus*, *Streptococcus*; and minor genera Proteobacteria-associated *Acinetobacter*, Enterobacteriaceae-associated *Pseudomonas*, and *Micrococcaceae* (Nahidul-Islam et al., 2018). On the other hand, predominant yeast genera revealed the presence of *Kodamaea*, *Clavispora*, *Candida*, and *Tricosporon*, with minor genera *Moniliera*, *Syncephalastrum*, and *Lichtheimia*; with predominant species *Kodamaea ohmeri*, followed by *Clavispora lusitaniae*, *Candida parapsilosis*, *Trichosporon* sp., and *Candida tropicalis* (Nahidul-Islam et al., 2018).

b. ***Dahi* (Pakistan)**- Culture-dependent analysis of dahi from Pakistan have reported the predominance of LAB species which comprises of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lacticaseibacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Lactococcus lactis*, *Enterococcus mundtii* (Soomro and Masud, 2007; Mahmood et al., 2013; Nawaz et al., 2019).

c. ***Dahi* (Bhutan)**- Dahi is also very popular in Bhutan which is prepared on the daily basis by spontaneous fermentation and back slopping process (Shangpliang et al., 2017). Culture-dependent analysis have reported the presence of potential probiotic strain viz., *Enterococcus faecium* (Shangpliang et al., 2017).

d. ***Dahi* (Nepal)**- In Nepal, *dahi* is also prepared daily by the Nepali communities. Culture-dependent bacterial study has reported the presence of LAB species which comprises of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Limosilactobacillus fermentum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Koirala et al., 2014; Bhattarai et al., 2016).

e. ***Dahi* (India)**- In India, dahi can be found in most households and is one of the important traditional fermented milk products mostly prepared from cow or buffalo's milk. When prepared from yak's milk, it is also known as shyow (commonly consumed by the Tibetans) (Dewan and Tamang, 2007). Culture-dependent study of dahi have reported the presence of *Loigolactobacillus bifermentans*, *Companilactobacillus alimentarius*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactiplantibacillus pentosus*, *Lactiplantibacillus paraplantarum*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *tructae*, *Leuconostoc mesenteroides* and *Enterococcus italicus* (Dewan and Tamang, 2007; Ghatani and Tamang,

2017; Rai, 2020; Tirwa et al., 2021). Culture-independent analysis using next-generation Illumina MiSeq-based amplicon sequencing revealed the predominance of Firmicutes viz., *Lactococcus lactis*, followed by *Lac. raffinolactis*, *Leuconostoc mesenteroides*, *Leu. pseudomesenteroides*, *Lactobacillus helveticus*, *Lb. gasseri*, and *Proteobacteria* viz., *Acetobacter pasteurianus*, *Acetobacter syzygii*, *A. lovaniensis*, and *Pseudomonas fluorescens* (Rai, 2020).

5) ***Gioddu***- It is an Italian acidulous milk (acid-alcoholic fermented milk) which is prepared from ovine or goat's milk (Maoloni et al., 2020). It is also known by different names viz., *miciuratu*, *mezzoraddu* or *latte ischidu*. Culture-independent analysis detected the presence of LAB species- *Lactobacillus delbrueckii* (predominant), followed by *Streptococcus thermophilus*, *Lactobacillus kefiri*, *Lactococcus lactis*; yeast species- *Kluyveromyces marxianus* (predominant), *Galactomyces candidum*, *Geotrichum Galactomyces*, *Pichia cactophila*, *Glomus hyderabadensis*, *Saccharomyces cerevisiae* and few other minor species belonging to *Alternaria*, *Cladosporium* and *Aerobasidium* were also reported (Maoloni et al., 2020).

6) ***Gwell***- It is a traditional mesophilic fermented milk product commonly prepared in Brittany region (France) by back-slopping (von Gastrow et al., 2020). Culture-dependent and culture-independent analysis revealed the predominance of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, and *Staphylococcus warnerii* and few eukaryotic species- *Geotrichum candidum* (fungi), *Kazachstania servazii* (yeast), and *Yarrowia lipolytica* (yeast) (von Gastrow et al., 2020).

7) ***Hurunge***- It is Mongolian fermented milk products which is usually prepared from cow, horse, camel, sheep, and goat's milk (Shuangquan et al., 2006). Culture-dependent study revealed the predominance of *Lactococcus raffinolactis*, followed by other LAB species which included *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *cremoris*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus* casei, *Lactobacillus kefiranofaciens*, *Lactobacillus acetotolerans*, and *Lactobacillus homohiochii*; and yeast species- *Candida kefyr*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* var. *lactis*, *Candida krusei* and *Candida valida* (Shuangquan et al., 2006).

8) ***Kashk zard***- It is a famous Persian fermented milk product, also commonly known as yellow curd (Pakroo et al., 2020). Culture-independent analysis revealed the predominance of Firmicutes LAB species which includes *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Limosilactobacillus reuteri*, *Limosilactobacillus vaginalis*, *Lacticaseibacillus zeae*, *Lactobacillus delbrueckii*, *Limosilactobacillus pontis*, *Limosilactobacillus fermentum*, *Pediococcus acidilactici*, and *Streptococcus thermophilus* (Pakroo et al., 2020).

9) ***Kefir***- It is one of the most popular fermented acidic-alcoholic milk drinks in the world which is composed of a symbiotic fermentation of lactic acid bacteria and yeasts within the kefir grain matrix (Prado et al., 2015; Bourrie et al., 2016). Kefir is probably one of the popular fermented milk products where huge microbial diversity study has been explored and is also found in many countries which includes Asia- Tibet (China) and Taiwan (China), Turkey; Africa- South Africa; Asia and Europe- Russia; Europe- Bosnia and Herzegovina, Belgium, Czech Republic/Czechia, Estonia, Greece, Hungary, Ireland, Italy, Latvia, Norway, Poland, Romania, Slovakia, and Ukraine; South America- Argentina and Brazil (Bengoa et

al., 2019). Predominance of lactobacilli have been reported from various studies which includes- *Lactobacillus acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. delbrueckii*, *Lb. gallinarum*, *Lb. garvieae*, *Lb. gasseri*, *Lb. helveticus*, *Lb. instestinalis*, *Lb. johnsonii*, *Lb. kalixensis*, *Lb. kefiranofaciens*, *Lb. kefirgranum*, *Furfurilactobacillus rossiae*, *Lacticaseibacillus casei*, *Lactic. paracasei*, *Lactic. rhamnosus*, *Lactiplantibacillus pentosus*, *Lactiplantibacillus plantarum*, *Latilactobacillus curvatus*, *Latilactobacillus sakei*, *Lentilactobacillus buchneri*, *Lentilactobacillus diolivorans*, *Lentilactobacillus kefiri*, *Lentilactobacillus otakiensis*, *Lentilactobacillus parabuchneri*, *Lentilactobacillus parafarraginis*, *Lentilactobacillus parakefiri*, *Lentilactobacillus rapi*, *Lentilactobacillus sunkii*, *Levilactobacillus brevis*, *Ligilactobacillus salivarius*, *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, and *Liquorilactobacillus satsumensis* (Bourrie et al., 2016). Among the other LAB species lactococcin- *Lactococcus garvieae*, *Lac. lactis* subsp. *lactis* biovar *diacetylactis*, *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *cremoris*, were reported followed by *Leuconostoc mesenteroides* subsp. cremoris, *Leu. mesenteroides* subsp. *mesenteroides*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, *Oenococcus oeni*, *Pediococcus claussenii*, *Ped. damnosus*, *Ped. halophilus*, *Ped. lolii*, *Ped. pentosaceus*, *Streptococcus thermophilus*, *Str. durans*, *Str. salivarius* subsp. *thermophilus*, and *Weissella viridescens* (Bourrie et al., 2016). Other fermenters reported from kefir includes- acetic acid bacteria- *Acetobacter sicerae*, *A. orientalis*, *A. lovaniensis*, and non-Firmicutes LAB species- *Bifidobacterium breve*, *B. choerinum*, *B. pseudolongum*, and *B. longum* (Bourrie et al., 2016). Yeast and fungal species reported from kefir includes- *Candida maris*, *C. holmii*, *C. inconspicua*, *C. kefyr*, *C. lipolytica*, *C. sake*, *C. friedrichii*, *Cryptococcus humicolus*, *Cr.* sp. Vega 039, *Cyberlindnera jadinii*, *Davidiella tassiana*, *D.*

bruxellensis, *Dekkera anomala*, Dioszegia *hungarica*, *Dipodascus capitatus*, *Eurotium amsteldami*, *Ganoderma lucidum*, *Geotrichum candidum*, *Heterbasidion annosum*, *Issatchenkia orientalis*, *Kazachstania aerobia*, *Ka. barnettii*, *Ka. exigua*, *Ka. servazzii*, *Ka. solicola*, *Kluyveromyces lactis*, *Kluv. marxianus*, *Kluv. marxianus* var. *lactis*, *Malassezia pachydermatis*, *Microdochium nivale*, *Naumovozyma castelli*, *Penicillium* sp. Vega 347, *Peziza campestris*, *Pichia fermentans*, *Saccharomyces cariocanus*, *Sac. cerevisiae*, *Sac. humaticus*, *Sac. turicensis*, *Sac. unisporus*, *Teratosphaeria knoxdaviesii*, *Torulaspora delbrueckii*, *Trichosporon coremiiforme*, *Wallemia sebi*, *Yarrowia lipolytica*, and *Zygosaccharomyces lentus* (Bourrie et al., 2016).

10) ***Krokmach***- It is a soft and creamy-like traditional Bulgarian fermented dairy product which contains high fat and salt content and resembles soft smear cheese rather than yoghurt (Dimov, 2021). It is predominated with LAB species with *Lactococcus lactis* subsp. *lactis* as the most abundant species followed by *Exiguobacterium* sp., *Kluyvera georgiana*, and few other minor bacteria including *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus garvieae* subsp. *garvieae*, *Enterococcus faecalis*, Aeromonas sp., *Apilactobacillus kunkeei*, *Megamonas*, *Klebsiella* sp., *Aeromonas* sp., and Escherichia-Shigella (Dimov, 2021).

11) ***Kurut***- It is a naturally fermented yoghurt-like product (which can also be made dried) which is prepared from yak's milk and is commonly found in Tibet (China) (Liu et al., 2012). Predominant LAB species identified from this product includes *Lactococcus lactis* ssp. *lactis*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Lacticaseibacillus casei* and *Acetobacter* sp. (Chen et al., 2010; Liu et al., 2012).

12) ***Långfil* and *Filmjölk***- *Långfil* is Swedish fermented yoghurt-like product which is of ropy and slimy characteristics (Widyastuti et al., 2021), which is due to the presence of exopolysaccharide-producing LAB species- *Lactococcus lactis subsp. lactis* and *Lactococcus lactis subsp. cremoris*. On the hand, *Filmjölk* is very similar to *långfil*, but little less sour in comparison to yoghurt, which is usually prepared by *Lactococcus lactis* and *Leuconostoc mesenteroides* (Hati et al., 2019).

13) ***Meekiri*/*mee-deekiri***- It is curd-like fermented milk product that is prepared and localized only to Sri Lanka which is prepared only from water buffalo's milk (Priyashantha et al., 2021). Culture-dependent study have revealed the presence of LAB species which includes *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. bulgaricus, *Limosilactobacillus fermentum*, *Latilactobacillus curvatus*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lactobacillus helveticus*, *Lacticaseibacillus casei*, *Streptococcus thermophilus*, *S. lactis*, *Micrococcus* spp., *Bacillus* spp. and yeast- *Saccharomyces cerevisiae* (Dekumpitiya et al., 2016; Adikari et al., 2021).

14) ***Shubat***- *Shubat* is a fermented camel milk, which is less thick than yoghurt commonly found in China (Xinjiang) and Kazakhstan, whereas in Turkey it is known as *chal* (Rahman et al., 2009; Shori, 2012). Culture-dependent and culture-independent analysis showed the predominance of LAB species which comprising of *Latilactobacillus sakei*, *Lactobacillus helveticus*, *Levilactobacillus brevis*, *Lactobacillus delbrueckii*, *Lactobacillus kefiri*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, and *Weissella hellenica* (Rahman et al., 2009; Zhadyra et al., 2021). Yeast species reported includes *Kluyveromyces marxianus*, *Kazachstania unispora*, and *Candida ethanolica* (Rahman et al., 2009).

15) ***Viili***- It is a mesophilic yoghurt-like fermented milk product commonly found in Finland, and is also present in Taiwan (Wang et al., 2008; Kahala et al., 2008). Predominant isolated LAB species includes- *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*; and predominant yeasts- *Kluyveromyces marxianus*, *Saccharomyces unisporus* and *Pichia fermentans* (Wang et al., 2008; Kahala et al., 2008).

Fermented creams

1) ***Jiaoke***- It is a traditional fermented cream of Inner Mongolia (China) made from the fat separated from the fermented milk (Gong et al., 2010; Fan et al., 2020). Microbiota associated with *Jiaoke* includes *Lactococcus lactis*, *Lactococcus garvieae*, *Lacticaseibacillus casei*, *Lactobacillus helveticus*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus italicus*, *Enterococcus gilvus*, *Streptococcus thermophilus*, *Streptococcus gallolyticus*, and *Leuconostoc lactis* (Fan et al., 2020).

2) ***Philu* (or *philuk*)**- It is indigenous cream-like milk product consumed and prepared by the Sikkimese (Bhutia and Sherpa communities) which is either prepared from either cow or yak's milk (Dewan and Tamang, 2007). Culture-dependent analysis reported the predominance of LAB species which includes *Loigolactobacillus bifermentans*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* (Dewan and Tamang, 2007; Rai, 2020).

Artisanal butter products

Gheu- It is an artisanal butter usually prepared in Nepal, Sikkim and Darjeeling Hills from cow or yak's milk by churning raw/boiled milk using a traditional equipment called *theki* (Tamang, 2010). It is also known by other names depending on the dialects, like *ghee* or *makhan* in Hindi, *maa* in Tibetan, and *mor* in Lepcha (Tamang, 2010). No culture-dependent study has been analyzed, however, through culture-independent analysis, *Lactococcus piscium* was the predominant species, which was followed by *Pseudomonas fluorescens*, *Lactococcus lactis*, *Lactobacillus helveticus*, *Lactococcus raffinolactis*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Lactobacillus gasseri*, and uncultured bacteria (Rai, 2020).

Buttermilks

Mohi- It is another popular fermented milk product commonly prepared in India which is traditional buttermilk and in some parts of India, it is also known as "*chaas*" (Mallappa et al., 2020). Culture-dependent study have revealed the presence of *Companilactobacillus alimentarius*, *Lacticaseibacillus rhamnosus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Leuconostoc mesenteroides* (Dewan and Tamang, 2007; Ansari et al., 2019; Rai, 2020). Pyrosequencing-based next generation sequencing study showed the predominance of *Lactobacillus delbrueckii* and *Streptococcus thermophilus*, followed by other minor species which comprises of *Limosilactobacillus fermentum*, *Lactobacillus johnsonii*, *Lactobacillus helveticus*, *Streptococcus mutans*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Dictyoglomus turgidum*, *Enterococcus faecalis*, *Macrococcus caseolyticus*, *Methylobacterium populi*, *Methylobacterium radiotolerans*, *Psychrobacter arcticus*, *Ralstonia solanacearum*,

Thermoanaerobacter sp. *Synechocystis* sp., and *Bartonella quintana* (Jayashree et al., 2013).

Artisanal cheese-like products

1) ***Chhurpi*** **(hard and soft)-** In the Himalayan regions- Sikkim (Sherpas calls it "*sherkam*"), Darjeeling, Bhutan, Arunachal Pradesh, Ladakh and Nepal, artisanal/cottage cheese is commonly known as *chhurpi* (either as soft or hard variety) (Tamang, 2021). In Bhutan, the soft-variety chhurpi and hard-variety are locally known as *datshi* and *chugo*/*churkam* respectively (Shangpliang et al., 2017). Bacterial-based culture-dependent studies have revealed the presence of *Lentilactobacillus kefiri*, *Lactiplantibacillus plantarum*, *Companilactobacillus alimentarius*, *Lentilactobacillus hilgardii*, *Latilactobacillus curvatus*, *Limosilactobacillus fermentum*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Enterococcus faecium*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *cremoris*, *Enterococcus faecalis*, *Enterococcus pseudoavium* (Tamang et al., 2000; Dewan and Tamang, 2007; Rai, 2020). Using culture-independent analysis, bacterial diversity reported includes the predominance of *Lactobacillus helveticus* and *Lactococcus lactis*, followed by *Acetobacter syzygii*, *Acetobacter pasteurianus*, *Acetobacter lovaniensis*, *Leuconostoc pseudomesenteroides*, *Lactobacillus delbrueckii*, *Staphylococcus cohnii*, *Hafnia* alvei, *Gluconobacter oxydans*, *Leuconostoc mesenteroides* and *Pseudomonas fluorescens* (Rai, 2020). Yeast species identified from soft-*chhurpi* includes *Kluyveromyces marxianus*, *Issatchenkia orientalis*, *Candida parapsilosis* and *Saccharomyces cerevisiae* (Rai et al., 2016).

2) ***Somar***- It is another form of chhurpi commonly prepared in Sikkim, which is usually fermented (anaerobic condition) for about 15 days (Dewan and Tamang, 2007). It is also a rare NFM product and only two LAB species have been reported through culture-dependent analysis *viz*., *Lacticaseibacillus paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *cremoris* (Dewan and Tamang, 2007).

3) ***Vrum***/***wrum***- It is type of an artisanal cottage cheese that is usually prepared in Inner Mongolia (China) (Yamei et al., 2019). The predominance of *Lactococcus lactis*, *Lactobacillus kefiranofaciens*, *Streptococcus salivarius*, *Lactobacillus helveticus*, *Lentilactobacillus kefiri*, *Acetobacter orientalis*, and *Lentilactobacillus diolivorans*; and *Acetobacter malorum* (acetic acid bacteria) was reported; with fungal species including- *Kazachstania unispora*, *Saccharomyces cerevisiae*, *Trichosporon asahii*, *Kluyveromyces marxianus*, *Mucor racemosus*, *Kluyveromyces lactis*, *Pichia fermentans*, and *Mucor circinelloides* (Yamei et al., 2019). Recently, culture-independent Illumina MiSeq amplicon sequencing revealed the predominance of species which includes- *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Pediococcus acidilactici*, *Leuconostoc mesenteroides*, *Lactococcus raffinolactis*, and *Pseudomonas brenneri* (Liang et al., 2021).

4) **Artisanal cheeses**- Cheeses are another popular fermented milk products, which are vastly prepared all around the world spanning different continents. The most predominant fermenting members in cheese are species belonging to the families- Lactobacillaceae, Streptococcaceae, Enterococcaceae, and Leuconostocaceae, with *Lactococcus lactis* and *Streptococcus thermophilus* as the most common two species commonly detected in most cheese varieties, both core and rind (Dugat-Bony et al., 2016; Choi et al., 2020). In *surk* (a traditional cheese which is usually prepared in the East Mediterranean region including Turkey, Syria, Lebanon, and the Middle

East), predominant LAB isolated comprises of *Lactiplantibacillus plantarum*, *Companilactobacillus alimentarius*, *Ligilactobacillus acidipiscis*; non-LAB *Staphylococcus sciuri*, *Bacillus* sp., *Staphylococcus lentus*, *Bacillus pumilus*, *Bacillus amyloliquefaciens* (Esen and Çetin, 2021). Queijo de Azeitão PDO cheese, a traditional cheese of Portugal revealed the predominance of *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lacticaseibacillus zeae* and *Lentilactobacillus kefiri* (Cardinali et al., 2021). The core bacterial genera reported from Historic Rebel (HR) cheese was predominantly observed (99%) by Firmicutes-associated genera including *Streptococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc* and *Pediococcus* (Turri et al., 2021), which is similar to that reported from cheddar cheese (Afshari et al., 2020; Camargo et al., 2021). In Kazak artisanal cheese, genera- *Lactobacillus*, *Lactococcus, Acinetobacter*, *Anoxybacillus*, *Macrococcus, Acetobacter*, *Kurthia*, *Lelliottia,* and *Leuconostoc* were reported to be the key fermenting bacteria (Zheng et al., 2021). Shotgun metagenomics sequencing analysis of artisanal cheese of Argentina showed the bacteriocinogenic potential of the cheese microbiome which correlate to the presence of *Lactococcus* and *Enterococcus* (*E. faecium*) (Suárez et al., 2020). Core microbiota of minas artisanal cheese (Brazil) showed the presence of core microbiota which includes *Lactococcus lactis, Streptococcus salivarius, S. thermophilus* and *Acinetobacter johnsonii* (Nero et al., 2021). Culture-dependent and PCR-DGGE-based culture-independent approach revealed the predominance of *Streptococcus gallolyticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus lutetiensis, Streptococcus* spp., and *Enterococcus hirae* in traditional Turkish goatskin *Tulum* cheese, with *Enterococcus* spp. and *Lactococcus* spp. were predominated in culture-based study of the cheese ripening (Demirci et al., 2021). LAB species- *Leuconostoc mesenteroides*, *Lacticaseibacillus casei*,

Limosilactobacillus fermentum, and *Enterococcus* sp. were also isolated and reported to be prevalent in all stages of Brazilian buffalo mozzarella cheese fermentation (Silva et al., 2021). Yeasts are also the other fermenting microbes commonly found in many artisanal cheeses. *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *Debaryomyces hansenii*, *Pichia fermentans* and *Candida zeylanoides* were reported from traditional mouldy civil cheese of Turkey (Yildiz et al., 2021). Species *Debaryomyces hansenii*, *Candida zeylanoides*, *Yarrowia lipolytica*, and *Kluyveromyces lactis* were also reported from skyr (Esen and Çetin, 2021). The detection of potential probiotic yeast- *Kluyveromyces marxianus*, isolated from *Fiore Sardo* cheese, Sardinia (Italy) is also of great importance of yeasts' presence in cheese fermentation (Fadda et al., 2017). *Pichia kudriavzevii*, *Kluyveromyces marxianus* and *Kluyveromyces lactis* were also reported to be the predominant species in Kazak artisanal cheese (Zheng et al., 2018). *Debaryomyces hansenii* (halotolerant yeast species) was the predominant yeast species in the salted "Pecorino di Filiano" cheese, with minor species including *Kluyveromyces lactis* and *Dekkera anomala* (Capece and Romano, 2009). Among fungal species, *Penicillium roqueforti* and *Debaryomyces hansenii* were reported to be predominant in traditional Turkish cheese (Onmaz et al., 2021). Additionally, unusual fungal species- *Geotrichum candidum* and *Cladosporium cladosporioides* were also detected in "Tomme d'Orchies" cheese of France (Ceugniez et al., 2017).

Rare traditional milk products of Europe and Middle Eastern Asia

In some parts of Europe, there are fermented milk which are rare and very few literatures are available on their microbial diversity. *Blaand* is a one of the rare NFM product traditionally prepared in Scotland, Iceland, and Norway which is acidic and has low alcohol content (Kaur et al., 2019). *Tätmjölk* is another Scandinavian traditional yoghurt-like product which is prepared by *Lactococcus lactis* and EPS-producing *Leuconostoc mesenteroides* subsp. *cremoris* (Fondén et al., 2006). *Surmjölk* and *filbunke* are also very similar to *tätmjölk* but are usually prepared without the EPS-producing bacteria; and *skyr* is mostly a thermophilic fermented milk which is prepared with *Streptococcus thermophilus*, *Lactobacillus* spp., yeasts and moulds (Fondén et al., 2006). Other traditional fermented milk products include buttermilk- *kärnmjölk*, *kjernemelk, kærnemælk,* and *kirnupiimä*; concentrated fermented milk- *lactofil*, *ymer*, and *kokkeli*; cultured cream- *gräddfil, rømme*, *crème fraîche*, and *kermapiimä*; cultured milk- *lättfil, kulturmelk, skummet kulturmelk, tykmælk, talouspiimä,* and *rasvatonpiimä*; and yoghurt-like, *tettemelk* (Duboc and Mollet, 2001). In Israel, *zivda* is a fermented milk product which is usually prepared using *Lactococcus lactis* (Keller et al., 1974; Karenzi et al., 2013).

The distribution of the traditional milk and milk products (including rare ones) mentioned above are depicted in the Figure D-F.

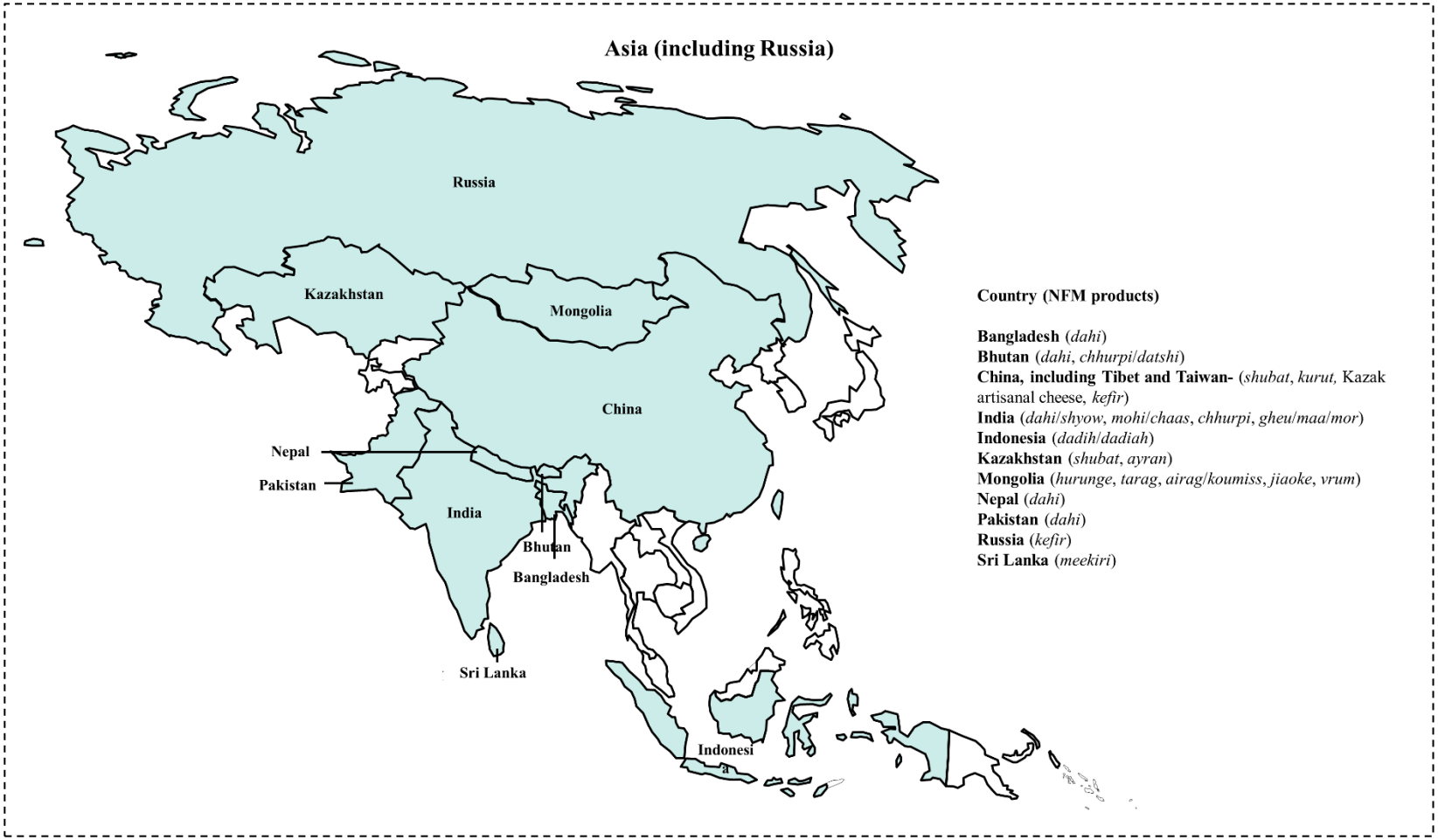


Figure D: Naturally/traditional fermented milk products prepared in the Asia countries (including Russia)

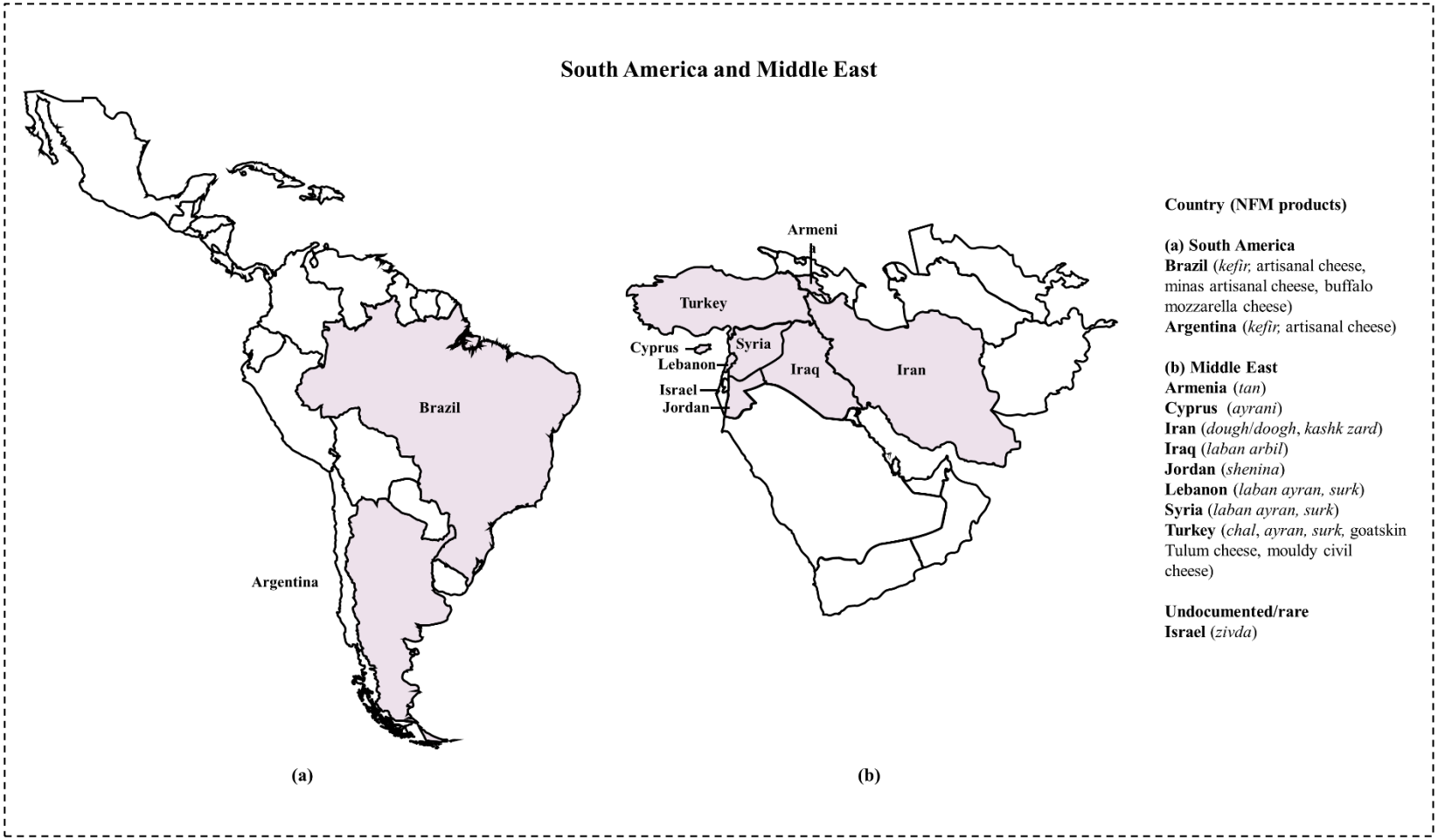


Figure E: Naturally/traditional fermented milk products prepared in (a) South America and (b) Middle East

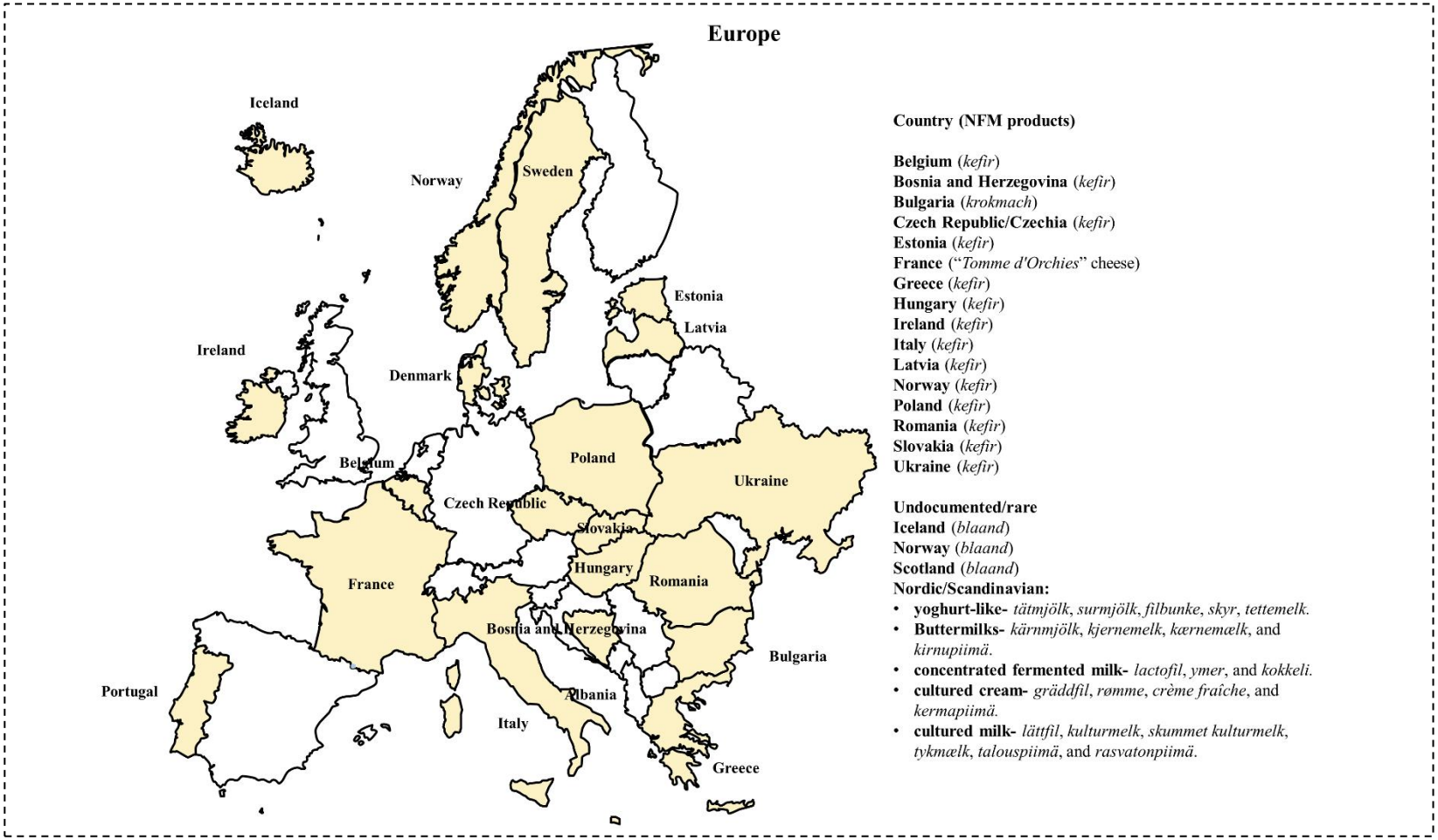


Figure F: Naturally/traditional fermented milk products prepared in Europe

Methods used in bacterial diversity study

Fermented milk products are the results of action of beneficial and harmless microorganisms (García-Burgos et al., 2020), making them good sources for isolating and identifying several health beneficial and industrially important microorganisms (Tamang et al., 2016). There are two categories of methods used in microbial diversity study of food samples *viz*., culture-dependent, and culture-independent methods. Culture-dependent methods depend on the laboratory conditions to simulate the microenvironment (of samples) for cultivating the desired microorganisms using general or specific culture media. On the other hand, culture-independent techniques rely on the technologies and the growing innovations of molecular techniques and bioinformatics. Though there are limitations to either of the methods, and at times, contradictory, however, they are significant in microbial taxonomy study (Cocolin et al., 2011; Tamang et al., 2016). Additionally, culture-independent methods have revolutionized the field of food microbiology with the advancement of high-throughput sequencing technologies which have been widely used in microbial diversity study (Ercolini, 2013) and its vast application in fermented milk products as reviewed by de Melo Pereira and group (de Melo Pereira et al., 2020). Despite this, however, culture-dependent remains the gold standard for species/strain characterization (Walsh et al., 2017).

MATERIALS AND METHODS

MATERIALS

Media used

***Lactobacillus* MRS Agar (M641, HiMedia, India)**

Ingredients	Gram/Litre
Proteose peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2
Agar	12
Final pH (at 25°C)	6.5±0.2

***Lactobacillus* MRS Broth, MRS Broth (M369, HiMedia, India)**

Ingredients	Gram/Litre
Proteose peptone	10
HM Peptone B (Equivalent to Beef Extract)	10
Yeast extract	5
Dextrose (Glucose)	20
Polysorbate 80 (Tween 80)	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium hydrogen phosphate	2
Final pH (at 25°C)	6.5±0.2

Nutrient Agar (MM012, HiMedia, India)

Ingredients	Gram/Litre
Peptone	10
Meat extract B (Equivalent to Beef extract)	10
Sodium chloride	5
Agar	12
pH after sterilization	7.3±0.1

Nutrient Broth (M002, HiMedia, India)

Ingredients	Gram/Litre
Peptone	5
Sodium chloride	5
HM peptone B#	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4±0.2

Arginine hydrolysis medium (Thornley 1960)

Ingredients	Gram/Litre
Peptone	10
Yeast extract	5
D (+) glucose	0.5
Potassium phosphate	2
Magnesium sulphate	0.1
Manganese sulphate	0.05
Sodium acetate	5
Tri-sodium citrate	20
Tween 80	1 mL
Arginine*	0.3%
Phenol red	0.01
pH	5

Note: For liquid, measurement is taken in mL. Ingredients marked "*" are calculated as percentage.

Carbohydrate fermentation media (Schillinger and Lucke, 1987)

Ingredients	Gram/Litre
Peptone	10
Yeast extract	5
Potassium phosphate	2
Tri-sodium phosphate	2
Carbohydrate*	0.5%
Tween 80 (liquid)	1mL
Sodium acetate	5
Magnesium sulphate	0.58
Manganese sulphate	0.28
Phenol red*	0.004 %

Note: For liquid, measurement is taken in mL. Ingredients marked "*" are calculated as percentage.

Mueller Hinton Agar (M173, HiMedia, India)

Ingredients	Gram/Litre
HM infusion B (Equivalent to Beef infusion)	300
Acicase (Equivalent to Casein acid hydrolysate)	17.5
Starch	1.5
Agar	17
Final pH (at 25°C)	7.3±0.1

Sheep Blood Agar Plate (MP1301, HiMedia, India)

Ingredients	Gram/Litre
Casein enzymic hydrolysate	14
Peptic digest of animal tissue	4.5
Yeast extract	4.5
Sodium chloride	5
Agar	12.5
Sheep Blood	5
Final pH (at 25°C)	7.3±0.2

Carbohydrates used	Product No.
L (+) Arabinose	GRM037, HiMedia, India
D(+) Cellobiose	RM098, HiMedia, India
D - Fructose	GRM196, HiMedia, India
D-(+)-Galactose	GRM101, HiMedia, India
D-Gluconic acid potassium salt	GRM466, HiMedia, India
Lactose monohydrate	RM565G, HiMedia, India
D-(+)-Maltose monohydrate	GRM3050, HiMedia, India
D-Mannitol	PCT0604, HiMedia, India
D-(+)-Mannose	RM104, HiMedia, India
D (+) Melibiose monohydrate	RM106, HiMedia, India
D-(+) Raffinose pentahydrate	RM107, HiMedia, India
D-(-)-Ribose	GRM197, HiMedia, India
D-(-)-Salicin	RM108, HiMedia, India
Sucrose, Certified	GRM601, HiMedia, India
D(+)-Trehalose dihydrate	GRM110, HiMedia, India
D(+)Xylose	GRM111, HiMedia, India

Chemicals/reagents/stains used	Product No.
Ethanol	MB106, HiMedia, India
Calcium carbonate, CaCO3	GRM397, HiMedia, India
Agar powder, Bacteriological Grade	RM026, HiMedia, India
Glycerol	MB060, HiMedia, India
Sodium chloride, NaCl	MB023, HiMedia, India
tri-Sodium citrate dihydrate	GRM3953, HiMedia, India
Petroleum ether	1.01769, Merck, Germany
EDTA (Ethylenediaminetetraacetic acid) disodium salt dihydrate	MB011, HiMedia, India
Mutanolysin from *Streptomyces globisporus* ATCC 21553	M9901, Sigma-Aldrich, USA
Lysozyme, From Chicken egg white	MB098, HiMedia, India
Lyticase from *Arthrobacter luteus*	SAE0098, Sigma-Aldrich, USA
Guanidine thiocyanate	G9277, Sigma-Aldrich, USA
Proteinase K, From *Pichia pastoris*	RM2957, HiMedia, India
Triton® X-100	MB031, HiMedia, India
SDS (Dodecyl sulphate sodium salt; Lauryl sulphate sodium salt)	MB010, HiMedia, India
20% SDS	ML007, HiMedia, India
Ammonium acetate	GRM295, HiMedia, India
Phenol saturated w/10mM Tris 1mM EDTA	MB082, HiMedia, India
Phenol:Chloroform:Isoamyl alcohol	P3803, Merck, Germany
Tris (hydroxylmethyl) aminomethane base	TC072, HiMedia, India
Isoamyl alcohol	1.00978, Merck, Germany
1X TE buffer (pH 8)	ML060, HiMedia, India
Agarose	MB080, HiMedia, India
100 bp DNA Ladder	MBT049, HiMedia, India
1 Kb DNA Ladder	MBT051, HiMedia, India
Ethidium Bromide	RM813, HiMedia, India
GoTaq® Green Master Mix	M7122, Promega, Wisconsin, USA
Poly (ethylene glycol) MW400	GRM3662, HiMedia, India
QIAquick gel extraction kit	28706, Qiagen, Germany
Qubit™ dsDNA HS and BR Assay Kits	Q32853, Invitrogen, USA
Potassium hydroxide pellets Hi-ARTM/ACS	GRM1015, HiMedia, India
Skim milk	RM1254, HiMedia, India
Ox Bile	CR010, HiMedia, India
Ringer solution	M525, HiMedia, India
Cholesterol	TC101, HiMedia, India
X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside)	MB069, HiMedia, India
IPTG (Isopropyl-b-D-1-thiogalactopyranoside)	RM2578, HiMedia, India
Calcium chloride, CaCl2	GRM710, HiMedia, India
Sodium Cholate	RM202, HiMedia, India

Chemicals/reagents/stains used	Product No. (Contd.)
Taurocholic acid sodium salt hydrate	T4009, Sigma-Aldrich, USA
Sodium deoxytaurocholate	RM9822, HiMedia, India
Sodium taurochenodeoxycholate	T6260, Sigma-Aldrich, USA
Ninhydrin	151173, Sigma-Aldrich, USA
Diethyl ether	1.00923, Merck, Germany
n-Hexane	1.04368, Merck, Germany
Toluene	1.08323, Merck, Germany
Chloroform	1.94506, Merck, Germany
Xylene	60868505001730, Merck, Germany
n-Hexadecane	RM2238 HiMedia, India
Butyl alcohol (1-Butanol)	1.01990, Merck, Germany
Acetic acid (glacial)	27221, Merck, Germany
Gram's Iodine solution	S013, HiMedia, India
Gram's Crystal Violet	S012, HiMedia, India
Gram's Decolorizer	S032, HiMedia, India
Safranin, 0.5% w/v	S027, HiMedia, India
Hydrogen peroxide 30%	1.07209, Merck, Germany
Nuclease-Free Water (10 x 50 ml)	129115, QIAGEN, Germany

Software/database used	Links
Sequence Scanner v2.0	https://www.thermofisher.com/in/en/home/life-science/sequencing/sanger-sequencing/sanger-dna-sequencing/sanger-sequencing-data-analysis.html
ChromasPro v1.34	http://technelysium.com.au/wp/chromas/
Mallard	http://www.softsea.com/review/Mallard.html
BLAST (basic local alignment search tool)	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch
EzTaxon	https://www.ezbiocloud.net/
clustalW	https://www.genome.jp/tools-bin/clustalw
Molecular Evolutionary Genetics Analysis version 7 (MEGA7.0.26)	https://www.megasoftware.net/
PAST v4 (Paleontological Statistics Software Package for Education and Data Analysis)	https://palaeo-electronica.org/2001_1/past/issue1_01.htm
MG-RAST	https://www.mg-rast.org/
QIIME	http://qiime.org/
MS-Excel v365	https://www.microsoft.com/en-in/microsoft-365/excel
Canoco software v4.52 (Wageningen University, The Netherlands)	https://www.wur.nl/en/Research-Results/Research-Institutes/show/Canoco-for-visualization-of-multivariate-data.htm
ClustVis	https://biit.cs.ut.ee/clustvis/

Software/database used	Links (Contd.)
PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)	https://github.com/picrust/picrust2/wiki
QIIME2-2020.6	https://docs.qiime2.org/2020.6/
Piphillin	https://piphillin.secondgenome.com/
SILVA v132	https://www.arb-silva.de/documentation/release-132/
KEGG: Kyoto Encyclopedia of Genes and Genomes	https://www.genome.jp/kegg/
BioCyc	https://biocyc.org/
STAMP-statistical analysis of taxonomic and functional profiles	https://beikolab.cs.dal.ca/software/STAMP
Statistical Package for the Social Sciences (SPSS) v 20	https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-20

Machines	Details
Analytical Weighing Balance	AX 204, Mettler, USA
Stomacher	400, Seward, London, UK
Biological Incubator	Accumax, CIS-24BL, India
Orbital Shaker Incubator	RSB-12, Remi, Mumbai, India
Autoclave	Instrumentation India, Mumbai, India
Laminar Air Flow	1386, Thermo Scientific, USA
Digital pH meter	GeNei™, Bangalore, India
Hot Air Oven	Instrumentation India, Kolkata
Water Bath	RIME-1322, Remi, Mumbai, India
Water Distillation unit	72240020, Riviera, Mumbai, India
Freezer (-20 ^{o}C)	ROFV-170, Remi, Mumbai, India
Freezer (-80 ^{o}C)	TSE240A, Thermo Fisher, USA
Compound Microscope	EX1000, Olympus, Japan
Phase contrast microscope	CKX41, Olympus, Japan
Centrifuge	CL21, Thermo Scientific, USA
Microcentrifuge	MicroCL 21R, ThermoFisher Scientific, Carlsbad, CA, USA
Microwave Oven	28L, Samsung, India
SimpliAmp™ Thermal Cycler	A24811, ThermoFisher Scientific, Carlsbad, CA, USA
Thermal Cyclers	2720, Applied Biosystems, USA
Electrophoresis Unit	Bio Rad, USA
Gel Doc™ EZ Imager	BioRad, Hercules, CA, USA
UV-Transilluminator	MD-25/HD-25, Wealtech, USA
Eppendorf BioSpectrometer	Eppendorf, Hamburg, Germany
Qubit 3.0 fluorometer	Invitrogen, Carlsbad, CA, USA
Automated DNA analyser	ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA
NGS Illumina-MiSeq	Illumina Platform, USA

Reference strains used	**Accession No.**
Lactobacillus plantarum (Basonym: *Lactiplantibacillus plantarum*)	MCC 2034
Lactobacillus plantarum subsp. *plantarum* (Basonym: *Lactiplantibacillus plantarum*)	MTCC 2974
Lactobacillus fermentum (Basonym: *Limosilactobacillus fermentum*)	MTCC 2760
Lactobacillus brevis (Basonym: *Levilactobacillus brevis*)	MTCC 2198
Lactococcus lactis subsp. *lactis*	MTCC 440
Leuconostoc mesenteroides subsp. mesenteroides	MTCC 867
Escherichia coli	MTCC 2413
Staphylococcus aureus subsp. *aureus*	MTCC 740
Bacillus cereus	MTCC 1272
Salmonella enteric subsp. *enterica* ser. *typhimurium*	MTCC 3223

Note:
Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.
Microbial Culture Collection (MCC), Pune, Maharashtra, India.

METHODS

Field survey and documentation

Documentation of the naturally fermented milk (NFM) products was carried in different districts of Arunachal Pradesh. With the help of a structured questionnaire (Table A), the indigenous knowledge of traditional preparation of the NFM products was recorded via a face-to-face interaction with the ethnic residents. Households were selected only from those ethnic groups where the practice of milk fermentation is associated with. During the time of survey, many criteria were questioned and sought, which includes- origin/source of milk, method of preparation, taboo and festivals associated with the preparation, sensory properties, culinary practices, mode of consumption, socio-economy, knowledge of preparation etc.

Table A: Questionnaire on Consumption of Naturally Fermented Milk Products in Arunachal Pradesh

I. General Information

1. Identification Number:
2. Name of the Informant:
3. Ethnic Group:
4. Name of (i) Village/Revenue Block:
   (ii) Sub-division:
   (iii) District:
8. Approximate number of (i) Households in the Village:
   (ii) Population of the Village:
9. Distance of the village from (i) Nearest Market (km):
   (ii) Nearest Town (km):

II. Information on Fermented Products

1. Kindly provide information on fermented foods

Fermented Products	Local Name	Raw or boiled	Milk (Cow/Yak)	If yes, consume (daily/ times per week/ occasionally)	Whether prepared at home/ market purchased/ both	Price

2. Name of each product in different ethnic dialect.
3. Method of preparation of each product (separately)
4. Flow sheet of traditional preparation of each product.
5. Do you have any taboo to prepare/consume the fermented products you mentioned? *Yes/No*

   If yes, provide details:
6. Do you have any preference to prepare/consume the fermented products of any type that you have mentioned during any particular season and/or social/religious ceremony? *Yes/No*

   If yes, provide details:
7. Do you perform any ritual or worship any particular god(s) or goddess(es) with fermented products, you consume? *Yes/No*

   If yes, provide details:
8. Do you think that fermented products, which you have mentioned, have medicinal value(s) or play a role in promoting health?
9. Are you economically dependent on any fermented products? *Yes/No*

   If yes, kindly name the product(s):
10. Do you prepare fermented foods you consume at home? If so, how did you know the traditional method of preparation of fermented foods?
11. What is the approximate quantity of monthly/annual production of fermented products? (ref: last month/year)
12. What is the estimated cost of production? (ref: last month/year)
13. What is the approximate income from the sale of such products?

Remarks:

Name and signature of Investigator:

Date:

Sample collection

A total of 30 samples of NFM products (6 cow-milk *mar*, 4 yak-milk *mar*, 6 cow-milk *chhurpi*, 4 yak-milk *chhurpi*, 6 cow-milk *churkam* and 4 yak-milk *churkam*) were collected from West Kameng and Tawang districts of Arunachal Pradesh in India (Table 1). All samples were collected in pre-sterilized containers and transported to the laboratory in an ice-box cooler and stored at 4 °C for immediate microbiological analysis.

Table 1: NFM sample collection sites in Arunachal Pradesh and their coordinates.

NFM products (n)	Source	Place of collection	Altitude (m)	Latitude (º N)	Longitude (º E)
Mar (7)	Cow	Cheghar, Tawang	1705	27.5742	91.9244
		Samchin, Tawang	1650	27.6325	91.7539
		Kudung, Tawang	1695	27.5481	91.8358
		Tawang, Tawang	2587	27.578	91.8757
		Bomdila, West Kameng	2339	27.3428	92.3024
		Dirang, West Kameng	2102	27.3584	92.2408
		Dirang, West Kameng*	2102	27.3584	92.2408
Mar (4)	Yak	Samchin, Tawang	1650	27.6325	91.7539
		Cheghar, Tawang	1705	27.5742	91.9244
		Dirang, West Kameng	2088	27.3584	92.2408
		Bomdila, West Kameng	2339	27.3428	92.3024
Chhurpi (6)	Cow	Cheghar, Tawang	1705	27.5742	91.9244
		Samchin, Tawang	1650	27.6325	91.7539
		Kudung, Tawang	1695	27.5481	91.8358
		Tawang, Tawang	2587	27.578	91.8757
		Bomdila, West Kameng	2339	27.3428	92.3024
		Dirang, West Kameng	2095	27.3584	92.2408
Chhurpi (4)	Yak	Samchin, Tawang	1650	27.6325	91.7539
		Cheghar, Tawang	1705	27.5742	91.9244
		Dirang, West Kameng	2061	27.3584	92.2408
		Bomdila, West Kameng	2340	27.3428	92.3024
Churkam (11)	Cow	Cheghar, Tawang	1705	27.5742	91.9244
		Samchin, Tawang	1650	27.6325	91.7539
		Samchin, Tawang*	1650	27.6325	91.7539
		Samchin, Tawang*	1650	27.6325	91.7539
		Kudung, Tawang	1695	27.5481	91.8358
		Kudung, Tawang*	1695	27.5481	91.8358
		Kudung, Tawang*	1695	27.5481	91.8358
		Tawang, Tawang	2587	27.578	91.8757
		Dirang, West Kameng	2095	27.3584	92.2408
		Bomdila, West Kameng*	2340	27.3428	92.3024
		Bomdila, West Kameng	2340	27.3428	92.3024
Churkam (4)	Yak	Samchin, Tawang	1650	27.6325	91.7539
		Cheghar, Tawang[#]	1705	27.5742	91.9244
		Dirang, West Kameng	2061	27.3584	92.2408
		Bomdila, West Kameng	2340	27.3428	92.3024

Note: 30 samples were used for culture-dependent analysis and 35 samples were used for NGS analysis (extra samples are denoted by "*"). Sample marked by "#" failed for NGS analysis but was only used for culture-dependent analysis.
n = number of samples, m = meter

pH analysis

One gram of sample was dissolved in 10 mL pre-sterilized physiological saline (0.85% NaCl) and the pH of all samples were determined using a pH meter (GeNei™, Bangalore, India) and calibrated with standard buffers. The pH value was represented as mean ± SD values of triplicates sets.

Culture-dependent Analysis

Enumeration and isolation of LAB

Hard samples (*churkam*) were first cut into small pieces with sterile scalpel before homogenisation and soft samples of *mar* and *chhurpi* samples were directly homogenised. All samples were homogenised in a stomacher (400, Seward, London, UK) using stomacher bags in a ratio of 10:100 (w/v) dissolved in physiological solution (0.85% NaCl) and serial dilution (10^{-1} to 10^{-8}) was made. One millilitre of homogenised mixture was transferred into *Lactobacillus* MRS (Man-Rogosa-Sharpe) agar plate (M641, HiMedia, India), pH 6.2 (Yang et al., 2018) with 1% $CaCO_3$ by pour plate method and incubated in an anaerobic jar for 48 h at 30 °C. The number of colonies was counted as colony forming units (cfu/g) presented as log values with mean ± SD values of triplicate sets. Colonies were randomly selected and purified twice using the streak plate method. Purified colonies were checked then stored in 20% glycerol at −80 °C.

Phenotypic and biochemical characterisation

A total of 307 isolates of LAB isolates were isolated from 30 samples of *mar*, *chhurpi* and *churkam*. Preliminary characterisation including colony morphology, cell morphology, Gram stain, catalase test and ability of the colonies to produce light halo zone in the *Lactobacillus* MRS media (M641, HiMedia, India) supplemented with 1%

CaCO3 (Dewan and Tamang, 2007) were performed for presumptive selection of LAB. The ability of LAB isolates to produce gas from glucose was used to differentiate homo-fermenters from hetero-fermenters (Carr et al., 2002). Homo-fermenters were then differentiated, based on the cellular morphology and the ability to grow at 10 °C, 15 °C, 45 °C and 6.5% NaCl. Hetero-fermenters were further differentiated by the arginine hydrolysis test. Sugar fermentation test was performed following the method described by (Holzapfel and Wood, 1995). Based on phenotypic tests, biochemical and physiological profiles, all 307 isolates were tentatively identified up to genus level or groups, out of which 76 representative strains were randomly selected for further identification.

Genotypic characterisation

DNA extraction

DNA of LAB was extracted using an enzymatic-heating lysis method as described by (Jeyaram et al., 2010) with slight modifications. A pure colony was inoculated in *Lactobacillus* MRS (Man-Rogosa-Sharpe) broth (GM369, HiMedia, India) and incubated at 30 °C for 16 to18 h. The 2 mL of the culture broth was then transferred to 2 mL micro-centrifuge tube and centrifuged (Microcentrifuge, MicroCL 21R, ThermoFisher Scientific, Carlsbad, CA, USA) at 8000 × *g* for 5 min. The supernatant was discarded, and the remaining pellet was then washed with sterile 0.5 m NaCl two times, followed by immediate washing with sterile deionised water (MilliQ H_2O). The pellet was suspended in 1X TE buffer (pH 8), and 10 µL of lysozyme (2 mg/mL) was added to the solution. The cell suspension was then incubated at 37 °C for 30 min for enzyme activation, followed by immediate heating at 98 °C for 15 min. The suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was transferred to

a sterile micro-centrifuge tube. DNA was quantified using Eppendorf BioSpectrometer (Hamburg, Germany). Quantified DNA was stored at −20 °C until required and DNA purity of 1.8–2.2 was used for PCR reaction.

PCR amplification

Identification of LAB isolates was carried out using Sanger sequencing of 16S rRNA gene (Heather and Chain, 2016). The PCR reaction was carried out in a 50 µL reaction volume using GoTaq® Green Master Mix (M7122, Promega, Wisconsin, USA) containing the required dNTPs (dATPs, dTTPs, dGTPs, dCTPs), MgCl2; primers 27F 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R 5′- GTTACCTTGTTACGACTT-3′ (Lane, 1991) and about 30–50ng of the DNA template. The PCR amplification was carried out using a SimpliAmp™ Thermal Cycler (Cat No. A24811, ThermoFisher Scientific, Carlsbad, CA, USA) with the following conditions: initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C (denaturation) for 1 min, 55 °C (annealing process) for 1 min and 72 °C (elongation process) for 1.5 min. Lastly, PCR amplification was set to a final elongation process of 72 °C for 10 min and a stoppage process at 4 °C.

Purification of the PCR amplicons

The PCR amplicons were purified using PEG (polyethylene glycol)-NaCl (sodium chloride), 20% (w/v) PEG, 2.5 m NaCl (Schmitz and Riesner, 2006) with slight modifications. Briefly, 0.6 volume of PEG-NaCl solution was mixed with the PCR amplicons and incubated at 37 °C for 30 min. The mixture was centrifuged at 10,000 × g, 4 °C for 30 min, the supernatant was carefully discarded, the pellet was then washed twice with freshly prepared and cold 70% ethanol and was allowed to air-dry overnight. Finally, 20 µL nuclease free water was used to suspend the purified DNA. Agarose

(1.2%) gel electrophoresis was visualised using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

16S rRNA gene sequencing

The purified PCR amplicons was subjected to sequencing using the primer pairs 27F 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R 5′- GTTACCTTGTTACGACTT-3′ (Lane, 1991). Two sequencing PCR reactions were carried out for each primer. A final volume of 50μL reaction volume containing 0.2 μM primer, 0.2 mM dNTPs (dATPs, dTTPs, dGTPs, dCTPs), 2.0 mM MgCl2, 0.5 mg/mL and 0.04 U/μL Taq DNA polymerase. The PCR conditions used for sequencing included an initial denaturation of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 1 min (denaturation), 40 ℃ for 2 min (annealing), 72 ℃ for 1 min (elongation) and a final elongation of 72 ℃ for 10 min. Sequencing was performed using an automated DNA analyser (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA).

Bioinformatics analysis

Raw sequences were checked for their quality using Sequence Scanner v2.0, a software from Applied Biosystems, https://www.thermofisher.com/in/en/home/life-science/sequencing/sanger-sequencing/sanger-dna-sequencing/sanger-sequencing-data-analysis.html**.** Good quality sequencing reads were then assembled using ChromasPro v1.34, http://technelysium.com.au/wp/chromas/**.** Chimera-check was performed using a programme called Mallard (Ashelford et al., 2006). Identity was acquired by aligning the sequences with BLAST (basic local alignment search tool) https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch (Altschul et al., 1990) and EzTaxon, https://www.ezbiocloud.net/ (Kim et al., 2012) databases.

Phylogenetic relationship of the identified species was carried out after aligning the sequences with clustalW (Thompson et al., 1994). Phylogenetic tree using neighbour-joining (Saitou and Nei, 1987) was constructed by Molecular Evolutionary Genetics Analysis version 7 (MEGA7.0.26) (Kumar et al., 2016).

Statistical analysis

Frequency of the isolates was calculated using MS Excel v365. Diversity indices [Simpson diversity index (H_{Si}), Shannon diversity index (H_{Sh})] were calculated using PAST v4 (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer et al., 2001).

Shannon's diversity index was given as follows:

$$H_{Sh} = -\sum_{i=1}^{S} p_i \ln (p_i)$$

Simpson's diversity index was given as follows:

$$D = \sum_{i=1}^{S} p_i^2$$

$$H_{Si} = 1 - D$$

Where, S is the number of species p_i is the number of the given species divided by the total number of isolates observed, and D is Simpson's index (Daly et al., 2018).

Culture-independent analysis

Metagenomic DNA extraction

Since the samples are of different nature, two methods for metagenomic DNA extraction were employed based on methods reported by (Keisam et al., 2016). *Mar* is lipid-rich sample whereas *chhurpi and churkam* are categorized as casein-based samples. For lipid-rich samples, the designated method I (Keisam et al., 2016) was followed with some modifications. Since *mar* is rich in lipid/fat, it was necessary to dissolve it before extraction of DNA, where in this case, an equal ratio of petroleum ether: hexane (1:1) was used. The use of hydrocarbons resolves the samples into two visible phases after vortexing rigorously. Briefly, *mar* was firstly melted by heating at low temperature and 2 mL of the sample was homogenized with 2 mL citrate buffer (2%). Four mL the combined hydrocarbons, petroleum ether: hexane (1:1), was added and vortexed at high speed. The mixture was then incubated at room temperature for 10 min. The fat-free lower portion of the homogenate was then transferred to a sterile 2 mL screw-cap tube containing 0.5g of zirconia/silica beads (0.1 mm) and 4 glass beads (2 mm) and were then centrifuged at 4 ^{o}C for 10 min at 14,000 × *g*. After discarding the supernatant, the pellet is then resuspended in 150 µl proteinase-K buffer [50 mM Tris-Cl, 10 mM EDTA (pH 8), 0.5% (w/v) SDS], with 25 µl proteinase K (25 mg/ml). Following an overnight (16 to 18 h) incubation at 65 ^{o}C, the mixture was then 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)]. Phenol (pH 8.0, 300 µL) was added, and the mixture was then vortexed at maximum speed for three times with an interval of 10 s for 1 min using a bead beater. Then, the aqueous phase was collected in a new sterile 1.5 mL microcentrifuge tube after centrifugation at 4 ^{o}C for 15 min at 14,000 × *g*. The resulting pellet was washed twice with 70% ethanol and centrifuged at 4 ^{o}C for 15 min at 14,000 × *g*. Lastly, the

pellet was then air-dried at room temperature and followed by dissolving it in 50µl of TE buffer (10 mM Tris, 1 mM EDTA).

For casein-based samples, *chhurpi* and *churkam*, metagenomic DNA extraction was followed as per the designated method V (Keisam et al., 2016) with some modifications. This method was selected as Keisam and group (Keisam et al., 2016) have reported it to have recovered maximum DNA yield from fermented milks. Briefly, solid samples of about 10 g were homogenized in 100 mL (w/v) of 2% sodium citrate buffer. For *chhurpi*, the samples were directly weighed and homogenized, however, in case of *churkam*, the samples were firstly grinded into fine pieces before homogenization. About 1.5 mL of the homogenate was used for the extraction procedure, by transferring into a sterile microcentrifuge tube and pellet was recovered after centrifugation for 10 min at 18,000 × *g*. About 400 µL of TES buffer [50 mM Tris, 1 mM EDTA, 8.7% sucrose] 50 KU lysozyme, 25 U mutanolysin and 20 U lyticase was added to the pellet and incubated at 37 ^{o}C for 1 h. This was followed by the addition of proteinase-K (25mg/mL) and further incubated at 65 ^{o}C for 1 h. Following this, 500µL of GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% sarkosyl) was added to the above mixture and was cooled in ice for 5 min. Precipitation of proteins was carried out by adding 250µL of 7.5 M ammonium acetate, followed by purification with 600µL of chloroform: isoamyl alcohol (24:1). The pellet was then washed twice using 70% ethanol, air-dried, and further suspended in 50 µL of 1X TE (10 mM Tris, 1 mM EDTA) buffer. The quality ($A_{260/280}$) and quantity of the extracted metagenomic DNA from both the methods was checked using a spectrometer (NanoDrop ND-1000, USA) and stored at -20 ^{o}C until further required.

Barcoded Illumina MiSeq Sequencing

In-depth analysis of bacterial community from NFM products of Arunachal Pradesh was carried out by barcoded Illumina MiSeq amplicon sequencing targeting the V4-V5 region of the 16S rRNA gene, using 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 which was used for sample multiplexing (Romi et al., 2015). PCR reaction was carried out in a total volume of 25 µL with a template-free reaction acting as a control, and the following PCR conditions was used for amplification: initial denaturation (98 ^{o}C for 5 min, denaturation (98 ^{o}C for 15 sec), annealing (55 ^{o}C for 30 sec), elongation (72 ^{o}C for 30 sec). Additionally, 28 PCR cycles was run with a final elongation of 72 ^{o}C for 5 min, and a stop reaction at 4 ^{o}C. The DNA quality was checked using 1.5 % agarose (w/v) gel for ~430 amplicon size. The target amplified bands were then carefully excised from the gel and with a sterile scalpel and then purified using QIAquick gel extraction kit (Qiagen, New Delhi, India) as per the manufacturer's instructions. Purified DNA was quantified using Qubit dsDNA BR Assay Kit (Invitrogen) in a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). Equimolar concentration of the individual DNA from each sample was then pooled and final DNA was sent to the NGS facility in Xcelris Genomics (Ahmedabad, India) for paired-end MiSeq sequencing (2 × 300 bp) using Illumina platform.

Bioinformatics analysis

Raw sequences generated from Illumina MiSeq platform was analysed using the default settings in MG-RAST, Metagenomic Rapid Annotations using Subsystems Technology, (Meyer et al., 2008) and an open-source bioinformatics pipelines QIIME v1.8.0,

Quantitative Insights into Microbial Ecology (Caporaso et al., 2010). Secondary quality filtering was applied to remove non-rRNA sequences before clustering into operational taxonomic units (OTUs) and taxonomic assignment was achieved using SILVA SSU (Quast et al., 2012) in MG-RAST database. The resulting OTU table was further collapsed into four different taxonomic levels *viz*., phylum, family, genus, and species and all eukaryote-specific and unassigned OTUs were removed before downstream analysis.

Statistical analysis

Raw reads were normalized to relative abundance (formula below) and was visualized by 100% stacked bar-chart using MS-Excel v365.

$$\text{Relative abundance of each OTU (\%)} = \left(\frac{\text{Raw read}}{\text{Total raw reads in a sample}}\right) \times 100$$

For understanding the microbial community variation among the NFM products, principal component analysis (PCA) of log transformed [$\log_{10}(x_i + 1)$] was plotted using Canoco software v4.52 (Wageningen University, The Netherlands). Significant differences in the bacterial diversity amongst the three samples was evaluated ANOSIM/PERMANOVA (10,000 permutations) using Bray-Curtis similarity index in PAST v2.17. Furthermore, significant differences in the abundance of the individual taxa at all the four taxonomic levels was tested using non-parametric Mann-Whitney U-Test. The data were represented as boxplots using MS-Excel v365 and significant p-values were denoted by * ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.001.

For alpha diversity calculation, species level-OTUs table was rarefied at a depth of 50 to 6482 sequences using multiple_rarefactions.py script in QIIME v1.8.0 (Caporaso et al., 2010) for generation of alpha rarefaction curves. Rarefaction plots were generated

for chao1, equitability, fisher_alpha, goods_coverage, shannon and simpson diversity indices using make_rarefaction_plots.py script and significant differences were calculated using compare_alpha_diversity.py in QIIME v1.8.0 (Caporaso et al., 2010).

Technological characterisation (Probiotics) of the isolated LAB strains

Here, two sets of experiments were conducted for screening the technological properties of the isolated and identified LAB strains, which are categorized into primary and secondary experimental tests. The primary tests contained the standard tests as per the ICMR-DBT guidelines (Ganguly et al., 2011), and subsequently, further secondary screening was employed using few extra tests of interest described as follows.

Acidification and coagulation

Acidifying and coagulating properties of the LAB strains was assayed according to (Olasupo et al., 2001). Briefly, 1% of the tested strains were inoculated in 10% skim milk (RM1254, HiMedia, Mumbai, India) and incubated at 30 ^{o}C for 72 h. For every 24 h within the next 3 days, monitoring of clotting formation and measurement of pH was also followed.

Tolerance to low pH (acid)

Tolerance to low pH (acid) was assayed following the method described by (Ramos et al., 2013) with few modifications. Overnight active cultures of LAB strains grown in *Lactobacillus* MRS (Man-Rogosa-Sharpe) broth (GM369, HiMedia, India) at 37 ^{o}C were harvested by centrifugation at 2800 × *g* for 5 min at room temperature. The collected pellet was washed twice with phosphate buffer solution, pH 7.0. An optical density of 0.08 to 0.1 ($\sim 10^{8}$ CFU/mL) at 600 nm was used as an inoculum and the cell suspension was resuspended freshly prepared *Lactobacillus* MRS (Man-Rogosa-Sharpe) broth (GM369, HiMedia, India) (pH 3.0, adjusted with 1N HCl), followed by acidic treatment for 3 h incubation at 37 ^{o}C. Percentage of survivors were determined by pour plating method and cfu count after 48 h of incubation at 37 ^{o}C.

Tolerance to bile

Tolerance to bile was followed as per (Ramos et al., 2013) with some modifications. Overnight of freshly cultured LAB strains (incubated at 37 ^{o}C) were used for exposure to bile treatment. Here, cells were harvested after 5 min of centrifugation (2800 × *g*) at room temperature and were washed twice using phosphate buffer solution (pH 7.0). An inoculum of 0.08 to 0.1 optical density at 600 nm, which is equivalent to ~10^8 CFU/mL was inoculated to a freshly prepared *Lactobacillus* MRS (Man-Rogosa-Sharpe) broth (GM369, HiMedia, India) containing 0.3% Ox Bile (CR010, HiMedia, India) and incubated at 37 ^{o}C for 3 h. Viable percentage count was determined by serial dilution and pour plating method after 48 h incubation at 37 ^{o}C.

Microbial attachments to hydrocarbons (MATH)

Cell surface properties of the LAB strains was assayed following the method described by (Mallappa et al., 2019) with few modifications. Five different hydrocarbons were used as solvents in this experiment, which includes- chloroform, diethyl ether, n-hexadecane, toluene, and xylene. Overnight culture of LAB strains was harvested by centrifuging at (2800 × *g*) for 5 in at room temperature. The pellet was then washed using phosphate buffer solution (pH 7) and an adjusted optical density of 0.08 to 0.1 at 600 nm (~10^8 CFU/mL) was maintained as inoculum, designated as '$A_{initial}$'. Three mL of the cell suspension was mixed with 1 mL of each of the hydrocarbons and vortexed for even mixing. The tubes were then briefly incubated at 37 ^{o}C for 10 min for temperature equilibrium, mixed by vortex and were incubated again at 37 ^{o}C for 3 h without agitation. After incubation, 1 mL of the upper layer (aqueous phase) was carefully taken and measured for its optical density at 600 nm. This reading was

designated as 'A_{Final}', and the percentage of cell surface hydrophobicity was calculated as follows:

$$\text{Hydrophobicity (\%)} = \left[1 - \left(\frac{A_{Final}}{A_{Initial}}\right) \times 100\right]$$

Auto-aggregation and Co-aggregation

Cell surface properties of the LAB strains was further tested for auto-aggregation and co-aggregation using spectrophotometric method (Mallappa et al., 2019). In this method, before subjecting the LAB strains to the respective two different experiments, the cells were prepared, and inoculum was maintained as described above. For auto-aggregation, the adjusted initial absorbance ($A_{Initial}$) of the tested LAB strains was measured at 600 nm and recorded. The mixture was then mixed by vortex and incubated at 37 ^{o}C for 3 h (in triplicates) without agitation. After 3 h of incubation (A_{Time}), the mixture was then measured for its absorbance and the percentage was calculated using the formula below:

$$\text{Auto-aggregation (\%)} = \left[1 - \left(\frac{A_{Time}}{A_{Initial}}\right) \times 100\right]$$

Furthermore, the LAB strains were tested for their ability to adhere to other bacteria (co-aggregation), particularly pathogenic strains (Mallappa et al., 2019) which includes *Escherichia coli* MCC 2413, *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272. Here, the cell suspension for both the LAB strains and the tested pathogens was processed and maintained as described above. Initially, both LAB suspension and pathogen suspension was measured spectrophotometrically at 600 and denoted by A_{LAB} and $A_{Pathogen}$, respectively. Equal volume (2 mL each) of the LAB and pathogen

suspension was mixed by vortexing, and the mixture was incubated for 3 h at 37 ^{o}C without agitation. After incubation, the absorbance (A_{Mix}) of the mixture was measured at 600 nm and co-aggregation percentage was calculated as follows:

$$\text{Co-aggregation (\%)} = \left[\left(\frac{A_{LAB} + A_{Pathogen}}{2}\right) - \frac{(A_{Mix})}{\left((A_{LAB}+A_{Pathogen}) / 2\right)}\right] \times 100$$

Antimicrobial property against standard cultures of pathogenic strains

Antimicrobial property of the LAB strains was followed as per (Yadav et al., 2016) and tested against the above indicator strains *viz*., *Escherichia coli* MCC 2413, *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272. Freshly prepared bacterial suspension of the pathogenic strains (100µL) was mixed onto soft agar, overlaid on Mueller Hinton Agar (M173, HiMedia, India). With the help of a cork borer, wells were made and 100 µL of overnight culture of LAB strains was poured into the wells. The plates were then incubated at 37 ^{o}C for 24 to 48 h. The zone of growth inhibition was measured and categorized as "+++" > 18 mm, "++" > 15 mm, and "+" > 10 mm.

Bile salt hydrolysis (BSH) activity

BSH activity was qualitatively assessed using a plate assay as described by (Mallappa et al., 2019). Here, freshly grown LAB strains were streaked on *Lactobacillus* MRS (Man-Rogosa-Sharpe) agar plate (M641, HiMedia, India) containing bile salts (0.3 %) and $CaCl_2$ (0.375 g/L) and the plates were incubated at 37 ^{o}C for 48 h. Positive activity for bile salt hydrolases resulted in visible halos around the streaking area.

Beta (β)-galactosidase activity

To determine the positive activity for β-galactosidase enzymes, freshly grown LAB strains were streaked on *Lactobacillus* MRS (Man-Rogosa-Sharpe) agar plate (M641, HiMedia, India) containing 60 µL of X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) and 10 ml of IPTG (iso-propyl-thio- β-D galactopyranoside) solution as inducer (Angmo et al., 2016). Positive strains showed greenish to bluish colonies after 24 to 48 h of incubation at 37 ^{o}C.

Exopolysaccharide production

Freshly grown LAB cultures were streaked onto the surface of skim milk agar (10 %), containing 1 % w/v sucrose and an indicator dye ruthenium red (0.08 g/L). Observation of white ropy colonies were regarded as positive (Angmo et al., 2016).

Cholesterol reduction

The ability of the LAB strains to reduce cholesterol was determined using the method described by (Shehata et al., 2016). Briefly, freshly grown LAB strains were inoculated in MRS broth supplemented with 0.3 % ox bile and filter sterilized water-soluble cholesterol (100µg/mL), and the tubes were incubation at 37 ^{o}C for 24 h. The cells were then removed by centrifugation at 9000 × *g* for 15 min after incubation, and the cell-free broth was mixed with 1 mL of Potassium Hydroxide (KOH, 33% w/v) and 2 mL of absolute ethanol. The mixture was then vortexed for 1 min and incubated at 37 ^{o}C for 15 min. To this, 2 mL of sterile distilled water and 3 mL of hexane were added. The mixture was again mixed by vortex for 1 min, followed by transfer of the hexane layer to a fresh glass tube. The tubes were then evaporated at 65 ^{o}C and 2 mL of o-phthalaldehyde reagent was added immediately. Thereafter, the mixture was mixed by

vortex and 500 uL of concentrated sulphuric acid (H_2SO_4) was added and vortexed again for 1 min. Lastly, cholesterol reduction was determined by measuring the absorbance (after 10 min) at 550 nm using Eppendorf BioSpectrometer (Hamburg, Germany) and the percentage of reduction was calculated as follows:

$$\text{Cholesterol reduction (\%)} = \left[\frac{(C_0 - C)}{C_0}\right] \times 100$$

where, C_0 is the uninoculated (control) broth and C is the inoculated broth.

Screening for ɣ-(gamma)-aminobutyric acid (GABA)

Screening for GABA production was carried out using the methods as described by (Villegas et al., 2016). Briefly, LAB strains were incubated in MRS broth supplemented with 53 mM MSG (monosodium glutamate) at 30 ^oC for 96 h (4 days). After incubation, the cultures were then centrifuged at 5000 × *g* for 15 min and the supernatant was transferred into fresh tubes. About 2 µL of the supernatant was spotted onto the thin layer chromatography (TLS) silica gel 60 F_{254} (105554, Merck Millipore, Germany), and a solvent mixture of n-butanol:acetic acid:distilled water (5:3:2) was used as a mobile phase. After the run, the plates were then immersed into 0.4% (w/v) ninhydrin solution, and subsequently heated for visualization of the spots.

Safety evaluation of the isolated LAB strains

Haemolysis of blood

Haemolytic reaction of the LAB strains was evaluated as per (Angmo et al., 2016). Here, fresh cultures were streaked on the surface of Sheep Blood Agar Plate (MP1301, HiMedia, India) and incubated for 24 to 48 h at 37 ^oC. After incubation, the plates were examined for haemolysis.

Screening of probiotic and functional genes

Based on the available literatures on genetic screening of predictive probiotic and functional mechanism, few genes were attempted to be detected using PCR-based method. Details of the probiotic marker genes with predictive gene functions includes- bile salt tolerance (Table 2), low pH tolerance (Table 3), bile salt hydrolase (Table 4), attachment/adherence (Table 5), bacteriocin production (Table 6); and functional genes includes- GABA production, Glutamate decarboxylase (Table 7) and vitamin synthesis (Table 8). Here, for the PCR amplification, each mixture of a total of 15 µL, containing using GoTaq® Green Master Mix (M7122, Promega, Wisconsin, USA) containing the required dNTPs (dATPs, dTTPs, dGTPs, dCTPs), MgCl2; and the required specific primer pairs. PCR amplification was carried out using a SimpliAmp™ Thermal Cycler (Cat No. A24811, ThermoFisher Scientific, Carlsbad, CA, USA). The PCR amplicons were then checked in 0.8% agarose gel, pre-stained with 4 µL/100 mL of 1 X TAE (*Tris*-acetate-EDTA) gel running buffer. The gel was then visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA). Positive amplification was confirmed by comparing the target amplicon size form the literatures against a 100 bp DNA Ladder (MBT049, HiMedia, India) or 1 Kb DNA Ladder (MBT051, HiMedia, India).

Table 2: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for bile salt tolerance.

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
Hypothetical protein	*Ir0085*	F-RCTTTGACCGRTGGGGCTRT R-NNNATGGCCGCATGGAAA	95°C for 5 min; 95°C for 30 s; 57.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	150	(Turpin et al., 2011)
ABC transporter	*LBA1679*	F-ATGACAACGTCGTCGGGAGA R-GCTCCTCGTTGTTGGGACCT	95°C for 5 min; 95°C for 30 s; 61 °C for 10 s; 72°C for 15 s; 72°C for 5 min	267	(Turpin et al., 2011)
Aggregation-promoting factors	*apf*	F- YAGCAACACGTTCTTGGTTAGCA R- GAATCTGGTGGTTCATAYWCAGC	95°C for 5 min; 95°C for 30 s; 53°C for 10 s; 72°C for 15 s; 72°C for 5 min	112	(Turpin et al., 2011)
Hypothetical protein	*LBA1432*	F-TCCCATTCATCAYATGGAACAA R-CTGGCCCACATATCCATWCC	95°C for 5 min; 95°C for 30 s; 53°C for 10 s; 72°C for 15 s; 72°C for 5 min	352	(Turpin et al., 2011)
Major facilitator superfamily permease	*LBA0552*	F-GTGATTGCCCTAGCCCTGGT R-GATCCCGATCACGATGCAAG	95°C for 5 min; 95°C for 30 s; 57.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	180	(Turpin et al., 2011)
Major facilitator superfamily permease	*LBA1429*	F-AATTTCAGGATGCCCCGGTA R-CCAAGCTCCCAACAATGCAC	95°C for 5 min; 95°C for 30 s; 58°C for 10 s; 72°C for 15 s; 72°C for 5 min	196	(Turpin et al., 2011)
Major facilitator superfamily permease	*LBA1429-F1/R1*	F-CTACAGCCCGCTGCTAACCA R-AGTTTGCATGGCAACCTGGA	95°C for 5 min; 95°C for 30 s; 58°C for 10 s; 72°C for 15 s; 72°C for 5 min	174	(Turpin et al., 2011)
Multidrug resistance protein	*LBA1446*	F-GCTGGAGCCACACCGATAAC R-CAACGGGATTATGATTCCCATTAGT	95°C for 5 min; 95°C for 30 s; 58°C for 10 s; 72°C for 15 s; 72°C for 5 min	275	(Turpin et al., 2011)
Major facilitator superfamily permease	*Ir1584*	F-TAYGCCRTTCGGWTGTTTGG R-TCAWRATGGCRGTCCCAATG	95°C for 5 min; 95°C for 30 s; 55°C for 10 s; 72°C for 15 s; 72°C for 5 min	151	(Turpin et al., 2011)
Putative esterase	*Ir1516*	F-TRACCACTYTCWCCATTCAACAA R-CCACTAGCRATGACYAATACKGGTT	95°C for 5 min; 95°C for 30 s; 56.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	143	(Turpin et al., 2011)

Table 3: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for low pH tolerance.

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
Glucan synthase	*gtf*	F-ACACGCAGGGCGTTATTTTG R-GCCACCTTCAACGCTTCGTA	95°C for 5 min; 95°C for 30 s; 59°C for 10 s; 72°C for 15 s; 72°C for 5 min	374	(Turpin et al., 2011)
D-Alanine transfer protein	*dltD*	F-TTCGCCTGTTCAAGCCACAT R-ACGTGCCCTTCTTTGGTTCC	95°C for 5 min; 95°C for 30 s; 58°C for 10 s; 72°C for 15 s; 72°C for 5 min	283	(Turpin et al., 2011)
Histidine decarboxylase	*hdc*	F- AGATGGTATTGTTTCTTATG R- AGACCATACACCATAACCTT	95°C for 5 min; 95°C for 30 s; 52°C for 10 s; 72°C for 15 s; 72°C for 5 min	367	(Turpin et al., 2011)
Agmatine deiminase	*aguA*	F-GAACGACTAGCAGCTAGTTAT R-CCAATAGCCGATACTACCTTG	95°C for 5 min; 95°C for 30 s; 60°C for 10 s; 72°C for 15 s; 72°C for 5 min	542	(Turpin et al., 2011)
Amino acid permease	*La995*	F-AACGAAGGTCCCGACAAAGG R-ACGACCTTCGGGCTGGTTAC	95°C for 5 min; 95°C for 30 s; 57.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	246	(Turpin et al., 2011)
ATPase	*clpL*	F-GCTGCCTTYAAAACATCATCTGG R-AATACAATTTTGAARAACGCAGCTT	95°C for 5 min; 95°C for 30 s; 50°C for 10 s; 72°C for 15 s; 72°C for 5 min	158	(Turpin et al., 2011)
Cyclopropane FA synthase	*LBA1272-F1/R1*	F-GGCTTACCAATGGCCACCTT R-GATCAAAAAGCCGGTCACGA	95°C for 5 min; 95°C for 30 s; 57.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	210	(Turpin et al., 2011)
Cyclopropane FA synthase	*LBA1272-F2/R2*	F-GGCCGGTGTTCCACTAGTCC R-ACGTTGGGTCGATTTGACGA	95°C for 5 min; 95°C for 30 s; 57.5°C for 10 s; 72°C for 15 s; 72°C for 5 min	203	(Turpin et al., 2011)
Heat shock protein 60	*groEl*	F- TTCCATGGCKTCAGCRATCA R- GCTAAYCCWGTTGGCATTCG	95°C for 5 min; 95°C for 30 s; 58°C for 10 s; 72°C for 15 s; 72°C for 5 min	168	(Turpin et al., 2011)

Table 3: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for low pH tolerance.

(contd.)

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
Ornithine decarboxylase	*odc*	F-TMTWCCAACHGATCGWAATGC R-CRCCCCAWGCACARTCRAA	95°C for 5 min; 95°C for 30 s; 52°C for 10 s; 72°C for 15 s; 72°C for 5 min	245	(Turpin et al., 2011)
Tyrosine decarboxylase	*tdc*	F-CCACTGCTGCATCTGTTTG R-CCRTARTCNGGNATAGCRAARTCNGTRTG	95°C for 5 min; 95°C for 30 s; 50°C for 10 s; 72°C for 15 s; 72°C for 5 min	370	(Turpin et al., 2011)

Table 4: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for bile salt hydrolase.

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
bile salt hydrolase	*bsh*	F- ATTGAAGGCGGAACSGGMTA R- ATWACCGGWCGGAAAGCTG	94°C for 5 min; 94°C for 1 min; 58°C for 1 s; 72°C for 1 min; 72°C for 10 min	155	(Turpin et al., 2011)
bile salt hydrolase	*bsh F1*	F-ATTCCWTGGWTWYTGGGACA R-AAAAGCRGCTCTNACAAAWCKAGA	94°C for 5 min; 94°C for 1 min; 53°C for 1 s; 72°C for 1 min; 72°C for 10 min	384	(Turpin et al., 2011)
bile salt hydrolase	*bsh F2*	F-GGTTGGTCGGCCAGTTCTTT R-CCAACATGCCCAAGTTCGAC	94°C for 5 min; 94°C for 1 min; 60°C for 1 s; 72°C for 1 min; 72°C for 10 min	205	(Turpin et al., 2011)

Table 5: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for attachment/adherence.

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product Size	References
Fibronectin-binding protein	*fbp*	F-AGTGCTGAAATYATGGGAAGA R-AATTGTCCACCTTGTTGCTG	95 °C for 5 min, 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, 72 °C for 10 min.	835	(Archer and Halami, 2015)
ATP binding-substrate protein	*sbp*	F-CAGTTCTTAGCCACAGTTTG R-GGTTCGCCGCTAATAGTAAG	95 °C for 5 min, 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, 72 °C for 10 min.	805	(Archer and Halami, 2015)
Sortase	*sor*	F-CCACCTTGTACTGGTTAGTG R-GACCATTCGTGTACTTGCCG	95 °C for 5 min, 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, 72 °C for 10 min.	672	(Archer and Halami, 2015)
Mucin-binding protein	*mub*	F-GAGCAGAAGATGGGCCAAC R-CTTCTGCGTCAACAACTTCG	95 °C for 5 min, 95 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min, 72 °C for 10 min.	922	(Archer and Halami, 2015)
Mannose-specific adhesin	*msa*	F-GCGATTAGGGGTGTGCAAG R-GCAGTTGGTGACGTAGGCA	95 °C for 5 min, 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, 72 °C for 10 min.	319	(Archer and Halami, 2015)

Table 6: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for bacteriocin production.

Predictive gene function	Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
Leucocin	*lcnA*	F-ATGATGAACATGAAACCTAC R-TTACCAGAAACCATTTCC	95 °C for 5 min; 95 °C for 1 min; 45 °C for 45 s; 72 °C for 1 min 30 s; 72 °C for 7 min	185	(Xiraphi et al., 2008)
Leucocin	*lcnB*	F-ATGAATAACATGAAATCTGC R-TTACCAGAAACCATTTCCACC	95 °C for 5 min; 95 °C for 1 min; 45 °C for 45 s; 72 °C for 1 min 30 s; 72 °C for 7 min	185	(Xiraphi et al., 2008)
Leucocin	*lcnK*	F-ATGAAAAAATTCAAAGAAC R-TTAATTGTTAATGGTTGAAG	95 °C for 5 min; 95 °C for 1 min; 45 °C for 45 s; 72 °C for 1 min 30 s; 72 °C for 7 min	158	(Xiraphi et al., 2008)
Leucocin	*mesB*	F-ATGCAAGATAAAACAAAA R-TTATTTGTGGTTCTTG	95 °C for 5 min; 95 °C for 1 min; 45 °C for 45 s; 72 °C for 1 min 30 s; 72 °C for 7 min	161	(Xiraphi et al., 2008)
Leucocin	*mesY*	F-ATGACGAATATGAAGTC R-TTACCAAAATCCATTTCC	95 °C for 5 min; 95 °C for 30 s; 45 °C for 10 s; 72 °C for 15 s; 72°C for 5 min	185	(Xiraphi et al., 2008)
Lactococcin A	*Lactococcin A*	F-CAATCAGTAGAGTTATTAACATTTG R-GATTTAAAAAGACATTCGATAATTAT	92 °C for 5 min; 92 °C for 2 min; 38 °C for 40 s; 72 °C for 2 min; 72 °C for 10 min	771	(Rodríguez et al., 2000)
Nisin	*nisR*	F-CTATGAAGTTGCGACGCATCA R-CATGCCACTGATACCCAAGT	92 °C for 5 min; 92 °C for 2 min; 41°C for 40 s; 72 °C for 2 min; 72 °C for 10 min	898	(Rodríguez et al., 2000)
Lacticin	*Lac481*	F-TCTGCACTCACTTCATTAGTTA R-AAGGTAATTACACCTCTTTTAT	92 °C for 5 min; 92 °C for 2 min; 51 °C for 40 s; 72 °C for 1 min; 72 °C for 10 min	366	(Rodríguez et al., 2000)
Durancin	*durA*	F-TACAAATTCATTCAAAAGGAGTGTG R-TTACATACAACCAAGAACAGCACTG	99 °C for 5 min; 95 °C for 30 s; 56 °C for 30 min; 72 °C for 2 min; 72 °C for 5 min.	251	(Du et al., 2012)

Table 7: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for GABA production (Glutamate decarboxylase).

Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	References
gadA4	F-AACACCAAGGTAAACGCACCA R-AAGCCTTCCACAGTAACTTC	94°C for 5 min; 94°C for 30 s; 55°C for 10 s; 72°C for 40 s; 72°C for 5 min	894	(Mancini et al., 2019)
gadA7	F-TTTTGCTCGTACTGGTGTTTC R-AAAGTAACCCTGGAGTTGAC	94°C for 5 min; 94°C for 30 s; 54°C for 10 s; 72°C for 40 s; 72°C for 5 min	576	(Mancini et al., 2019)
gadA8	F-TAGCGTAAAGACGCCCATTT R-GCCGTGATAGTGCCTTGGTA	94°C for 5 min; 94°C for 30 s; 57°C for 10 s; 72°C for 40 s; 72°C for 5 min	711	(Mancini et al., 2019)
gadB4	F-GGGTTGTTTGAACTATTGGC R-TAGTTGAAGAGGGTGTCACGGA	94°C for 5 min; 94°C for 30 s; 54°C for 10 s; 72°C for 40 s; 72°C for 5 min	458	(Mancini et al., 2019)
gadB6	F-AGTTTCCCTGGCCTCATTCTA R-ACTGAATTGACGGGTAGTTGG	94°C for 5 min; 94°C for 30 s; 57°C for 10 s; 72°C for 40 s; 72°C for 5 min	662	(Mancini et al., 2019)
gadB7	F-ACAACCAGTGCGGTCTAAT R-AAGCCGCAAATGGTAGTAAC	94°C for 5 min; 94°C for 30 s; 55°C for 10 s; 72°C for 40 s; 72°C for 5 min	696	(Mancini et al., 2019)
gadR2	F-CGAAAGGTTTTGATCGGCAAA R-TTTTAGGCCGAAACCAGCAA	94°C for 5 min; 94°C for 30 s; 56°C for 10 s; 72°C for 40 s; 72°C for 5 min	408	(Mancini et al., 2019)
gadR3	F-GGGGTCGGAAAGGAATCAAGA R-GCTGTTGACCGACCGATCAAT	94°C for 5 min; 94°C for 30 s; 60°C for 10 s; 72°C for 40 s; 72°C for 5 min	218	(Mancini et al., 2019)
gadR4	F-AGCAAGGTGTTAGTCTAGCT R-CGTTAACCGGTACAATAATCT	94°C for 5 min; 94°C for 30 s; 51°C for 10 s; 72°C for 40 s; 72°C for 5 min	208	(Mancini et al., 2019)

Table 8: Details of genes studied, and their respective primers used for the detection of the predictive mechanism of action for vitamin synthesis.

Target gene	Sequence (5′→3′)	PCR conditions	Product size (bp)	Reference
folP	F- CCASGRCSGCTTGCATGAC R -TKACGCCGGACTCCTTTTWY	95 °C for 5 min; 95 °C for 30 s; 59.5 for 10 s; 72 °C for 15 s, 72 °C for 5 min	261	(Turpin et al., 2011)
ribA	F- TTTACGGGCGATGTTTTAGG R- CGACCCTCTTGCCGTAAATA	95 °C for 5 min; 95 °C for 30 s; 60 for 10 s; 72 °C for 15 s, 72 °C for 5 min	121	(Turpin et al., 2011)

Statistics and visualization

Based on the experimental results, the LAB strains were grouped using *in silico* analysis. LAB strains with similarities were grouped/clustered using principal component analysis (PCA) in PAST v4 (Paleontological Statistics Software Package for Education and Data Analysis). Furthermore, heatmap analysis was also plotted using ClustVis (*https://biit.cs.ut.ee/clustvis/*) (Metsalu and Vilo, 2015). Significant differences in experiments were also checked using paired Student's t-test.

From PCA and heatmap analysis mentioned above, two best strains were selected based on the highest average percentage of all the cumulative experimental tests performed which also reflected on both PCA plot and heatmap.

Microbial gene prediction using PICRUSt2 and Piphillin

Prediction of gene functions was carried using two bioinformatics pipelines- PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) **(**https://github.com/picrust/picrust2/wiki) **(**Douglas et al., 2020**)** and Piphillin **(**https://piphillin.secondgenome.com/**)** (Narayan et al., 2020). Prior to the predictive gene function analysis, raw sequences (Shangpliang et al., 2018) were accessed from NCBI/MG-RAST database server and were processed using QIIME2-2020.6 (https://docs.qiime2.org/2020.6/) (Bolyen et al., 2019). After importing into the QIIME2 environment, quality filtering and denoising was performed by both DADA2 (Callahan et al., 2016) via "qiime dada2 denoise-paired" plugin. Quality-filtered sequences were then clustered against SILVA v132 database (Quast et al., 2012) and taxonomic assignment was performed using "qiime-vsearch-cluster-features-closed-reference" plugin (Rognes et al., 2016).

(a) PICRUSt2 analysis

PICRUSt2 is a pipeline for predicting functional abundances based only on marker gene sequences, 16S rRNA gene **(**Douglas et al., 2020**).** Here, the quality-filtered sequences were fed into PICRUSt2 algorithm with the default parameters. The representative sequences were first clustered in QIIME2 against SILVA v132 database (Quast et al., 2012). Functional prediction in PICRUSt2 involves three main steps- phylogenetic placement of reads, hidden state prediction, pathway inference. Firstly for phylogenetic placement of reads, multiple assignment of the exact sequence variants (ESVs) was performed using HMMER **(**http://www.hmmer.org/**);** and placements of the ESVs in the reference tree was performed using evolutionary placement-ng (EPA-ng) (Barbera et al., 2019) and Genesis Applications for Phylogenetic Placement Analyses (GAPPA) (Czech

and Stamatakis, 2019). Secondly, hidden state prediction of the gene families was run by castor R package (Louca and Doebeli, 2018) with the default "maximum parsimony" algorithm. Lastly, for pathway inference, a modified version of MinPath packaged within PICRUSt2 is used and metagenome prediction was achieved using the default "metagenome_pipeline.py" script (Ye and Doak, 2009). The output features were then mapped against KEGG (Kyoto Encyclopaedia of Genes and Genomes) database for systematic analysis of gene functions (Kanehisa et al., 2012). Furthermore, the KEGG pathway information was then collapsed into three different levels- Category (Level-1), Super Pathway (Level-2) and Pathways (Level-3) (Scala et al., 2019).

(b) Piphillin analysis

Alternatively, functional prediction was also performed using Piphillin software (Narayan et al., 2020). Piphillin is a straightforward independent algorithm which predicts gene functionality from the structural 16S rRNA gene without the use of any proposed phylogenetic tree unlike PICRUSt or PICRUSt2 (Iwai et al., 2016). Most importantly, Piphillin is a web-based analysis software which is simplified, user-friendly and has been shown to have better accuracy in predicting genome function from 16S rRNA gene content (Narayan et al., 2020). Piphillin uses KEGG and BioCyc database as reference databases. Here, gene copy numbers within each genome were retrieved and formatted by KO. Inference of gene function or metagenomic content was achieved by simply matching each representative of OTU/ASVs directly to the nearest sequenced genome without placing the sequence on the phylogenetic tree (Iwai et al., 2016). The representative OTU abundance table is then transformed into genome abundance table by using USEARCH with global alignment (Edgar, 2010) and the resulting closest matched genome to the 16S rRNA gene copy of each representative OTUs/ASVs above

identity threshold is considered as the inferred genome for each OTU/ASV (Iwai et al., 2016). In Piphillin analysis, DADA2-clustered representative sequences (.fasta) and ASV abundance frequency table (.csv) were required to upload to the Piphillin server **(**https://piphillin.secondgenome.com/**)** via a web-browser. Inferred metagenomic content output was then collapsed into three different levels- Category (Level-1), Super Pathway (Level-2) and Pathways (Level-3) (Scala et al., 2019).

Statistical analysis and data visualization

Normalization of the predictive Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) profiles, or simply KO features, from both PICRUSt2 and Piphillin analyses was performed using Metagenomic Universal Single-Copy Correction (MUSiCC) (Manor and Borenstein, 2015). MUSiCC is a normalization paradigm which combines universal single-copy genes with machine learning tools to correct biases and to obtain accurate biological measure of gene abundance in metagenomic studies (Manor and Borenstein, 2015). Error-corrected functional abundance table was then used for downstream analysis and rleative abundances (%) was plotted in MS-Excel v365 as stacked bar-plot for both PICRUSt2 and Piphillin predictive outputs. To check the significant differences between the functional content as predicted by both PICRSUt2 and Piphillin, White's non-parametric with Benjamini-Hochberg FDR (false discovery rate) was applied in STAMP-statistical analysis of taxonomic and functional profiles (Parks et al., 2014) and visualized as extended error-bar chart with alpha significance of 0.05 (*q*-value) for all the functional levels – category (level-1), super pathway (level-2) and pathway (level-3). Furthermore, relationship between bacteria (lactic acid bacteria, LAB; acetic acid bacteria, AAB; and non-LAB/AAB) and functionalities were analyzed using non-parametric Spearman's correlation in Statistical Package for the Social

Sciences (SPSS) v20 and the correlation matrix was visualized as heatmap using ClustVis (Metsalu and Vilo, 2015). Significant interaction between bacteria and function are denoted with "*" <0.05 and "**" <0.01.

RESULTS

DOCUMENTATION OF THE ETHNIC FERMENTED MILK PRODUCTS OF ARUNACHAL PRADESH

Survey on naturally fermented milk products

A survey was conducted for the documentation of various common, rare and artisan naturally fermented milk (NFM) products in different regions of Arunachal Pradesh in India using structured questionnaire (Table A). Information obtained from questionnaire were compiled and recorded on the traditional methods of preparation, culinary, mode of consumption and socio-economy of various NFM products as follows.

Ethnic NFM products

Naturally fermented milk products were found to be confined or popular to only two districts in Arunachal Pradesh *viz*., West Kameng and Tawang. These NFM products are mostly prepared on a daily basis from both milk of cow and yak, where yaks are commonly found in the higher altitudes and cows are available in the lower altitudes. The practice of milk fermentation in these regions are associated only with the sub-ethnic groups belonging to the *Monpa* communities commonly known as the *Brokpas* (cattle herders). Some of the commonly found NFM products includes *mar*, *chhurpi* and *churkam*. Details of the collected sampled are shown in Fig. 1.

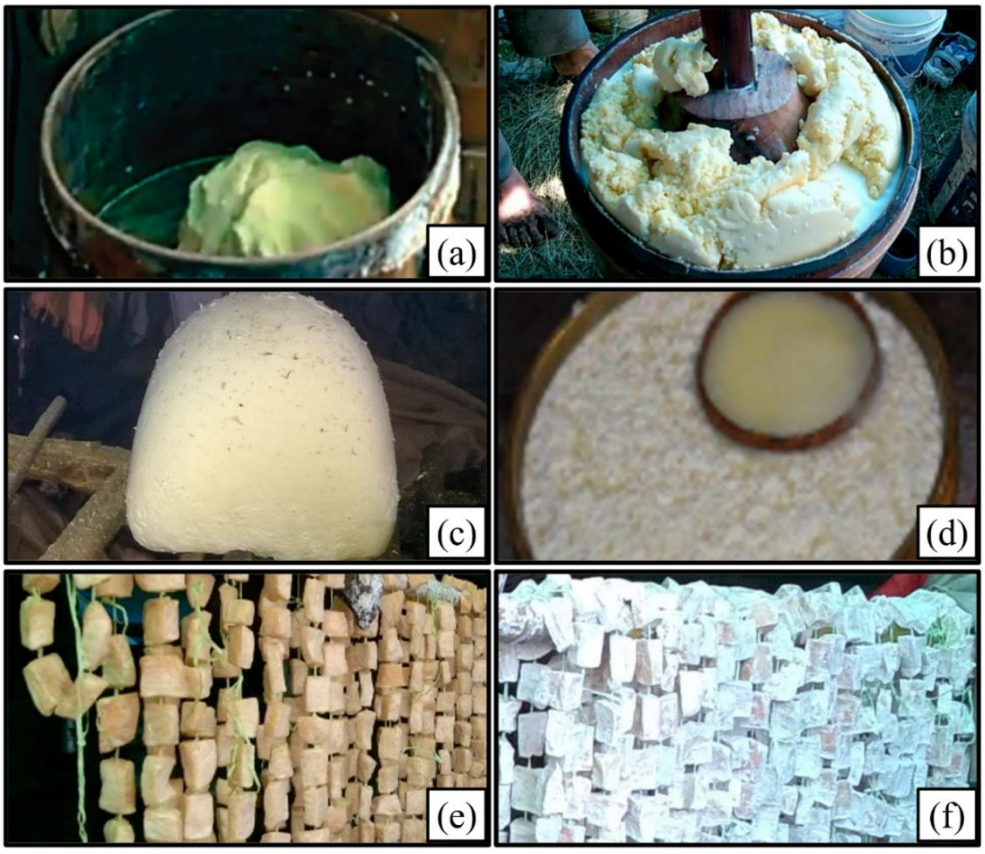


Figure 1: Ethnic naturally fermented milk (NFM) products of Arunachal Pradesh. *Mar*, a butter-like oily product: (a) cow-milk *mar*, (b) yak-milk *mar; Chhurpi*, a soft cottage cheese-like: (c) cow-milk *chhurpi*, (d) yak-milk *chhurpi*; *Churkam*, a hard masticator: (e) cow-milk *churkam*, (f) yak-milk *churkam*.

Indigenous method of preparation

In Arunachal Pradesh, fermentation of milk contains a series of steps where different final products are obtained. However, there is no difference in the preparation of these products whether it is from cow or yak's milk. Here, the collected milk is churned in a specially crafted wooden vessel, locally known as a *sop*/*shoptu*/*zopu*. It is also observed to be a common practice to warm up the milk before churning, in the cold season. Sometimes, when the climate is little warmer, the collected milk is collected and stored in cold place until the time to churn. Basically, NFM products consists of two types

depending on their chemical nature, whether lipid-rich or casein-based. *Mar*, an artisanal butter, is a lipid-rich product which is separated from the whole milk upon churning (Fig. 2a). After the separation of *mar*, the remaining buttermilk, locally known as *dhara*, is further boiled for 25-30 min till a visible formation of clumps appeared. *Mar* can be stored at room temperature for a longer period, and it does not get spoiled that easily. Following this, the traditional cheese clumps, locally known as *chhurpi*, are collected and the remaining whey (local term: *churku*) is drained off. *Chhurpi* is naturally and spontaneously fermented at room temperature for days to few weeks (Fig. 2b). On the other hand, *chhurpi* is also used to prepare another fermented milk, known as *churkam.* *Churkam* results from freshly prepared *chhurpi*, where it is immediately collected after boiling and dried by hanging in a cloth for 2-3 days. An extra step is usually taken to completely dry off the remaining liquid whey by placing *chhurpi* (hung in a cloth) in between two slabs of stones for 4-5 h. Following this step, is the careful uncovering of the cloth wrapping the *chhurpi*, and the semi-dried product is cut into small cubes which are then boiled along with the *churku* (whey) until they are almost dried. These cubes are usually sewn together in a thread with a total of 20 pieces in one roll, which are further dried by hanging for 3-4 days at room temperature (Fig. 2c). *Churkam* can be stored and kept for days to weeks in room temperature, and it does not get spoiled easily.

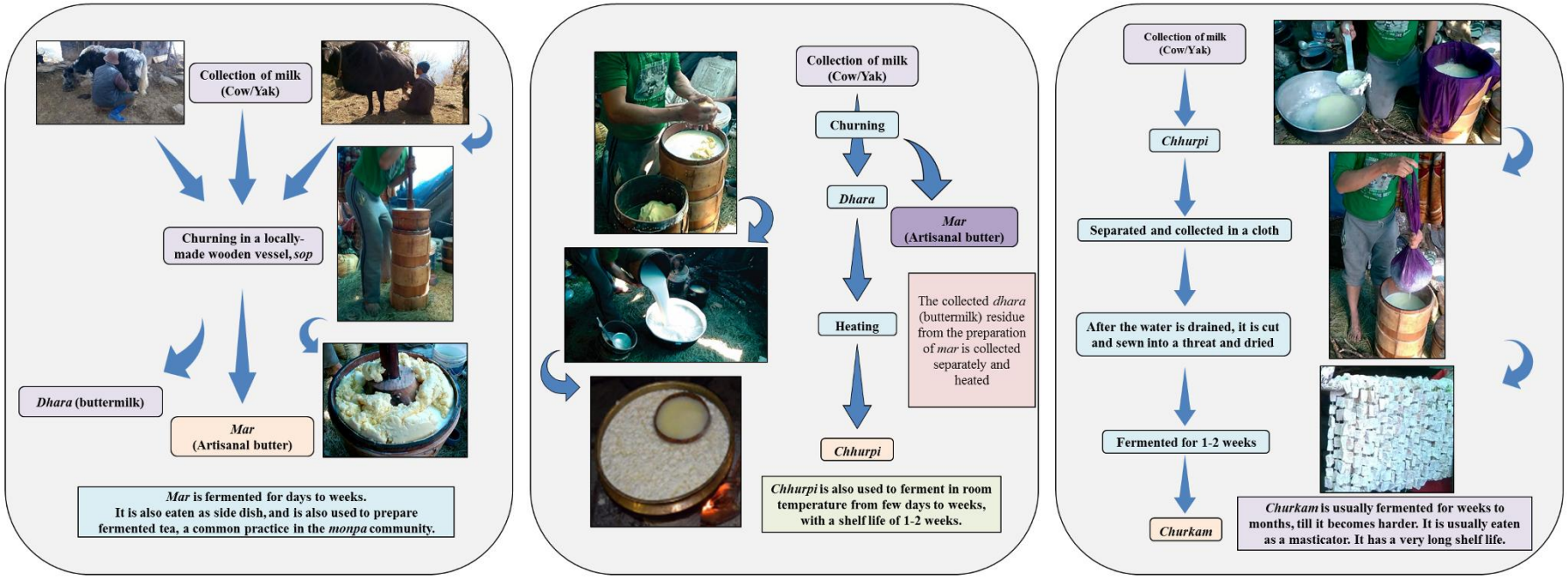


Figure 2: Indigenous method of preparation of the ethnic NFM products of Arunachal Pradesh from both cow and yak milk. (a) *mar*, (b) *chhurpi*, and (c) *churkam*.

Mode of consumption and ethnic values

Mar (artisanal butter) is used as an ingredient for beverage made of tea and salt, locally known as *shui zha*/*maar zha*, which is commonly known as *namak* tea (butter tea). Sometimes, it is also mixed in the preparation of dishes and can also be eaten with rice. In the local markets, *mar* is priced for ₹250 per kg. *Chhurpi* is usually eaten fresh and sometimes can be consumed raw without needing to be prepared as a dish. However, it can also be mixed or cooked with vegetables like tomatoes, potatoes, and even soybeans. It is sold in the market at the rate of ₹400 per kg. On the other hand, *churkam* is the only one among the three products which is eaten as masticator/mouth freshener by locals and especially by herders grazing their cattle in the higher regions. It is also used as greetings for families and loved ones. *Churkam* is sold at the rate of ₹120-150 per roll in the local markets.

NFM products are one of the essential milk products which are widely prepared by the *Brokpa* community also for income generation. Based on the survey documentation, it was observed that there is no taboo in the preparation of the NFM products among the ethnic communities in Arunachal Pradesh. Since these NFM products are one of the main sources of income, these NFM products are prepared daily. Sometimes, NFM products are used for religious ceremonies. In local beliefs and practices, the NFM products are known to have several health benefits: mostly with minor stomach ailments. *Mar* and *chhurpi* are known to cure stomach indigestion and diarrhoea. *Chhurpi* is also mixed with beverages and given to people with stomach-ache. *Churkam*, on the other hand, as it is often used as a masticator, it helps in gum protection and maintain body heat in colder regions and high altitudes. A good income from selling these NFM products was also recorded by the sellers where a profit of about ₹4000-₹9000 per month, depending on the market, demand, and export to other states.

MICROBIAL DIVERSITY ANALYSIS, PHENOTYPIC CHARACTERIZATION AND 16S rRNA-BASED CULTURE-DEPENDENT ANALYSIS

pH of the samples and microbial analysis

The pH values of naturally fermented milk of cow products (*mar, chhurpi* and *churkam*) were ranging from 5.32±0.01 to 6.55±0.01; and that of yak products (*mar, chhurpi* and *churkam*) from 5.40±0.01 to 6.62±0.01, respectively (Table 9). The microbial populations in NFM of cow products and yak products were ranging from 6.27±0.01 cfu/g to 6.40±0.01 cfu/g, and 6.27±0.01 cfu/g to 6.49±0.02 cfu/g, respectively (Table 9).

Table 9: Sample details, pH, and lactic acid bacterial load of the ethnic NFM products of Arunachal Pradesh.

NFM	Source of milk	Nature of samples	Place of collection	pH (Mean±SD)	Log cfu g^{-1} (Mean±SD)
Mar	Cow	Butter-like	Cheghar, Tawang	6.52±0.01	6.29±0.03
Mar	Cow	Butter-like	Samchin, Tawang	6.52±0.01	6.39±0.04
Mar	Cow	Butter-like	Kudung, Tawang	6.53±0.01	6.40±0.01
Mar	Cow	Butter-like	Tawang, Tawang	6.53±0.01	6.35±0.08
Mar	Cow	Butter-like	Bomdila, West Kameng	6.53±0.02	6.38±0.01
Mar	Cow	Butter-like	Dirang, West Kameng	6.55±0.01	6.39±0.04
Mar	Yak	Butter-like	Samchin, Tawang	6.56±0.02	6.47±0.02
Mar	Yak	Butter-like	Cheghar, Tawang	6.61±0.01	6.49±0.02
Mar	Yak	Butter-like	Dirang, West Kameng	6.62±0.01	6.48±0.03
Mar	Yak	Butter-like	Bomdila, West Kameng	6.62±0.01	6.43±0.03
Chhurpi	Cow	Soft, cheese-like	Cheghar, Tawang	5.32±0.01	6.28±0.02
Chhurpi	Cow	Soft, cheese-like	Samchin, Tawang	5.32±0.01	6.29±0.01
Chhurpi	Cow	Soft, cheese-like	Kudung, Tawang	5.32±0.02	6.32±0.02
Chhurpi	Cow	Soft, cheese-like	Tawang, Tawang	5.33±0.01	6.27±0.01
Chhurpi	Cow	Soft, cheese-like	Bomdila, West Kameng	5.33±0.02	6.33±0.02
Chhurpi	Cow	Soft, cheese-like	Dirang, West Kameng	5.35±0.01	6.29±0.01
Chhurpi	Yak	Soft, cheese-like	Samchin, Tawang	5.35±0.01	6.30±0.03
Chhurpi	Yak	Soft, cheese-like	Cheghar, Tawang	5.41±0.01	6.27±0.01
Chhurpi	Yak	Soft, cheese-like	Dirang, West Kameng	5.42±0.01	6.34±0.05
Chhurpi	Yak	Soft, cheese-like	Bomdila, West Kameng	5.42±0.02	6.36±0.03
Churkam	Cow	Hard-mass, masticator	Cheghar, Tawang	5.71±0.01	6.29±0.02
Churkam	Cow	Hard-mass, masticator	Samchin, Tawang	5.71±0.01	6.35±0.04
Churkam	Cow	Hard-mass, masticator	Kudung, Tawang	5.71±0.01	6.34±0.03
Churkam	Cow	Hard-mass, masticator	Tawang, Tawang	5.71±0.01	6.3±0.04
Churkam	Cow	Hard-mass, masticator	Dirang, West Kameng	5.72±0.01	6.38±0.03
Churkam	Cow	Hard-mass, masticator	Bomdila, West Kameng	5.72±0.01	6.34±0.11
Churkam	Yak	Hard-mass, masticator	Samchin, Tawang	5.82±0.01	6.31±0.06
Churkam	Yak	Hard-mass, masticator	Cheghar, Tawang	5.82±0.01	6.28±0.04
Churkam	Yak	Hard-mass, masticator	Dirang, West Kameng	5.87±0.02	6.34±0.03
Churkam	Yak	Hard-mass, masticator	Bomdila, West Kameng	5.85±0.02	6.36±0.04

Phenotypic and biochemical characterization

Based on the phenotypic characteristics and sugar fermentation, five groups of presumptive lactic acid bacteria (LAB) genera were identified from *mar* samples which included *Enterococcus*, lactobacilli (Hetero group I), lactobacilli, (Homo group I). *Lactococcus* (group I) and *Leuconostoc* (Table 10). Seven genera of LAB isolated from samples of *chhurpi* were tentatively grouped into lactobacilli (Hetero group I), lactobacilli (Hetero group II), lactobacilli (Homo group I), lactobacilli (Homo group II), *Lactococcus* (group I), *Lactococcus* (group II) and *Leuconostoc* (Table 11). Similarly, six genera of LAB isolated from samples of *churkam* were tentatively grouped into *Enterococcus*, lactobacilli (Hetero group I), *Lactococcus* (group I), *Lactococcus* (group II), *Lactococcus* (group III) and *Leuconostoc* (Table 12).

Grouping and identification using 16S rRNA gene sequencing

Based on similar phenotypic and sugar fermentation tests, 76 representative strains of LAB, comprising of major genera *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc* were selected for molecular identification. Sequencing of the 16S rRNA gene (~1500kb) was targeted for lactic acid bacteria identification, after PCR amplification and validation in 1.2% agarose gel electrophoresis (Fig. 3). Phylogenetic analysis was carried out using good quality sequences ranging from 1,048 bp-1,471 bp, which were aligned using EzTaxon and NCBI database (Table 13-15). All matched bacterial type strains were then used for phylogenetic tree construction using the Neighbour-joining method, with a bootstrap value of 1000 replicates using MEGA7 (Fig. 4). Based on the 16S rRNA sequencing method and phylogenetic tree, 17 species of LAB were identified from samples of cow-milk products, and 10 species of LAB from samples of yak-milk products, respectively. An overall representation of all

identified LAB species suing both grouping-based phenotypic tests and 16SrRNA gene-based sequencing is given in Table 16.

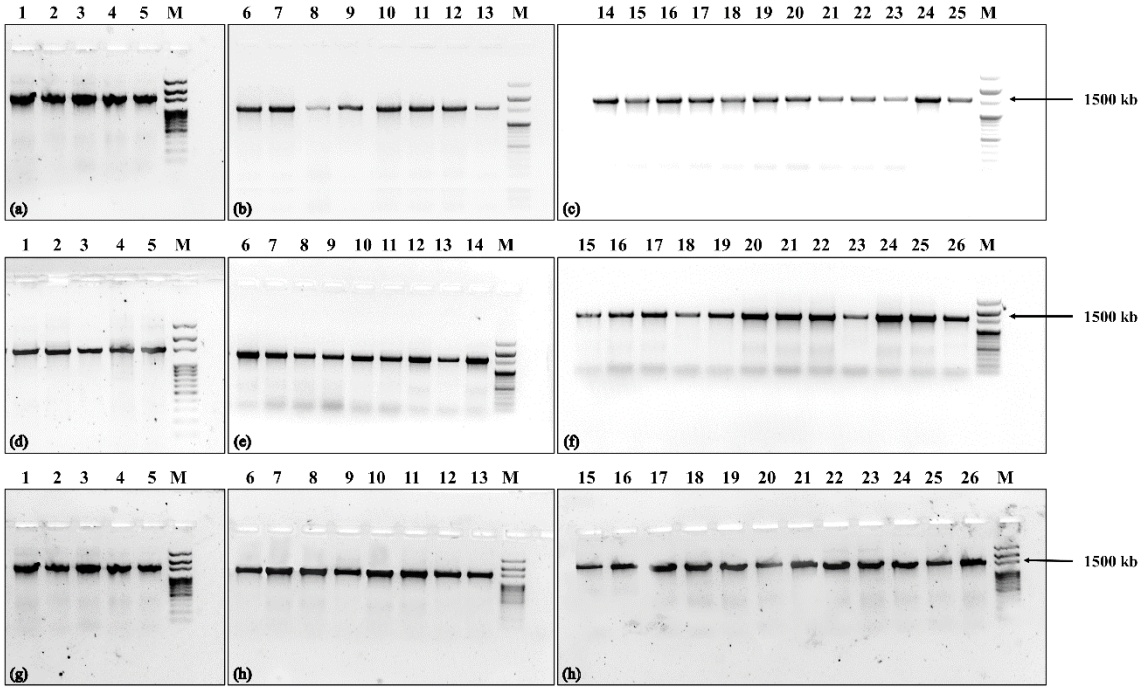


Figure 3: Confirmation of the targeted amplified 16S rRNA gene (~1500kb) of the total 76 LAB representatives isolated from (a-c) *mar* (25 representative LAB strains), (d-f) *chhurpi* (26 representative LAB strains) and (g-i) *churkam* (25 representative LAB strains). All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 1.2% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

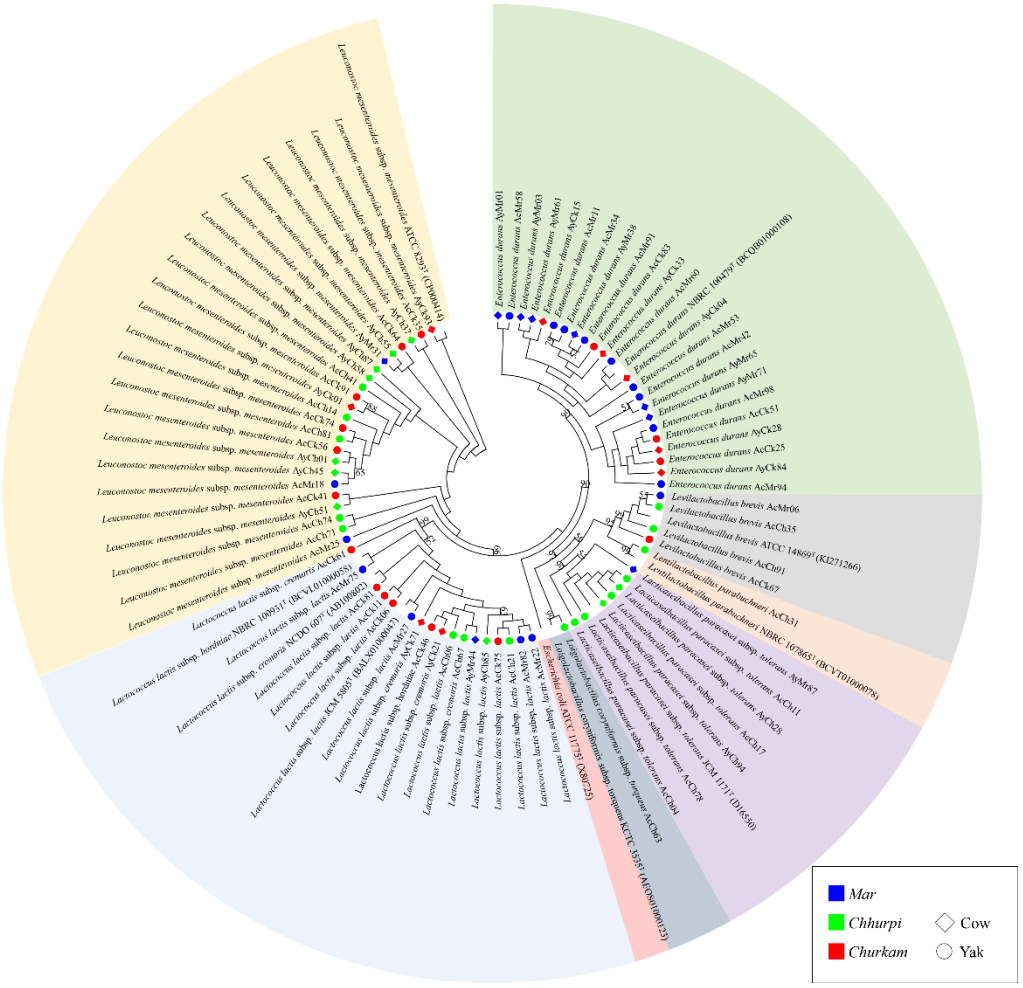


Figure 4: Phylogenetic diversity analysis of lactic acid bacteria species isolated from NFM products of Arunachal Pradesh. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. The analysis involved 86 nucleotide sequences (including type strains). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. The isolates were also depicted in colours where they were originally isolated from, blue- *mar*, green- *chhurpi* and red- *churkam*, diamond for cow and circle for yak products. *Escherichia coli* ATCC 11775(T) was used as an outgroup for phylogenetic tree construction.

Table 10: Phenotypic characterization and groupings of LAB isolated from *mar* (cow and yak milk) of Arunachal Pradesh.

Characteristic	***Enterococcus***		**lactobacilli (Hetero group I)**		**lactobacilli (Homo group I)**		***Lactococcus* (group I)**		***Leuconostoc***	
	Number of Isolates									
	Cow (31)	**Yak (19)**	**Cow (5)**	**Yak (0)**	**Cow (0)**	**Yak (3)**	**Cow (20)**	**Yak (5)**	**Cow (13)**	**Yak (6)**
Cell morphology	Cocci (20)/ coccoid (11)	Cocci (10)/ coccoid (9)	rods	-	-	rods	cocci	cocci	cocci(10)/coccoid(3)	short-rods
Cellular arrangement/shape	short-chains (7)/ single (8)/pairs (16)	short chains (5)/ single (5)/pairs (9)	pairs	-	-	chains	short-chains(10)/ chains(5)/pairs(5)	pairs	pairs(10)/chains(3)	chains
Gas from Glucose	-ve	-ve	+ve	-	-	-ve	-ve	-ve	+ve	+ve
Homo/Hetero-fermentation	Homo	Homo	Hetero	-	-	Homo	Homo	Homo	Hetero	Hetero
Growth at 6.5 NaCl	+ve(25)/-ve(6)	+ve(14)/-ve(5)	-ve	-	-	+ve	-ve	-ve	-ve	-ve
Growth at 45oC	+ve(23)/-ve(8)	+ve(8)/-ve(11)	-ve	-	-	+ve	-ve	-ve	+ve	+ve
arginine hydrolysis	+ve	+ve	+ve	-	-	-ve	+ve(15)/-ve(5)	-ve	-ve	-ve
mannitol	-ve	-ve	-ve	-	-	-ve	-ve	-ve	+ve(12)/-ve(1)	-ve
arabinose	-ve	-ve	+ve	-	-	-ve	-ve	-ve	+ve	+ve
cellobiose	+ve	+ve	-ve	-	-	-ve	+ve	+ve	+ve(8)/-ve(5)	+ve
gluconate	-ve	-ve	+ve	-	-	-ve	-ve	-ve	-ve	-ve
lactose	+ve	+ve	-ve	-	-	+ve	+ve	+ve	+ve(12)/-ve(1)	-ve
maltose	+ve	+ve	+ve	-	-	-ve	+ve	+ve	+ve	+ve

mannose	+ve	+ve	-ve	-	-	-ve	+ve	+ve	+ve(5)/-ve(8)	-ve
melibiose	-ve	-ve	+ve	-	-	-ve	-ve	-ve	+ve(5)/-ve(8)	-ve
raffinose	+ve(6)/-ve(25)	+ve(4)/-ve(15)	+ve	-	-	-ve	-ve	-ve	+ve(1)/-ve(12)	+ve
ribose	+ve	+ve	+ve	-	-	-ve	+ve	+ve	-ve	-ve
sucrose	+ve(9)/-ve(22)	+ve(3)/-ve(16)	-ve	-	-	-ve	-ve	-ve	+ve	+ve
trehalose	+ve	+ve	-ve	-	-	-ve	+ve	+ve	+ve	+ve
xylose	-ve	-ve	-ve	-	-	-ve	-ve	-ve	+ve(12)/-ve(1)	-ve
salicin	+ve	+ve	-ve	-	-	-ve	+ve	+ve	+ve(1)/-ve(12)	+ve
Representatives	9	6	1	0	0	1	4	1	2	1

Note: All isolates were Gram-positive, catalase positive, grew at 10^{o}C, utilized fructose, glucose, galactose and rhamnose. Number within parentheses denotes the number of strains with similar phenotype. Originally, 102 LAB strains were isolated, out of which 69 are from cow products and 33 are from yak products of *mar*. Based on random selection, we have collectively grouped all the isolated strains (from both cow and yak products) and further identified by 16S rRNA gene sequencing.

Table 11: Phenotypic characterizations and groupings of LAB isolated from *chhurpi* (cow and yak milk) of Arunachal Pradesh.

Characteristic	**lactobacilli (Hetero group I)**		**lactobacilli (Hetero group II)**		**lactobacilli (Homo group I)**		**lactobacilli (Homo group II)**		***Lactococcus* (group I)**		***Lactococcus* (group II)**		***Leuconostoc***	
	Number of isolates													
	Cow (14)	Yak (0)	Cow (2)	Yak (0)	Cow (16)	Yak (7)	Cow (3)	Yak (0)	Cow (10)	Yak (3)	Cow (5)	Yak (0)	Cow (17)	Yak (24)
Cellular morphology	rods (7)/short-rods(7)	-	rods	-	short-rods(10)/ rods (6)	short-rods(4)/rods(3)	rods	-	cocci(5)/coccoid(5)	cocci	cocci	-	short-rods(5)/ cocci(4) /coccoid (8)	short-rods(11)/ cocci(10)/coccoid(3)
Cellular arrangement/shape	pairs(7) /short-chains(7)	-	single	-	single(5)/ pairs(5) chains (6)	pairs(3)/ chains(4)	single	-	pairs(5)/chains(5)	pairs	pairs	-	pairs(8)/ chains(9)	pairs(8)/ chains(16)
Gas from Glucose	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve	+ve
Homo/Hetero-fermentation	Hetero	-	Hetero	-	Homo	Homo	Homo	-	Homo	Homo	Homo	-	Hetero	Hetero
Growth at 45oC	-ve	-	+ve	-	+ve	+ve	-ve	-	-ve	-ve	-ve	-	+ve	+ve
Arginine hydrolysis	+ve	-	+ve	-	-ve	-ve	-ve	-	+ve(7)/ -ve(3)	-ve	+ve	-	-ve	-ve
mannitol	-ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve(10)/ -ve(7)	+ve(12)/ -ve(12)
arabinose	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	+ve	-	+ve	+ve
cellobiose	-ve	-	-ve	-	-ve	-ve	+ve	-	+ve	+ve	-ve	-	+ve(13)/ -ve(4)	+ve(9)/-ve(15)

fructose	+ve	-	+ve	-	+ve	+ve	-ve	-	+ve	+ve	-ve	-	+ve	+ve
galactose	+ve(7)/-ve(7)	-	+ve	-	+ve	+ve	-ve	-	+ve	+ve	+ve	-	+ve	+ve
gluconate	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	-ve	-ve
lactose	-ve	-	-ve	-	+ve	+ve	+ve	-	+ve	+ve	-ve	-	+ve(5)/-ve(12)	+ve(6)/-ve(18)
maltose	+ve	-	+ve	-	-ve	-ve	-ve	-	+ve	+ve	-ve	-	+ve	+ve
mannose	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve	+ve	+ve	-	+ve(15)/-ve(2)	+ve(21)/-ve(3)
melibiose	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve(6)/-ve(11)	+ve(8)/-ve(16)
raffinose	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve(11)/-ve(6)	+ve(19)/-ve(5)
ribose	+ve	-	+ve	-	-ve	-ve	-ve	-	+ve	+ve	-ve	-	-ve	-ve
sucrose	+ve(7)/-ve(7)	-	-ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve	+ve
trehalose	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve	+ve	-ve	-	+ve	+ve
xylose	+ve(7)/-ve(7)	-	-ve	-	-ve	-ve	-ve	-	-ve	-ve	-ve	-	+ve(7)/-ve(10)	+ve(9)/-ve(15)
salicin	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve	+ve	-ve	-	+ve(8)/-ve(9)	+ve(8)/-ve(16)
Representatives	2	0	1	0	4	2	1	0	2	1	1	0	5	7

Note: All isolates were Gram-positive, catalase positive, grew at/in 10^{o}C and 6.5% NaCl, utilized glucose and rhamnose. Originally, 101 LAB strains were isolated, out of which 67 are from cow products and 34 are from yak products of *chhurpi*. Based on random selection, we have collectively grouped all the isolated strains (from both cow and yak products) and further identified by 16S rRNA gene sequencing.

Table 12: Phenotypic characterizations and groupings of LAB isolated from *churkam* (cow and yak milk) of Arunachal Pradesh.

Characteristic	***Enterococcus***		**lactobacilli (Hetero group I)**		**Lactococcus (group I)**		***Lactococcus* (group II)**		***Lactococcus* (group III)**		***Leuconostoc***	
	Number of isolates											
	Cow (13)	Yak (23)	Cow (5)	Yak (0)	Cow (15)	Yak (0)	Cow (4)	Yak (10)	Cow (3)	Yak (0)	Cow (23)	Yak (8)
Cellular morphology	coccoid(2)/cocci(11)	coccoid(8)/cocci(15)	rod	-	cocci(10)/coccoid(5)	-	coccoid	coccoid(8)/cocci(2)	cocci	-	short-rods(15)/cocci(5)/coccoid(3)	short-rods(2)/cocci(3)/coccoid(3)
Cellular arrangement/shape	pairs(8)/single(3)/short-chains(2)	pairs(11)/single(7)/short-chains(5)	pairs	-	pairs(2)/chains(13)	-	chains	pairs(1)/chains(9)	pairs	-	pairs(8)/chains(15)	pairs(5)/chains(3)
Gas from Glucose	-ve	-ve	+ve	-	-ve	-	-ve	-ve	-ve	-	+ve	+ve
Homo/Hetero fermentation	Homo	Homo	Hetero	-	Homo	-	Homo	Homo	Homo	-	Hetero	Hetero
Growth at 6.5 NaCl	+ve	+ve	-ve	-	-ve	-	+ve	+ve	-ve	-	-ve	-ve
Growth at 10^{o}C	+ve	+ve	-ve	-	+ve	-	+ve	+ve	+ve	-	+ve	+ve
Growth at 45^{o}C	+ve	+ve	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve	+ve
Arginine hydrolysis	+ve	+ve	+ve	-	+ve(9)/-ve(6)	-	-ve	+ve(7)/-ve(3)	+ve	-	-ve	-ve

mannitol	-ve	-ve	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve(7)/-ve(16)	+ve(7)/-ve(1)
arabinose	-ve	-ve	+ve	-	-ve	-	+ve	+ve(8)/-ve(2)	-ve	-	+ve	+ve
cellobiose	+ve	+ve	-ve	-	+ve	-	-ve	-ve	-ve	-	+ve(15)/-ve(8)	+ve(3)/-ve(5)
fructose	+ve	+ve	+ve	-	+ve	-	-ve	-ve	+ve	-	+ve	+ve
galactose	+ve	+ve	+ve	-	+ve	-	+ve	+ve	-ve	-	+ve	+ve
gluconate	-ve	-ve	+ve	-	-ve	-	-ve	-ve	-ve	-	-ve	-ve
lactose	+ve	+ve	-ve	-	+ve	-	-ve	-ve	-ve	-	+ve(7)/-ve(16)	+ve(6)/-ve(2)
maltose	+ve	+ve	+ve	-	+ve	-	-ve	-ve	-ve	-	+ve	+ve
mannose	+ve	+ve	-ve	-	+ve	-	+ve	+ve	+ve	-	+ve(18)/-ve(5)	+ve(5)/-ve(3)
melibiose	-ve	-ve	+ve	-	-ve	-	-ve	-ve	-ve	-	+ve(16)/-ve(7)	+ve(7)/-ve(1)
raffinose	+ve(7)/-ve(6)	+ve(6)/-ve(17)	+ve	-	-ve	-	-ve	-ve	-ve	-	+ve(7)/-ve(16)	+ve(4)/-ve(4)
ribose	+ve	+ve	+ve	-	+ve	-	-ve	-ve	-ve	-	-ve	-ve
sucrose	+ve(2)/-ve(11)	+ve(5)/-ve(18)	+ve	-	-ve	-	-ve	-ve	+ve	-	+ve	+ve
trehalose	+ve	+ve	-ve	-	+ve	-	-ve	-ve	+ve	-	+ve	+ve

xylose	-ve	-ve	-ve	-	-ve	-	-ve	-ve	-ve	-	+ve(15)/-ve(8)	+ve(3)/-ve(5)
salicin	+ve	+ve	-ve	-	+ve	-	-ve	-ve	+ve	-	+ve(14)/-ve(9)	+ve(3)/-ve(5)
Representatives	3	5	1	0	4	0	1	2	1	0	6	2

Note: All isolates were Gram-positive, catalase positive, utilized glucose and rhamnose. Originally, 104 LAB strains were isolated, out of which 63 are from cow products and 41 are from yak products of *churkam*. Based on random selection, we have collectively grouped all the isolated strains (from both cow and yak products) and further identified by 16S rRNA gene sequencing.

Table 13: Identification of LAB isolated from *mar* (cow and yak) by the 16S rRNA gene sequence.

	Isolates	Sequence size (bp)	Similarity (%)	Top-hit taxon (EzTaxon)	Top-hit type strain	Accession Number (NCBI)
Cow	AcMr06	1,380	99.78	*Levilactobacillus brevis*	ATCC 14869(T)	MK182839
	AcMr11	1,353	99.48	*Enterococcus durans*	NBRC 100479(T)	MT305922
	AcMr18	1,396	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182840
	AcMr22	1,405	99.57	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305923
	AcMr25	1,323	99.92	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK203744
	AcMr27	1,419	99.72	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305924
	AcMr34	1,356	99.48	*Enterococcus durans*	NBRC 100479(T)	MT305925
	AcMr42	1,267	99.84	*Enterococcus durans*	NBRC 100479(T)	MT305927
	AcMr53	1,399	99.71	*Enterococcus durans*	NBRC 100479(T)	MT305929
	AcMr58	1407	99.57	*Enterococcus durans*	NBRC 100479(T)	MT305930
	AcMr60	1,356	99.34	*Enterococcus durans*	NBRC 100479(T)	MT305931
	AcMr75	1,393	99.78	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305933
	AcMr82	1,427	99.65	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305934
	AcMr91	1,390	99.57	*Enterococcus durans*	NBRC 100479(T)	MT305936
	AcMr94	1,149	99.91	*Enterococcus durans*	NBRC 100479(T)	MK203741
	AcMr98	1,074	99.81	*Enterococcus durans*	NBRC 100479(T)	MK203740
Yak	AyMr01	1,379	99.42	*Enterococcus durans*	NBRC 100479(T)	MT305920
	AyMr03	1,223	100	*Enterococcus durans*	NBRC 100479(T)	MT305921
	AyMr31	1,393	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182841
	AyMr38	1,436	99.58	*Enterococcus durans*	NBRC 100479(T)	MT305926
	AyMr44	1,380	99.64	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305928
	AyMr61	1,385	99.42	*Enterococcus durans*	NBRC 100479(T)	MT305932
	AyMr65	1,360	98.97	*Enterococcus durans*	NBRC 100479(T)	MK203743
	AyMr71	1,293	99.92	*Enterococcus durans*	NBRC 100479(T)	MK203742
	AyMr87	1,436	99.72	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 11(T)71	MT305935

Note: Sequences were compared against EzTaxon to find the closest relatives.

Table 14: Identification of LAB isolated from *chhurpi* (cow and yak) by the 16S rRNA gene sequence.

	Isolates	Sequence size (bp)	Similarity (%)	Top-hit taxon (EzTaxon)	Top-hit type strain	Accession Number (NCBI)
Cow	AcCh04	1,300	99.53	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305880
	AcCh06	1,419	99.65	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305881
	AcCh11	1,420	99.79	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305882
	AcCh14	1,471	99.07	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305883
	AcCh17	1,428	99.86	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305884
	AcCh21	1,413	99.86	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305885
	AcCh31	1,427	99.79	*Lentilactobacillus parabuchneri*	NBRC 107865(T)	MT305887
	AcCh35	1,452	99.72	*Levilactobacillus brevis*	ATCC 14869(T)	MK182827
	AcCh41	1,330	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8294(T)	MT305888
	AcCh63	1,393	99.57	*Loigolactobacillus coryniformis* subsp. *torquens*	KCTC 3535(T)	MT305892
	AcCh67	1,324	99.77	*Lactococcus lactis* subsp. *cremoris*	NCDO 607(T)	MT305893
	AcCh71	1,048	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182830
	AcCh74	1,391	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305894
	AcCh78	1,446	100	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305895
	AcCh81	1,355	99.93	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182831
	AcCh91	1,421	99.93	*Levilactobacillus brevis*	ATCC 14869(T)	MK182832
Yak	AyCh01	1,366	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305879
	AyCh28	1,389	99.49	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305886
	AyCh37	1,354	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182828
	AyCh45	1,355	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8294(T)	MK182829
	AyCh51	1,400	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8294(T)	MT305889
	AyCh55	1,431	99.57	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8294(T)	MT305890
	AyCh58	1,316	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8294(T)	MT305891
	AyCh85	1,413	99.65	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305896
	AyCh87	1,434	99.79	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305897
	AyCh94	1,419	99.79	*Lacticaseibacillus paracasei* subsp. *tolerans*	JCM 1171(T)	MT305898

Note: Sequences were compared against EzTaxon to find the closest relatives.

Table 15: Identification of LAB isolated from *churkam* (cow and yak) by the 16S rRNA gene sequence.

	Isolates	Sequence size (bp)	Similarity (%)	Top-hit taxon (EzTaxon)	Top-hit type strain	Accession Number (NCBI)
Cow	AcCk06	1,379	99.78	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305903
	AcCk11	1,363	100	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305904
	AcCk25	1,345	99.7	*Enterococcus durans*	NBRC 100479(T)	MT305907
	AcCk35	1,369	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182833
	AcCk41	1,356	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182834
	AcCk46	1,427	99.23	*Lactococcus lactis* subsp. *hordniae*	NBRC 100931(T)	MT305910
	AcCk51	1,343	99.11	*Enterococcus durans*	NBRC 100479(T)	MT305911
	AcCk56	1,344	100	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182835
	AcCk61	1,293	98.99	*Lactococcus lactis* subsp. *cremoris*	NCDO 607(T)	MT305912
	AcCk64	1,362	99.78	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182836
	AcCk67	1,381	99.93	*Levilactobacillus brevis*	ATCC 14869(T)	MK182837
	AcCk74	1,333	99.85	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MK182838
	AcCk75	1,425	99.72	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305914
	AcCk81	1,420	99.65	*Lactococcus lactis* subsp. *lactis*	JCM 5805(T)	MT305915
	AcCk83	1,394	99.71	*Enterococcus durans*	NBRC 100479(T)	MT305916
	AcCk91	1,216	99.01	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305918
Yak	AyCk01	1,271	99.84	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305901
	AyCk04	1,384	99.93	Enterococcus *durans*	NBRC 100479(T)	MT305902
	AyCk15	1,408	99.72	*Enterococcus durans*	NBRC 100479(T)	MT305905
	AyCk21	1,417	99.22	*Lactococcus lactis* subsp. *cremoris*	NCDO 607(T)	MT305906
	AyCk28	1,285	99.21	*Enterococcus durans*	NBRC 100479(T)	MT305908
	AyCk33	1,374	99.93	*Enterococcus durans*	NBRC 100479(T)	MT305909
	AyCk71	1,424	99.79	*Lactococcus lactis* subsp. *cremoris*	NCDO 607(T)	MT305913
	AyCk84	1,370	99.27	*Enterococcus durans*	NBRC 100479(T)	MT305917
	AyCk93	1,396	99.93	*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293(T)	MT305919

Note: Sequences were compared against EzTaxon to find the closest relatives.

Table 16: Overall representation of the total isolates of LAB strains, tentative grouping based on phenotypic characteristics and identification using 16S rRNA gene sequencing.

NFM products	Total strains isolated	Cow products isolates	Yak products isolates	Tentative genera/groups	Cow-products (Tentative random grouping)	Yak-products (Tentative random grouping)	16S rRNA gene sequencing
	50	31	19	*Enterococcus*	9	6	*Enterococcus durans*
	5	5	0	lactobacilli (Hetero group I)	1	0	*Levilactobacillus brevis*
Mar	3	0	3	lactobacilli (Homo group I)	0	1	*Lacticaseibacillus paracasei* subsp. *tolerans*
	25	20	5	*Lactococcus* (group I)	4	1	*Lactococcus lactis* subsp. *lactis*
	19	13	6	*Leuconostoc*	2	1	*Leuconostoc mesenteroides* subsp. *mesenteroides*
Total	102	69	33		16	9	
	14	14	0	lactobacilli (Hetero group I)	2	0	*Levilactobacillus brevis*
	2	2	0	lactobacilli (Hetero group II)	1	0	*Loigolactobacillus coryniformis* subsp. *torquens*
	23	16	7	lactobacilli (Homo group I)	4	2	*Lacticaseibacillus paracasei* subsp. *tolerans*
Chhurpi	3	3	0	lactobacilli (Homo group II)	1	0	*Lentilactobacillus parabuchneri*
	13	10	3	*Lactococcus* (group I)	2	1	*Lactococcus lactis* subsp. *lactis*
	5	5	0	*Lactococcus* (group II)	1	0	*Lactococcus lactis* subsp. *cremoris*
	41	17	24	*Leuconostoc*	5	7	*Leuconostoc mesenteroides* subsp. *mesenteroides*
Total	101	67	34		16	10	
	36	13	23	*Enterococcus*	3	5	*Enterococcus durans*
	5	5	0	lactobacilli (Hetero group I)	1	0	*Levilactobacillus brevis*
Churkam	15	15	0	*Lactococcus* (group I)	4	0	*Lactococcus lactis* subsp. *lactis*
	14	4	10	*Lactococcus* (group II)	1	2	*Lactococcus lactis* subsp. *cremoris*
	3	3	0	*Lactococcus* (group III)	1	0	*Lactococcus lactis* subsp. *hordniae*
	31	23	8	*Leuconostoc*	6	2	*Leuconostoc mesenteroides* subsp. *mesenteroides*
Total	104	63	41		16	9	

Relative abundances of the identified culturable LAB strains

Enterococcus durans (32.87%) was the most dominant species followed by *Leuconostoc mesenteroides* subsp. *mesenteroides* (30.76%), *Lactococcus lactis* subsp. *lactis* (13.94%), *Lacticaseibacillus paracasei* subsp. *tolerans* (9.35%), *Lactococcus lactis* subsp. *cremoris* (5.79%), *Levilactobacillus brevis* (4.17%), *Loigolactobacillus coryniformis* subsp. *torquens* (1.04%), *Lentilactobacillus parabuchneri* (1.04%), and *Lactococcus lactis* subsp. *hordniae* (1.04%) (Fig. 5). Higher distributions of species (17 LAB species) were reported in cow-milk products, whereas only 10 species of LAB were observed in yak-milk products (Fig. 6-8.). Using iGraph-R-package, we performed a simple network analysis showing the distribution of the identified LAB species. *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis*, and *Levilactobacillus brevis* were present in all three NFM products. *Enterococcus durans* was not present in *chhurpi* samples (Fig. 9).

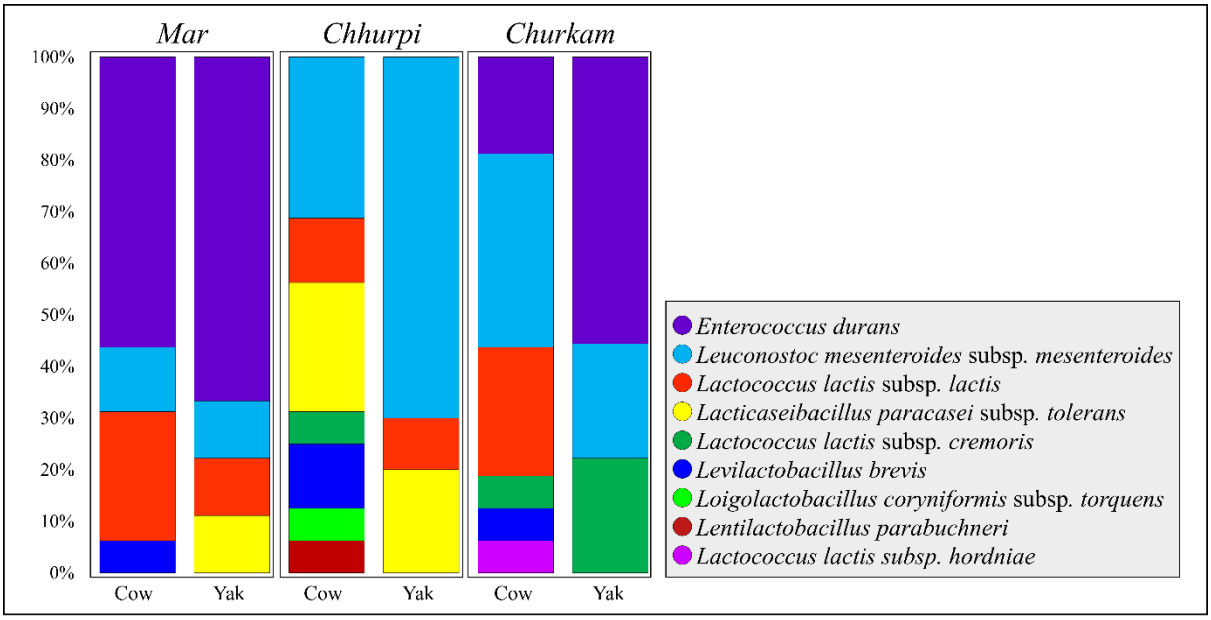
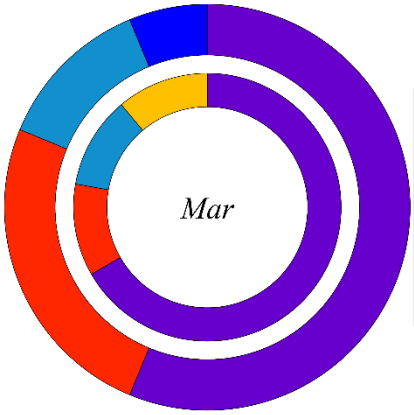
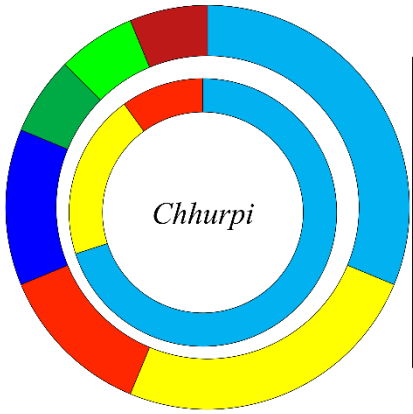


Figure 5: Overall bar-graph representation of identified LAB strains isolated from *mar*, *chhurpi* and *churkam* samples from both cow-milk and yak-milk products.



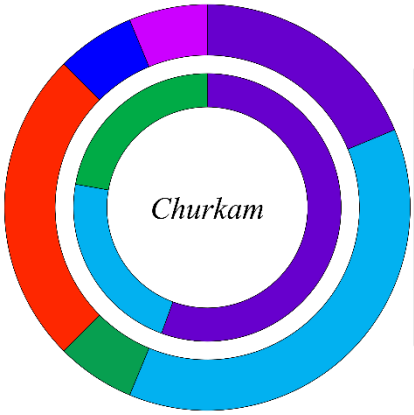
	Cow	Yak
Enterococcus durans	56.25%	66.67%
Leuconostoc mesenteroides subsp. *mesenteroides*	12.5%	11.11%
Lactococcus lactis subsp. *lactis*	25.0%	11.11%
Levilactobacillus brevis	6.25%	-
Lacticaseibacillus paracasei subsp. *tolerans*	-	11.11%

Figure 6: Doughnut-chart representation of the microbial diversity composition identified from *mar* products using 16S rRNA gene sequencing.



	Cow	Yak
Leuconostoc mesenteroides subsp. *mesenteroides*	31.25%	70%
Lacticaseibacillus paracasei subsp. *tolerans*	25%	20%
Lactococcus lactis subsp. *lactis*	12.5%	10%
Levilactobacillus brevis	12.5%	-
Lactococcus lactis subsp. *cremoris*	6.25%	-
Loigolactobacillus coryniformis subsp. *torquens*	6.25%	-
Lentilactobacillus parabuchneri	6.25%	-

Figure 7: Doughnut-chart representation of the microbial diversity composition identified from *chhurpi* products using 16S rRNA gene sequencing.



	Cow	Yak
Enterococcus durans	18.75%	55.56%
Leuconostoc mesenteroides subsp. *mesenteroides*	37.5%	22.22%
Lactococcus lactis subsp. *cremoris*	6.25%	22.22%
Lactococcus lactis subsp. *lactis*	25%	-
Levilactobacillus brevis	6.25%	-
Lactococcus lactis subsp. hordniae	6.25%	-

Figure 8: Doughnut-chart representation of the microbial diversity composition identified from *churkam* products using 16S rRNA gene sequencing.

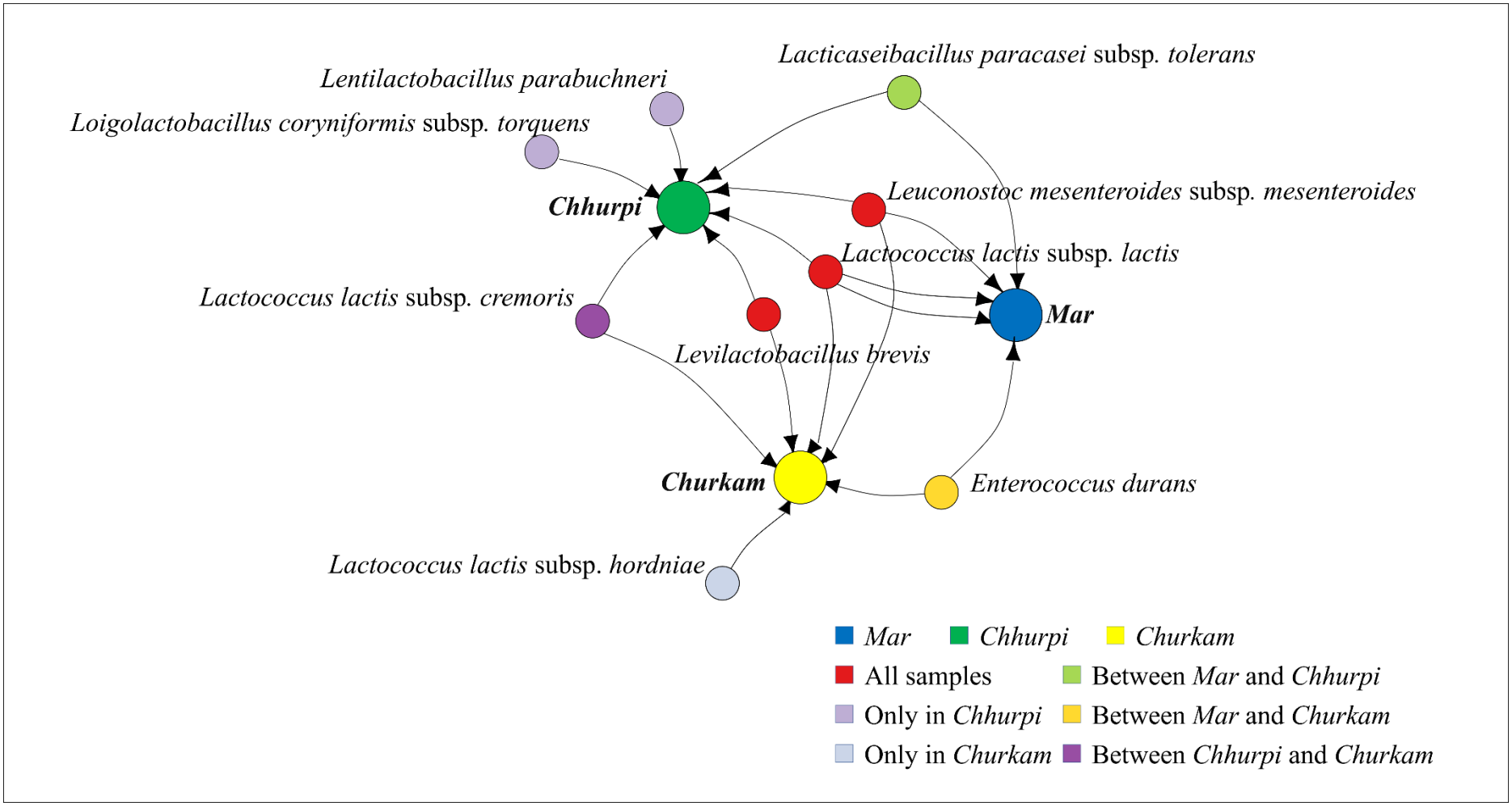


Figure 9: Simple network analysis using iGraph representation showing the shared and unique identified LAB species among the NFM products-viz., *mar*, *chhurpi*, and *churkam*.

Alpha diversities

Samples of *churkam* and *chhurpi* showed comparatively higher diversity index H_{Sh} = 1.43 and 1.40 for Shannon's diversity index, respectively. However, *mar* showed the lowest diversity with H_{Sh} = 1.06 and H_{Si} = 0.61. Additionally, we observed a higher diversity for cow-milk products with H_{Sh} = 1.73 and H_{Si} = 0.82 in comparison to yak products H_{Sh} = 1.28 and H_{Si} = 0.72 (Table 17).

Table 17: Frequency and species diversity indices of LAB strains isolated from the exotic NFM products (cow and yak) of Arunachal Pradesh.

	Mar		*Chhurpi*		*Churkam*	
	Cow (%)	Yak (%)	Cow (%)	Yak (%)	Cow (%)	Yak (%)
Enterococcus durans	56.25	66.67	0.00	0.00	18.75	55.56
Leuconostoc mesenteroides subsp. *mesenteroides*	12.50	11.11	31.25	70.00	37.50	22.22
Lactococcus lactis subsp. *lactis*	25.00	11.11	12.50	10.00	25.00	0.00
Lacticaseibacillus paracasei subsp. *tolerans*	0.00	11.11	25.00	20.00	0.00	0.00
Lactococcus lactis subsp. *cremoris*	0.00	0.00	6.25	0.00	6.25	22.22
Levilactobacillus brevis	6.25	0.00	12.50	0.00	6.25	0.00
Loigolactobacillus coryniformis subsp. *torquens*	0.00	0.00	6.25	0.00	0.00	0.00
Lentilactobacillus parabuchneri	0.00	0.00	6.25	0.00	0.00	0.00
Lactococcus lactis subsp. *hordniae*	0.00	0.00	0.00	0.00	6.25	0.00
	Species diversity indices					
Simpson diversity index (D)	0.64	0.58	0.85	0.51	0.80	0.67
Shannon diversity index (H)	1.01	0.84	1.56	0.70	1.39	0.88

CULTURE-INDEPENDENT ANALYSIS

Sequencing reads details

A total of 5,100,791 base pairs of raw reads were obtained from 35 samples of NFM products including *mar*, *chhurpi* and *churkam* (from both cow and yak's milk) using Illumina MiSeq sequencing platform.

Overall microbial composition

The overall microbial composition observed in the NFM products was represented at four different taxonomic levels- Phylum, Family, Genus and Species. The overall structure was predominated by Firmicutes (61.24%) and Proteobacteria (31.43%); about 6.93% were matched as unclassified (derived from Bacteria) and 0.40% were categorized as others (<1%) (Fig. 10). At the family level, the predominant families belong to Acetobacteraceae (27.88%), Streptococcaceae (26.71%), Lactobacillaceae (16.03%), Staphylococcaceae (8.95%), unclassified (derived from Bacteria) (6.93%), Leuconostocaceae (6.47%), Bacillaceae (1.94%), Pseudomonadaceae (1.87%) and those <1% are categorized as others (3.22%) (Fig. 11). On the other hand, at the genus level, the predominant genera include *Lactococcus* (26.16%), *Acetobacter* (19.09%), *Lactobacillus* (16.03%), *Staphylococcus* (8.94%), *Gluconobacter* (8.66%), unclassified (derived from Bacteria) (6.93%), *Leuconostoc* (6.11%), *Pseudomonas* (1.87%), *Bacillus* (1.64%) and those <1% are grouped as others (4.57%) (Fig. 12). Similarly, at the species level, we observed the predominance of *Lactococcus lactis* (23.28%), followed by, *Lactobacillus helveticus* (9.03%), *Staphylococcus cohnii* (7.26%), *Gluconobacter oxydans* (7.26%), uncultured bacterium (6.87%), *Acetobacter lovaniensis* (6.05%), *Leuconostoc mesenteroides* (4.67%), *Acetobacter pasteurianus* (4.24%), *Acetobacter syzygii* (3.68%), *Acetobacter tropicalis*(3.65%), Lactococcus *raffinolactis* (1.98%),

Lactobacillus delbrueckii (1.69%), *Pseudomonas fluorescens* (1.53%), *Lactobacillus acidophilus* (1.48%), *Bacillus cereus* (1.33%), *Gluconobacter cerinus* (1.27%), *Staphylococcus kloosii* (1.15%), *Lactobacillus sakei* (0.96%) and those <1% are categorized as others (12.62%) (Fig. 13).

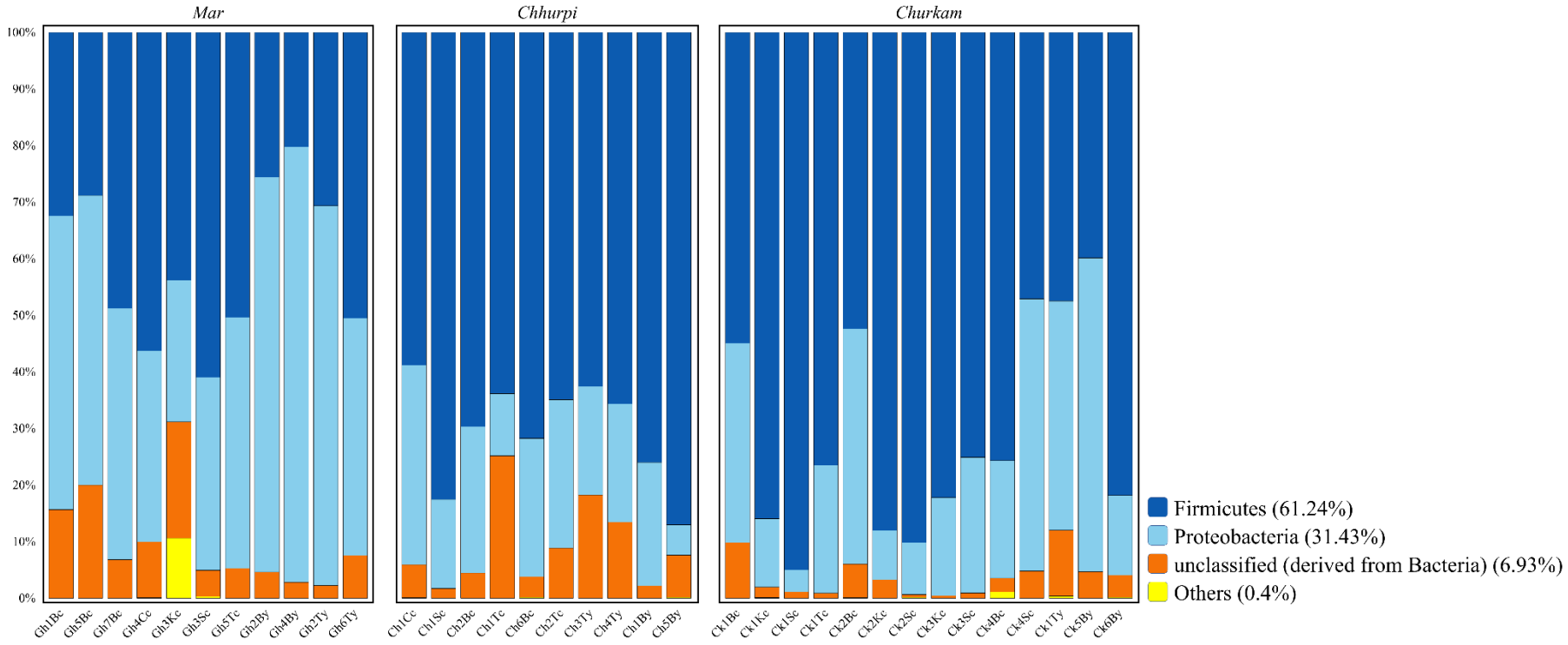


Figure 10: OTU-based classification of bacteria distributed at the phylum level that was analyzed by Illumina MiSeq amplicon sequencing in NFM products of Arunachal Pradesh: *mar*, *chhurpi* and *churkam* prepared from cow and yak's milk.

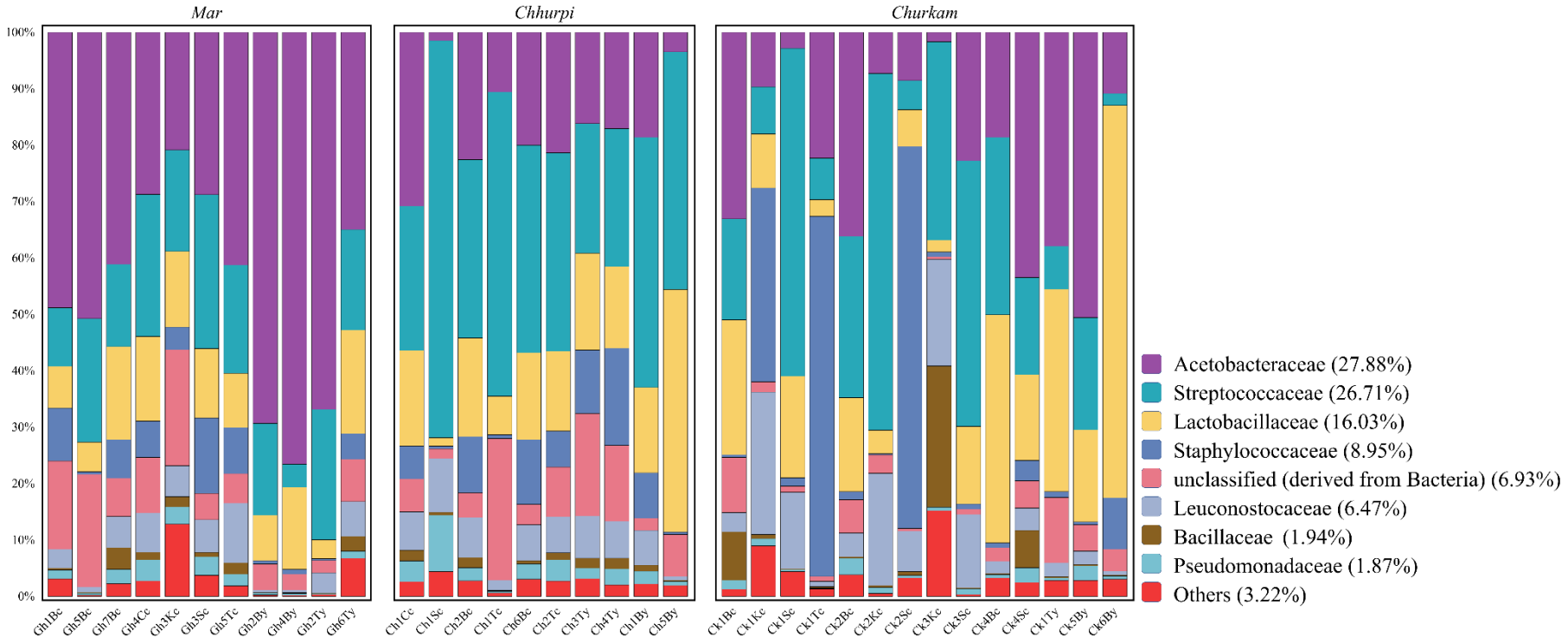


Figure 11: OTU-based classification of bacteria distributed at the family level that was analyzed by Illumina MiSeq amplicon sequencing in NFM products of Arunachal Pradesh: *mar*, *chhurpi* and *churkam* prepared from cow and yak's milk.

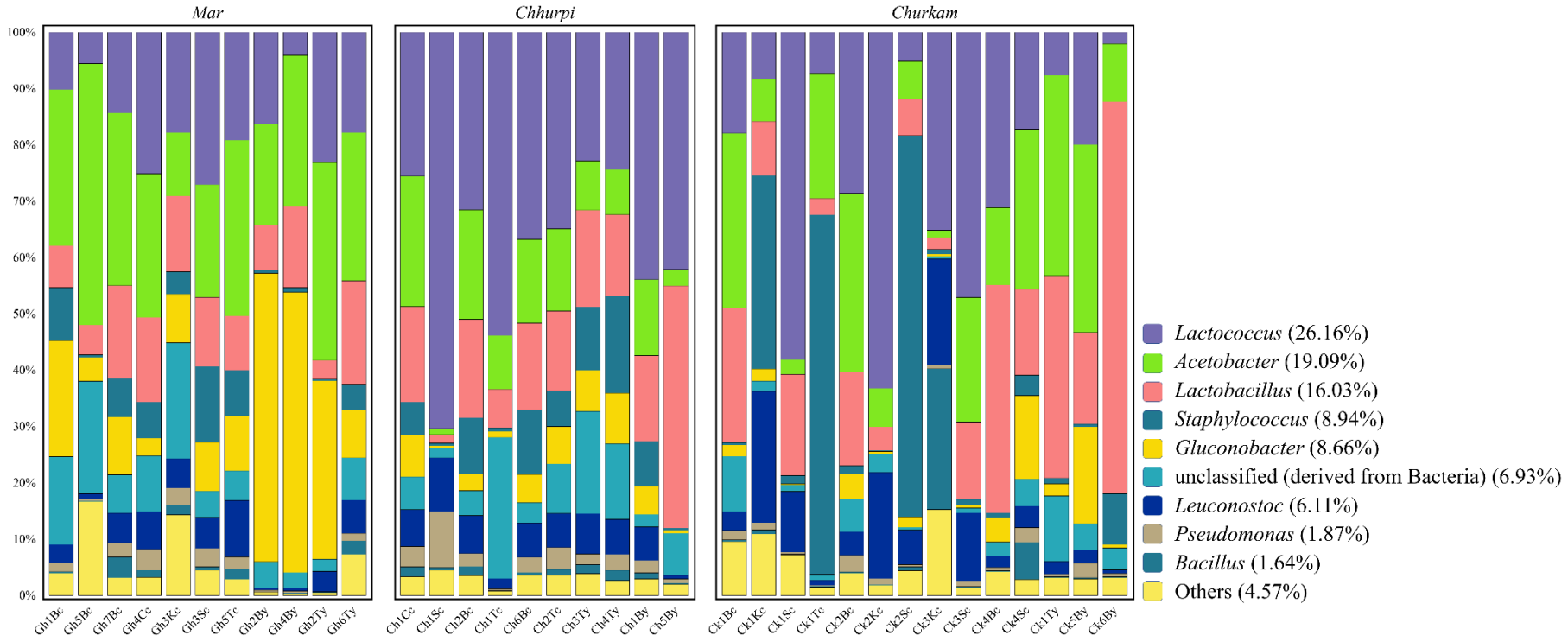


Figure 12: OTU-based classification of bacteria distributed at the genus level that was analyzed by Illumina MiSeq amplicon sequencing in NFM products of Arunachal Pradesh: *mar*, *chhurpi* and *churkam* prepared from cow and yak's milk.

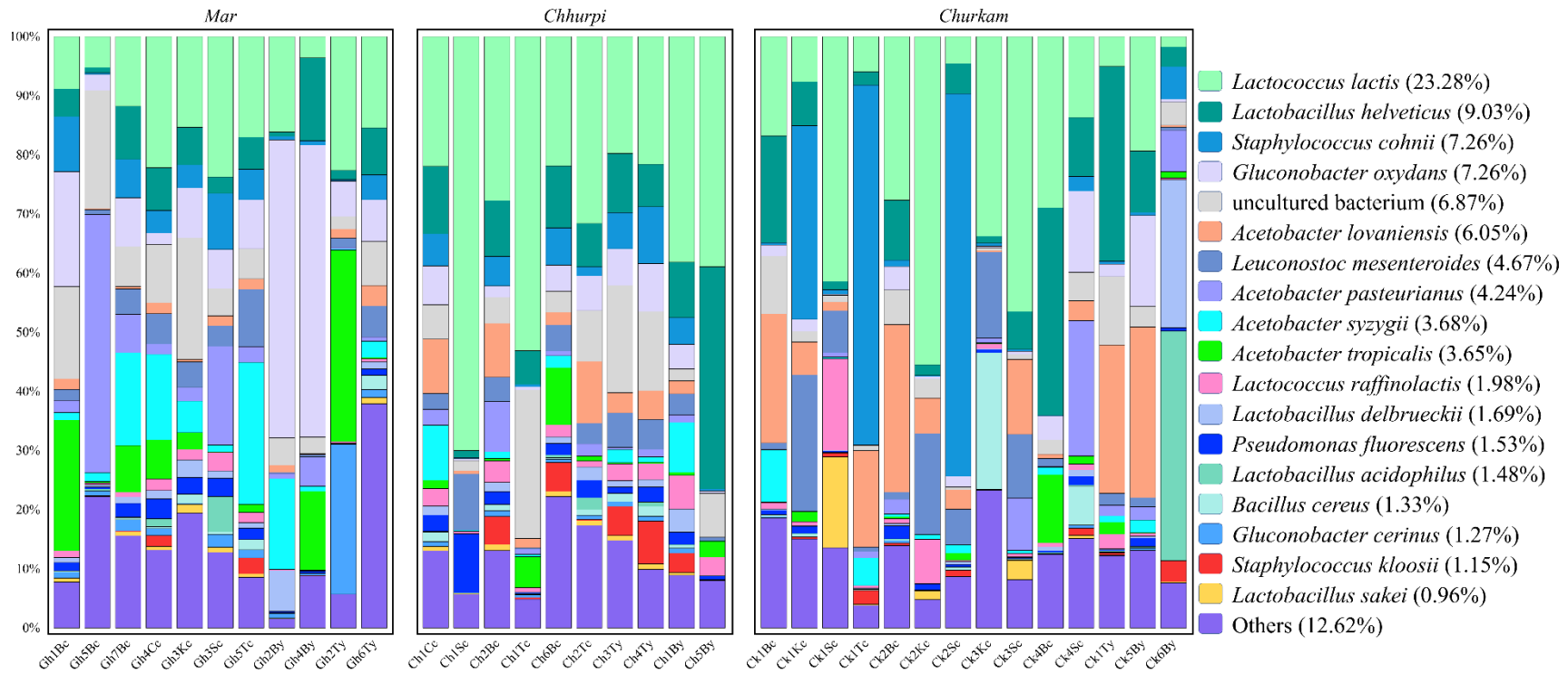


Figure 13: OTU-based classification of bacteria distributed at the species level that was analyzed by Illumina MiSeq amplicon sequencing in NFM products of Arunachal Pradesh: *mar*, *chhurpi* and *churkam* prepared from cow and yak's milk.

Microbial content of each NFM products

Microbial diversity of *mar*

In *mar* products, Proteobacteria and Firmicutes were at almost equal distribution of (49.15%) and (40.76%) respectively, followed by 9.07% of unclassified (derived from bacteria) and others those <1%, were classified as others (1.02%). At the family level, Acetobacteraceae (46.18 %) was predominant, followed by Streptococcaceae (17.99 %), Lactobacillaceae (11.26 %), unclassified (derived from bacteria) (9.07 %), Staphylococcaceae (5 %), Leuconostocaceae (4.49 %), Pseudomonadaceae (1.69 %), Bacillaceae (1.21 %), and those <1% were classified as others (3.11 %). At the genus level, *Acetobacter* (27.18 %) was predominant, followed by *Gluconobacter* (18.77 %), *Lactococcus* (16.4 %), *Lactobacillus* (11.26 %), unclassified (derived from bacteria) (9.07 %), *Staphylococcus* (5 %), *Leuconostoc* (4.32 %), *Pseudomonas* (1.69 %), *Bacillus* (1.11 %), and those <1% were classified as others (5.2 %). Similarly, at the species level, *Gluconobacter oxydans* (15.32 %) was the predominant species followed by *Lactococcus lactis* (14.7 %), uncultured bacterium (9.04 %), *Acetobacter tropicalis* (7.93 %), *Acetobacter syzygii* (7.51 %), *Acetobacter pasteurianus* (7.48 %), *Lactobacillus helveticus* (5.52 %), *Staphylococcus cohnii* (4.02 %), *Leuconostoc mesenteroides* (3.37 %), *Gluconobacter cerinus* (3.29 %), *Lactobacillus delbrueckii* (1.56 %), *Pseudomonas fluorescens* (1.52 %), *Acetobacter lovaniensis* (1.31 %), *Lactococcus raffinolactis* (1.07 %), *Lactobacillus acidophilus* (0.73 %), *Bacillus cereus* (0.66 %), *Lactobacillus sakei* (0.56 %), *Staphylococcus kloosii* (0.46 %), and those <1% were classified as others (13.95 %).

Microbial diversity of *chhurpi*

In *chhurpi* products, the predominant phylum was observed to be *Firmicutes* (70.28%), followed by Proteobacteria (20.58%), unclassified (derived from Bacteria) (9.07%), and

those <1% were classified as others (0.07%). At the family level, we observed the predominance of Streptococcaceae (38.75%), followed by Acetobacteraceae (16.21%), Lactobacillaceae (16.21%), unclassified (derived from bacteria) (9.08%), Staphylococcaceae (7.17%), Leuconostocaceae (5.89%), Pseudomonadaceae (3.05%), Bacillaceae (1.1%), and those <1% were classified as others (2.54%). Additionally, at the genus level, *Lactococcus* (38.62 %) was the predominant genera, followed by *Lactobacillus* (16.21%), *Acetobacter* (11.59%), unclassified (derived from bacteria) (9.08%), *Staphylococcus* (7.17%), *Leuconostoc* (5.67%), *Gluconobacter* (4.52%), *Pseudomonas* (3.05%), *Bacillus* (1.01%), and those <1% were classified as others (3.08%). Similarly, at the species level, *Lactococcus lactis* (34.46%) was the predominant species, followed by *Lactobacillus helveticus* (10.97 %), uncultured bacterium (9.02%), *Acetobacter lovaniensis* (4.34%), *Leuconostoc mesenteroides* (3.95%), *Staphylococcus cohnii* (3.94%), *Gluconobacter oxydans* (3.86%), *Pseudomonas fluorescens* (2.62%), *Staphylococcus kloosii* (2.56%), *Lactococcus raffinolactis* (2.51%), *Acetobacter syzygii* (2.48%), *Acetobacter tropicalis* (2.11%), *Acetobacter pasteurianus* (1.78%), *Lactobacillus delbrueckii* (1.3%), *Bacillus cereus* (0.76%), *Lactobacillus sakei* (0.59%), *Gluconobacter cerinus* (0.59%), *Lactobacillus acidophilus* (0.38%), and those <1% were classified as others (11.78%).

Microbial diversity of *churkam*

Firmicutes was the predominant phylum in *churkam* products with 70.87%, followed by Proteobacteria (25.25%), unclassified (derived from bacteria) (3.72%), and those <1% were classified as others (0.16%). At the family level, Streptococcaceae (24.96%) was the predominant family, followed by Acetobacteraceae (21.83%), Lactobacillaceae (19.64%), Staphylococcaceae (13.31%), Leuconostocaceae (8.43 %), unclassified

(derived from bacteria) (3.72 %), Bacillaceae (3.12%), Pseudomonadaceae (1.17%), and those <1% were classified as others (3.82%). Similarly, *Lactococcus* (24.92 %) was the predominant genus, followed by *Lactobacillus* (19.64%), *Acetobacter* (18.09%), *Staphylococcus* (13.31%), *Leuconostoc* (7.83%), unclassified (derived from bacteria) (3.72%), *Gluconobacter* (3.67%), *Bacillus* (2.49%), *Pseudomonas* (1.16 %), and those <1% were classified as others (5.17 %). Lastly, at the species level, we observed the predominance of *Lactococcus lactis* (22.02%), followed by *Staphylococcus cohnii* (12.18%), *Acetobacter lovaniensis* (11.01%), *Lactobacillus helveticus* (10.4 %), *Leuconostoc mesenteroides* (6.21 %), uncultured bacterium (3.62%), *Acetobacter pasteurianus* (3.45%), *Gluconobacter oxydans* (3.35%), *Lactobacillus acidophilus* (2.85%), *Lactococcus raffinolactis* (2.33%), *Bacillus cereus* (2.27%), *Lactobacillus delbrueckii* (2.08%), *Lactobacillus sakei* (1.54%), *Acetobacter syzygii* (1.54%), *Acetobacter tropicalis* (1.4%), *Pseudomonas fluorescens* (0.74%), *Staphylococcus kloosii* (0.69 %), *Gluconobacter cerinus* (0.16%), and those <1% were classified as others (12.16%).

Alpha diversities

Alpha diversity (chao1, equitability, Fisher alpha, Goods coverage, Shannon and Simpson) was calculated for the different NFM products (Table 18, Fig. 14-15.). Significant difference was observed between *churkam* and *mar* for Equitability (p=0.0263). Additionally, significant difference was observed between *chhurpi* and *churkam* for Fisher alpha (p=0.031). Similarly, significant difference was observed between *mar* and *churkam* (Shannon, p=0.0373; Simpson, p=0.0388). It is also noteworthy to observe the sequencing depth coverage, as indicated by Goods coverage

index. However, no significant differences were observed between the cow-based and yak-based products.

Table 18: Alpha diversity matrices comparison among the NFM products using script-based analysis in QIIME1 for NGS-based amplicon sequencing study.

Chao1:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	134.26	31.77	129.41	34.99	0.32	0.75
Churkam	*Mar*	108.46	27.94	129.41	34.99	-1.56	0.13
Chhurpi	*Churkam*	134.26	31.77	108.46	27.94	1.98	0.06
Equitability:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	0.53	0.11	0.55	0.08	-0.47	0.64
Churkam	*Mar*	0.48	0.05	0.55	0.08	-2.44	0.03
Chhurpi	*Churkam*	0.53	0.11	0.48	0.05	1.38	0.18
Fisher Alpha:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	14.61	4.01	13.34	4.07	0.68	0.49
Churkam	*Mar*	11.46	2.25	13.34	4.07	-1.37	0.18
Chhurpi	*Churkam*	14.61	4.01	11.46	2.25	2.28	0.03
Goods Coverage:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	1.00	0.00	1.00	0.00	-0.35	0.73
Churkam	*Mar*	1.00	0.00	1.00	0.00	1.60	0.12
Chhurpi	*Churkam*	1.00	0.00	1.00	0.00	-2.02	0.06
Shannon:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	3.43	0.89	3.50	0.74	-0.17	0.87
Churkam	*Mar*	2.94	0.39	3.50	0.74	-2.25	0.04
Chhurpi	*Churkam*	3.43	0.89	2.94	0.39	1.71	0.10
Simpson:							
Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Chhurpi	*Mar*	0.80	0.14	0.84	0.09	-0.68	0.52
Churkam	*Mar*	0.76	0.09	0.84	0.09	-2.17	0.04
Chhurpi	*Churkam*	0.80	0.14	0.76	0.09	0.91	0.38

Note: significant differences are marked as underline.

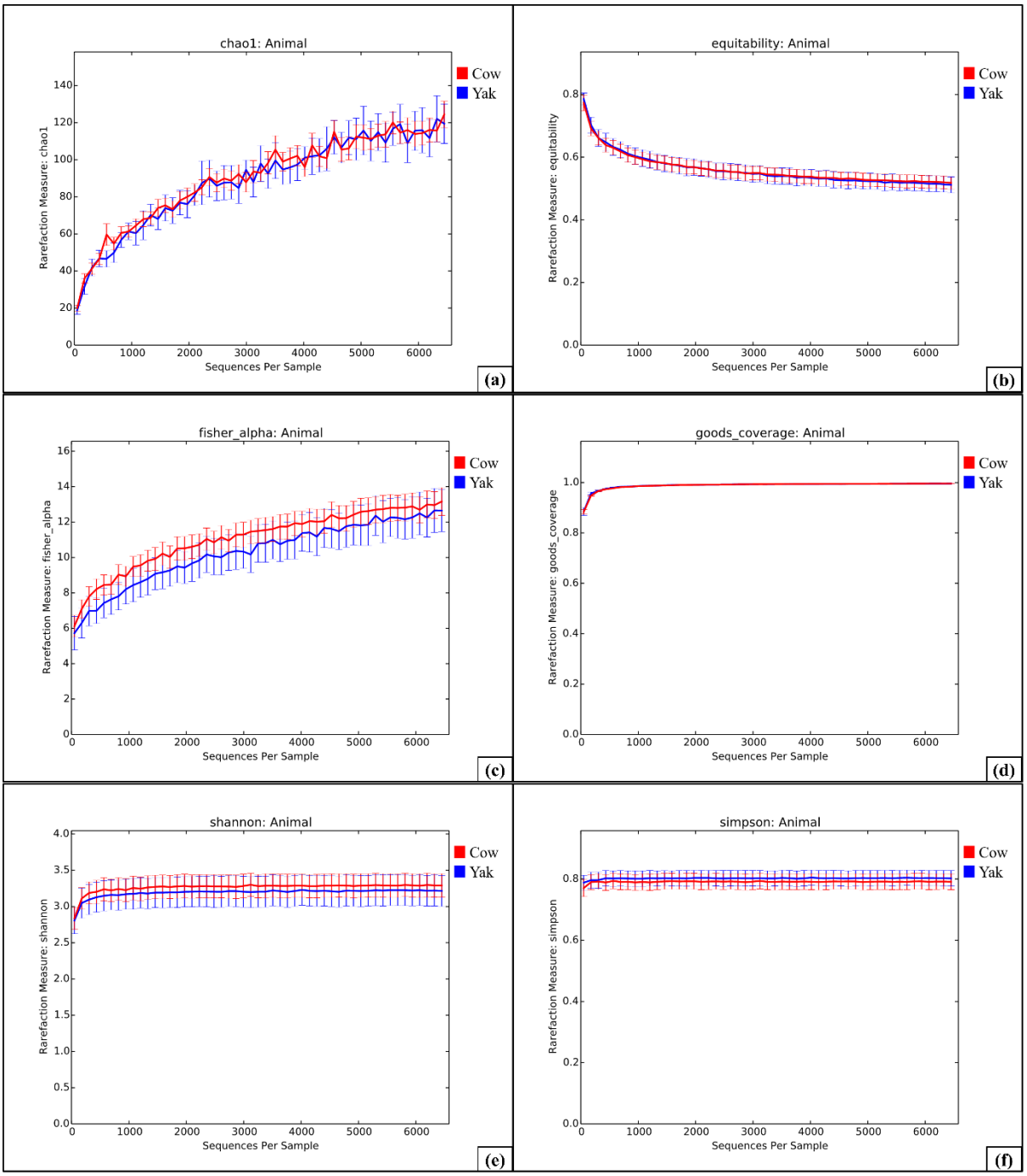


Figure 14: Rarefaction curve calculated as per Chao1, Equitability, Goods coverage, Fisher alpha, Shannon, and Simpson index (alpha diversity) for determining the differences between cow-based and yak-based products. No significant differences were observed.

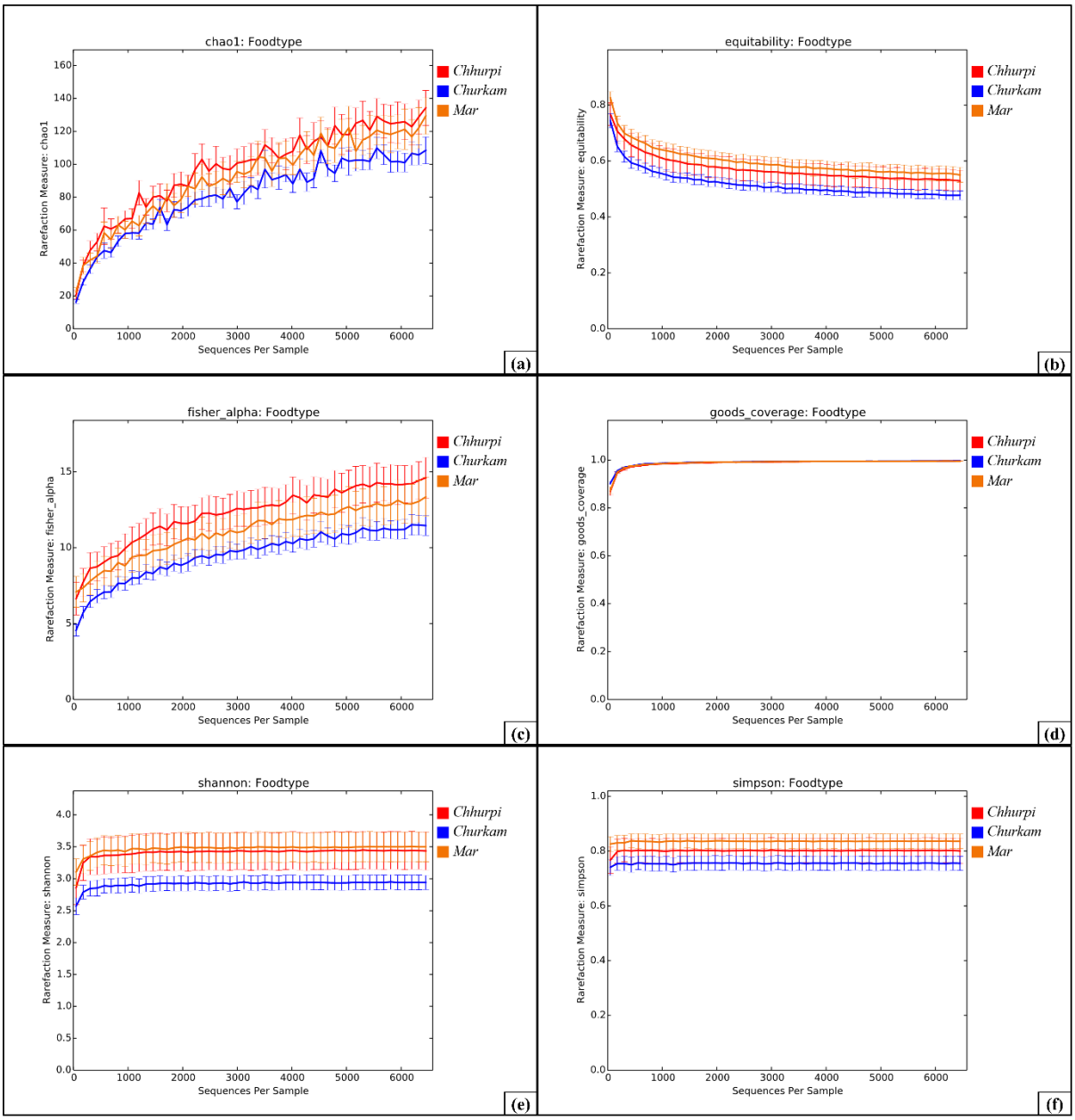


Figure 15: Rarefaction curve calculated as per Chao1, Equitability, Goods coverage, Fisher alpha, Shannon, and Simpson index (alpha diversity) for determining the differences between food types- *mar*, *chhurpi* and *churkam*. Significant differences were mentioned in the main text.

Multivariate analysis

ANOSIM (p=0.013; R=0.1078) and PERMANOVA-based (p=0.0004; F=2.78) multivariate analysis showed significant differences among the NFM products. Bonferroni-corrected analysis showed a significant difference between *mar* and *chhurpi* (ANOSIM; p=0.0068 and PERMANOVA; p=0.00009) and between *mar* and *churkam* (ANOSIM; p=0.0168 and PERMANOVA; p=0.00059). Detailed information can be referred to Table 19, and PCA-based representation (Fig. 16). No significant difference was observed between *chhurpi* and *churkam*.

Table 19: Multivariate statistical analysis of the species-level OTUs data as calculated by ANOSIM and PERMANOVA with Bonferroni-corrected p-values.

ANOSIM

Similarity Index	Bray-Curtis	Bonferroni-corrected *p*-values			
Permutation N:	10000		*Mar*	*Chhurpi*	*Churkam*
Mean rank within:	276.2	*Mar*		0.0068	0.016
Mean rank between:	308.3	*Chhurpi*	0.0068		1
R:	0.1078	*Churkam*	0.016	1	
p (same):	0.013				

PERMANOVA

Similarity Index	Bray-Curtis	Bonferroni-corrected *p*-values			
Permutation N:	10000		*Mar*	*Chhurpi*	*Churkam*
Total sum of squares:	6.602	*Mar*		0.0003	0.00059
Within-group sum of squares:	5.625	*Chhurpi*	0.0003		0.24
F:	2.78	*Churkam*	0.00059	0.24	
p (same):	0.0004				

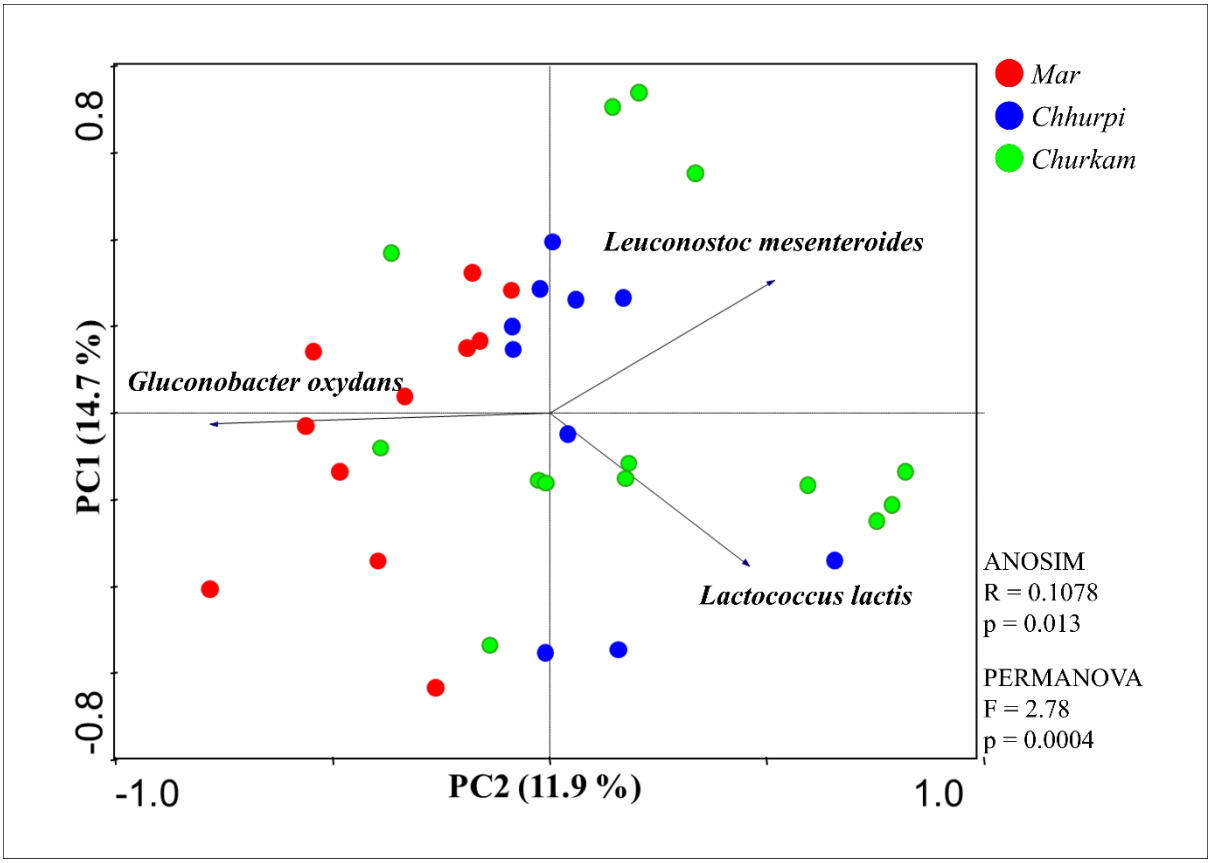


Figure 16: PCA plot showing the difference in bacterial community structure among the NFM products of Arunachal Pradesh. Arrow indicates the species direction. Significance difference is shown by ANOSIM/PERMANOVA analyzed with 10,000 permutations using Bray-Curtis distances. Significance was observed between *mar* with both *chhurpi* and *churkam*.

Significant microbial composition among the NFM products

OTU-based bacterial diversity of different taxonomical groups was compared among different designated groups based on (a) food types- *mar*, *chhurpi* and *churkam*; (b) animal source of milk- cow and yak; and (c) nature of the samples- lipid-rich and casein-based products, using a non-parametric Mann-Whitney U-test.

Phylum level comparison

At the phylum level, Firmicutes was significantly abundant in *chhurpi* (p=0.00016) and *churkam* (p=0.002) in comparison to *mar*. Contrastingly, Proteobacteria was significantly abundant in *mar* in comparison to both *chhurpi* (p=0.00048) and *churkam* (p=0.00338). On the other hand, significant differences were observed between *mar* and *churkam* (p=0.0173) and *chhurpi* and *churkam* (p=0.04338) (Fig. 17). Comparison of phyla based on the animal milk source (cow or yak) where the products are prepared from showed no significant differences (Fig. 18). Paired non-parametric comparison based on the nature of the products showed a significance difference in Firmicutes where it was significant abundant in casein-based products (p=0.00008) in comparison to lipid-rich products. On the contrary, Proteobacteria was significantly abundant in lipid-rich products (p=0.00024) in comparison to casein-based products. No significant difference was observed for unclassified sequences (derived from bacteria) (Fig.19).

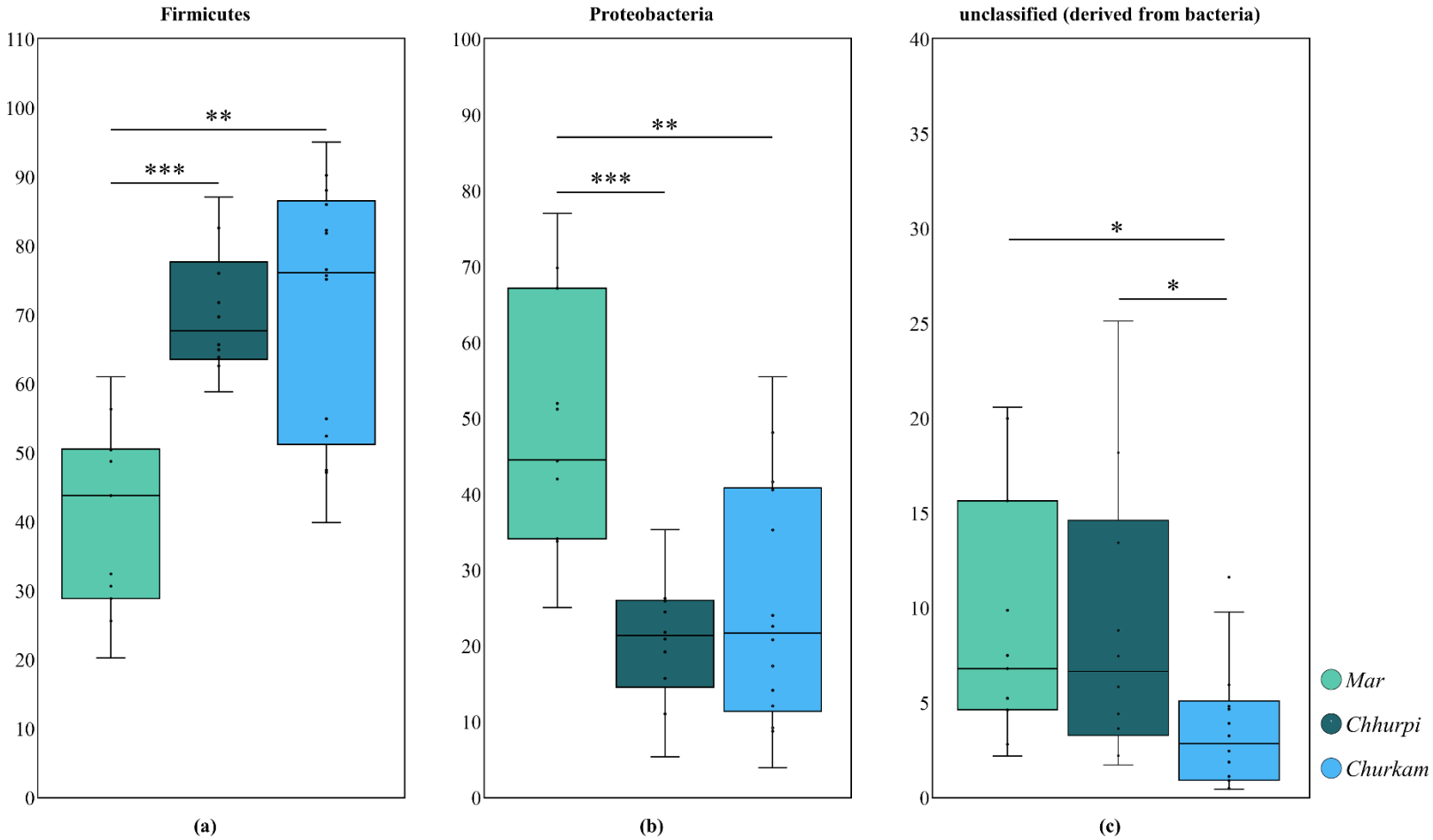


Figure 17: Pairwise comparison of bacterial phyla- (a) Firmicutes, (b) Proteobacteria and (c) unclassified (derived from bacteria) based on the food types- *mar*, *chhurpi*, and *churkam*, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

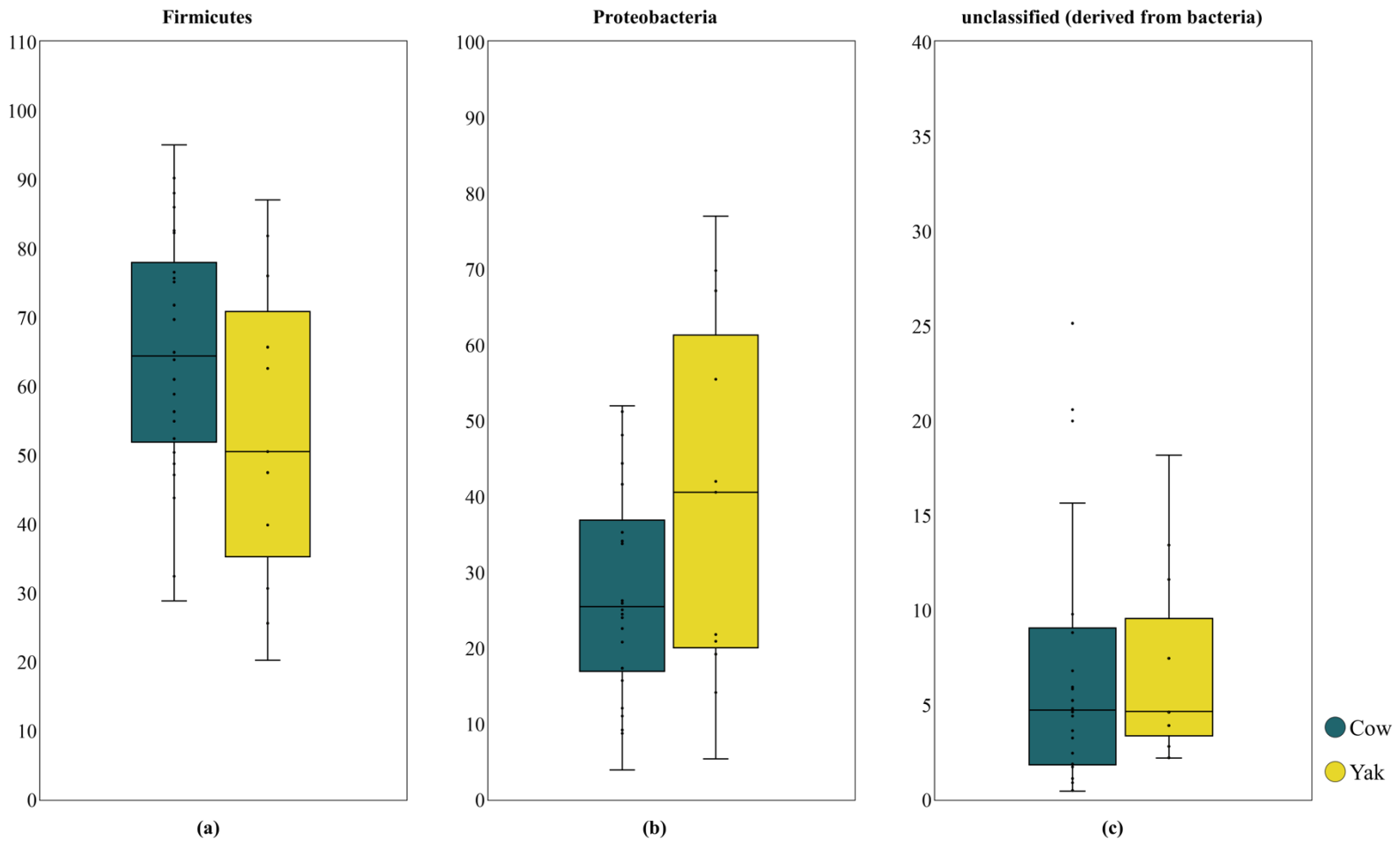


Figure 18: Pairwise comparison of bacterial phyla- (a) Firmicutes, (b) Proteobacteria and (c) unclassified (derived from bacteria) based on the animal milk source- cow and yak. No significant difference was observed.

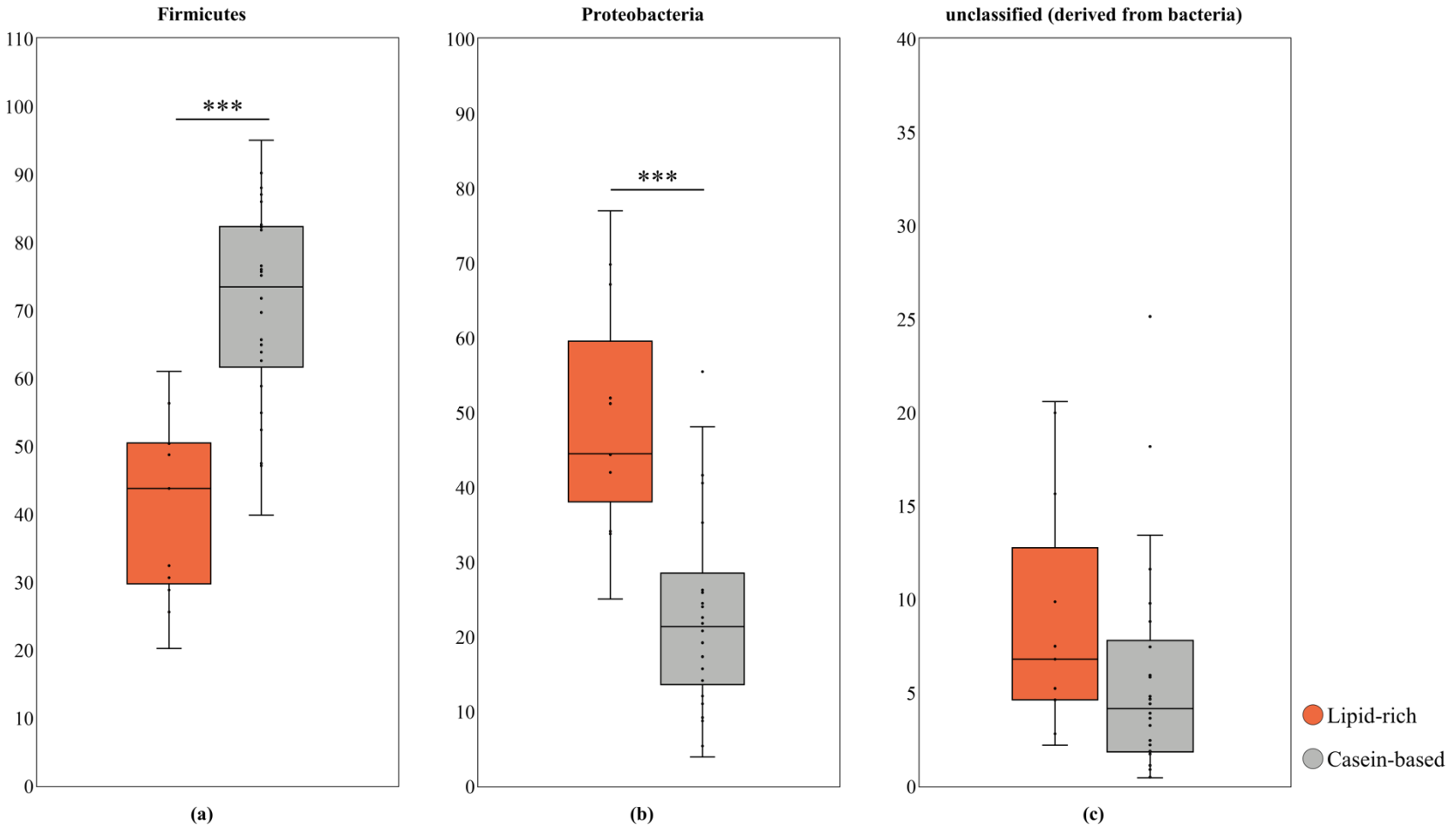


Figure 19: Pairwise comparison of bacterial phyla- (a) Firmicutes, (b) Proteobacteria and (c) unclassified (derived from bacteria) based on the nature of the products- lipid-rich and casein-based, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

Family level comparison

At the family level, Acetobacteraceae was significantly abundant in *mar* in comparison to both *chhurpi* (p=0.00048) and *churkam* (p=0.00578). *Streptococcaceae* was significantly abundant in *chhurpi* in comparison to *mar* (p=0.00062). Unclassified (derived from bacteria) was observed to be significantly abundant in *mar* (p=0.01732) and *chhurpi* (p=0.04338) in comparison to *churkam*, and Pseudomonadaceae was observed to be significantly abundant in *chhurpi* in comparison to *churkam* (p=0.02444) (Fig. 20). Among products from different animal milk source- cow or yak, only Leuconostocaceae was significantly abundant in cow compared to yak (p=0.0198) (Fig. 21). Comparison of products based on the nature of the samples showed significant differences only for Acetobacteraceae and Streptococcaceae, where the former was significantly abundant in lipid-rich products (p=0.00036) and contrastingly the latter was significantly abundant in casein-based products (p=0.04136) (Fig. 22).

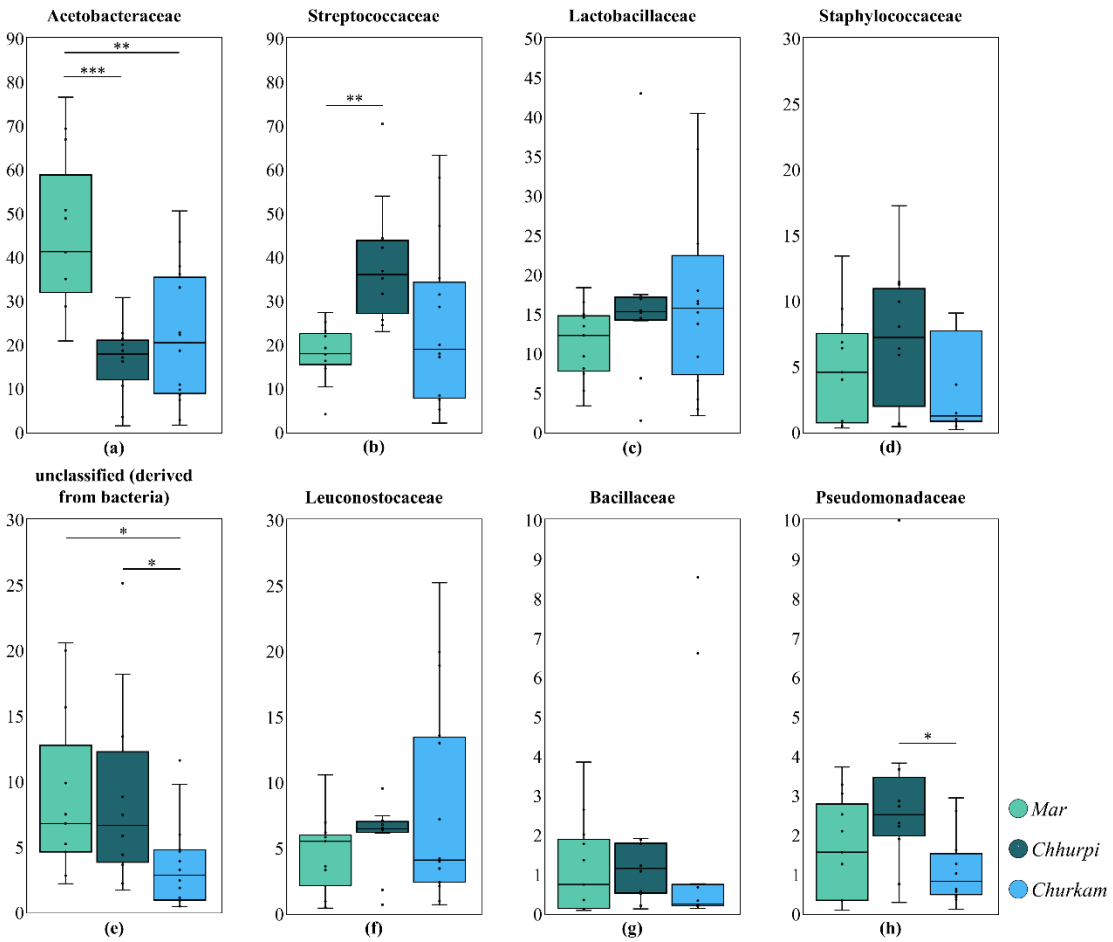


Figure 20: Pairwise comparison of bacterial families- (a) Acetobacteraceae, (b) Streptococcaceae, (c) Lactobacillaceae, (d) Staphylococcaceae, (e) unclassified (derived from Bacteria), (f) Leuconostocaceae, (g) Bacillaceae, and (h) Pseudomonadaceae based on the food types- *mar*, *chhurpi*, and *churkam*, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

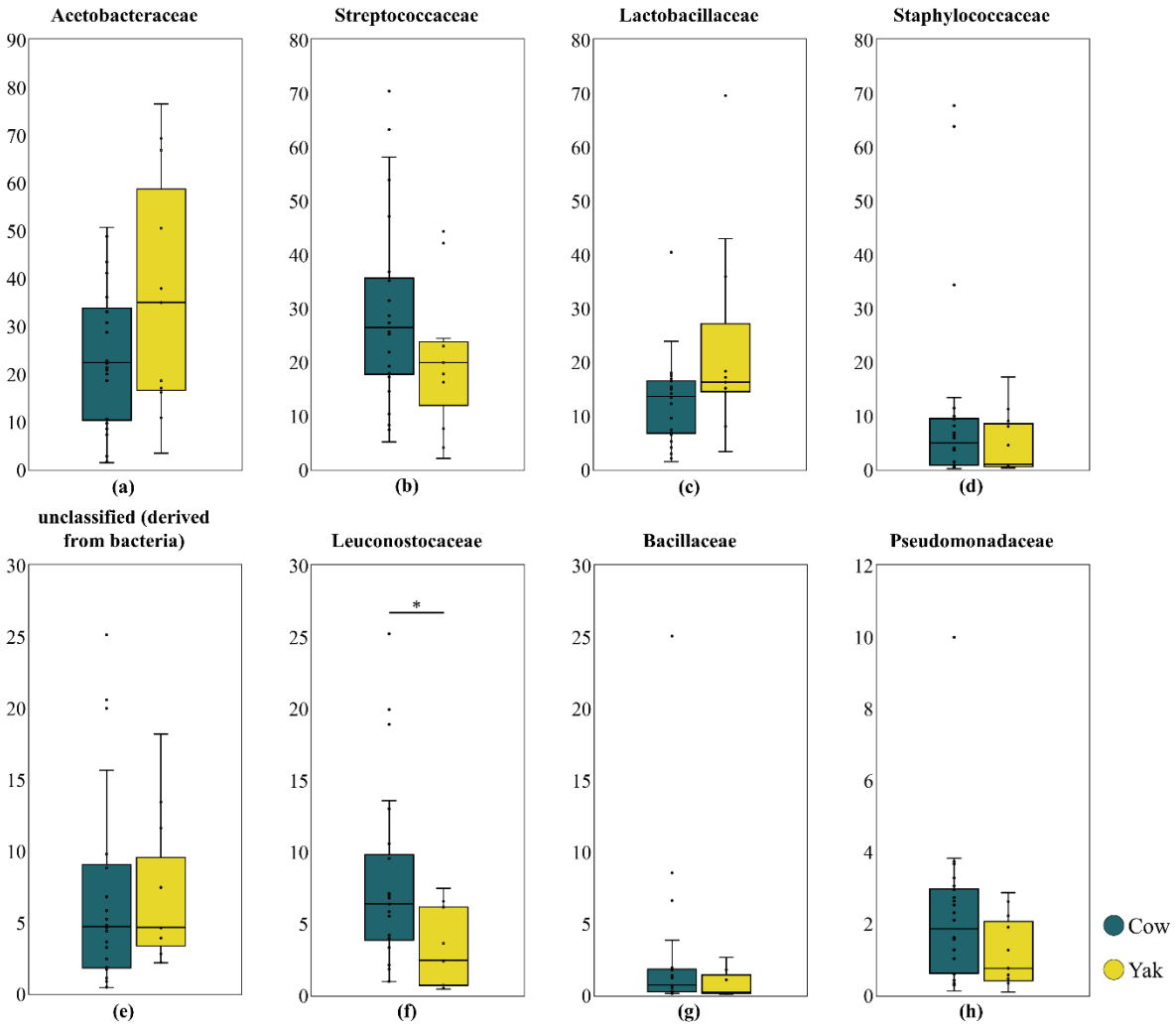


Figure 21: Pairwise comparison of bacterial families-(a) Acetobacteraceae, (b) Streptococcaceae, (c) Lactobacillaceae, (d) Staphylococcaceae, (e) unclassified (derived from Bacteria), (f) Leuconostocaceae, (g) Bacillaceae, and (h) Pseudomonadaceae based on the animal milk source- cow and yak, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

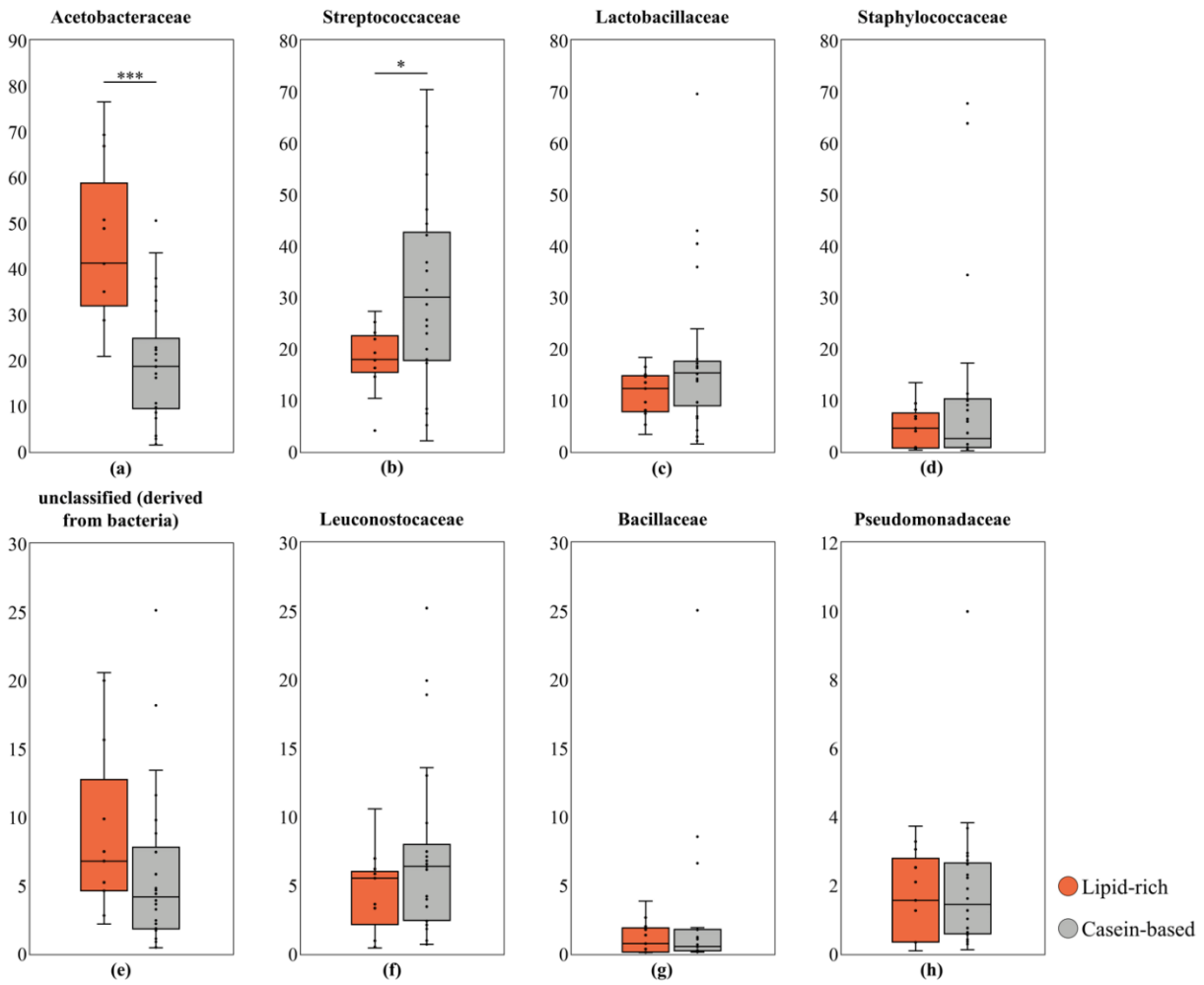


Figure 22: Pairwise comparison of bacterial families- (a) Acetobacteraceae, (b) Streptococcaceae, (c) Lactobacillaceae, (d) Staphylococcaceae, (e) unclassified (derived from Bacteria), (f) Leuconostocaceae, (g) Bacillaceae, and (h) Pseudomonadaceae based on the nature of the products- lipid-rich and casein-based, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

Genus level comparison

At the genus level, *Lactococcus* was significantly abundant in *chhurpi* in comparison to *mar* (p=0.00062) but however it was not significantly abundant compared to *churkam* (p=0.05744). *Acetobacter* was significantly abundant in *mar* compared to *chhurpi* (p=0.00108) and not significant to *churkam* (p=0.14706). *Gluconobacter* was significantly abundant in *mar* in comparison to both *chhurpi* (p=0.00544) and *churkam* (p=0.00138). A significant abundance of unclassified sequences (derived from bacteria) was also observed in *mar* (p=0.01732) and *chhurpi* (p=0.04338) in comparison to *churkam*. Lastly, *Pseudomonas* was significantly abundant in *chhurpi* in comparison to *churkam* (p=0.02444) (Fig. 23). When comparing the products based on the animal milk source, only *Leuconostoc* was significantly abundant in cow products in comparison to yak products (p=0.02202) (Fig. 24). Additionally, a significant abundance of *Lactococcus* was observed in casein-based products when compared to that of lipid-based products (p=0.02202). However, Acetobacteraceae-based *Acetobacter* and *Gluconobacter* are significantly abundant in lipid-rich in comparison to casein-based products with p-values of 0.00906, and 0.00046, respectively (Fig. 25).

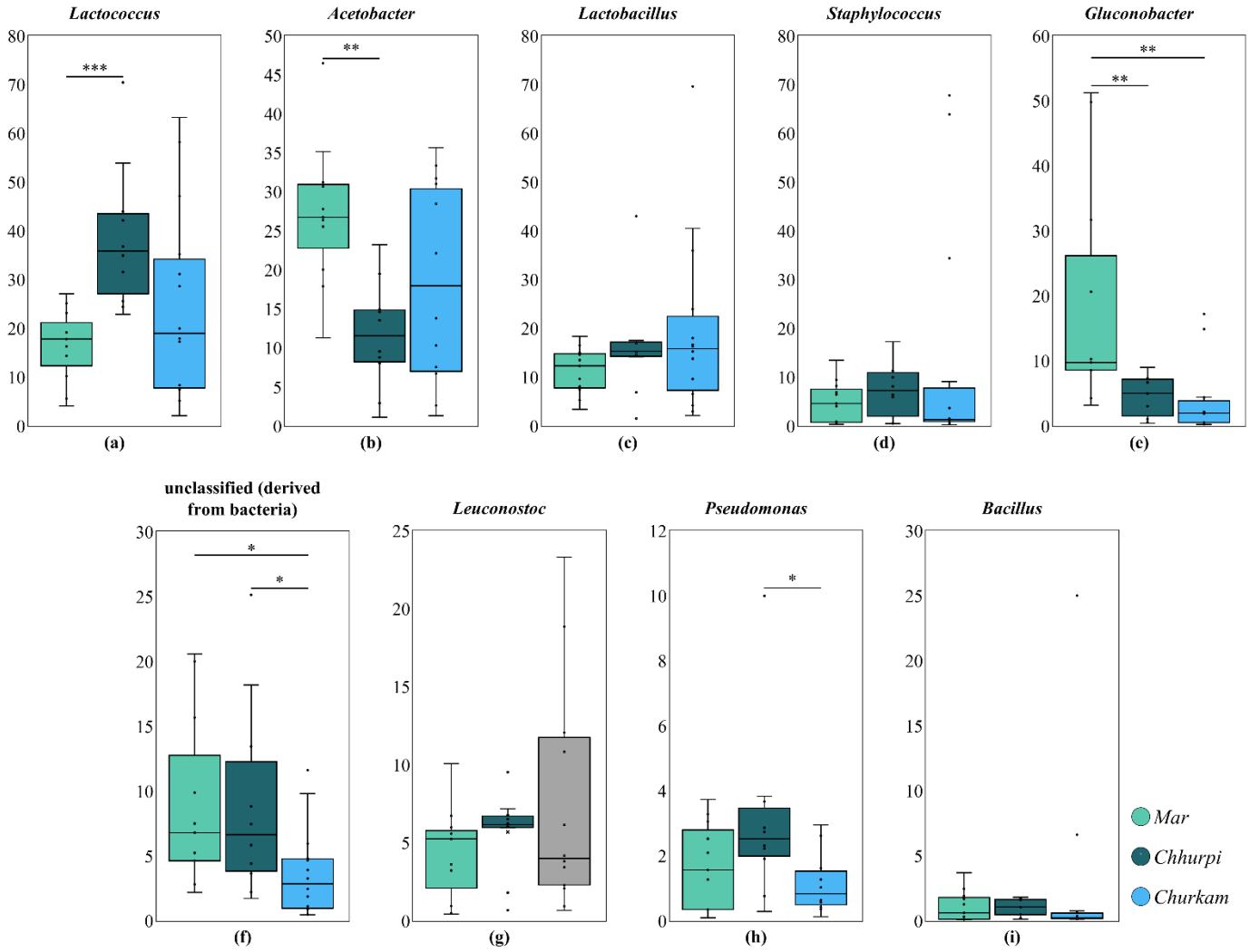


Figure 23: Pairwise comparison of bacterial genera- (a) *Lactococcus*, (b) *Acetobacter*, (c) *Lactobacillus*, (d) *Staphylococcus*, (e) *Gluconobacter*, (f) unclassified (derived from Bacteria), (g) *Leuconostoc*, (h) *Pseudomonas*, and (i) *Bacillus*, based on the food types- *mar*, *chhurpi*, and *churkam*, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

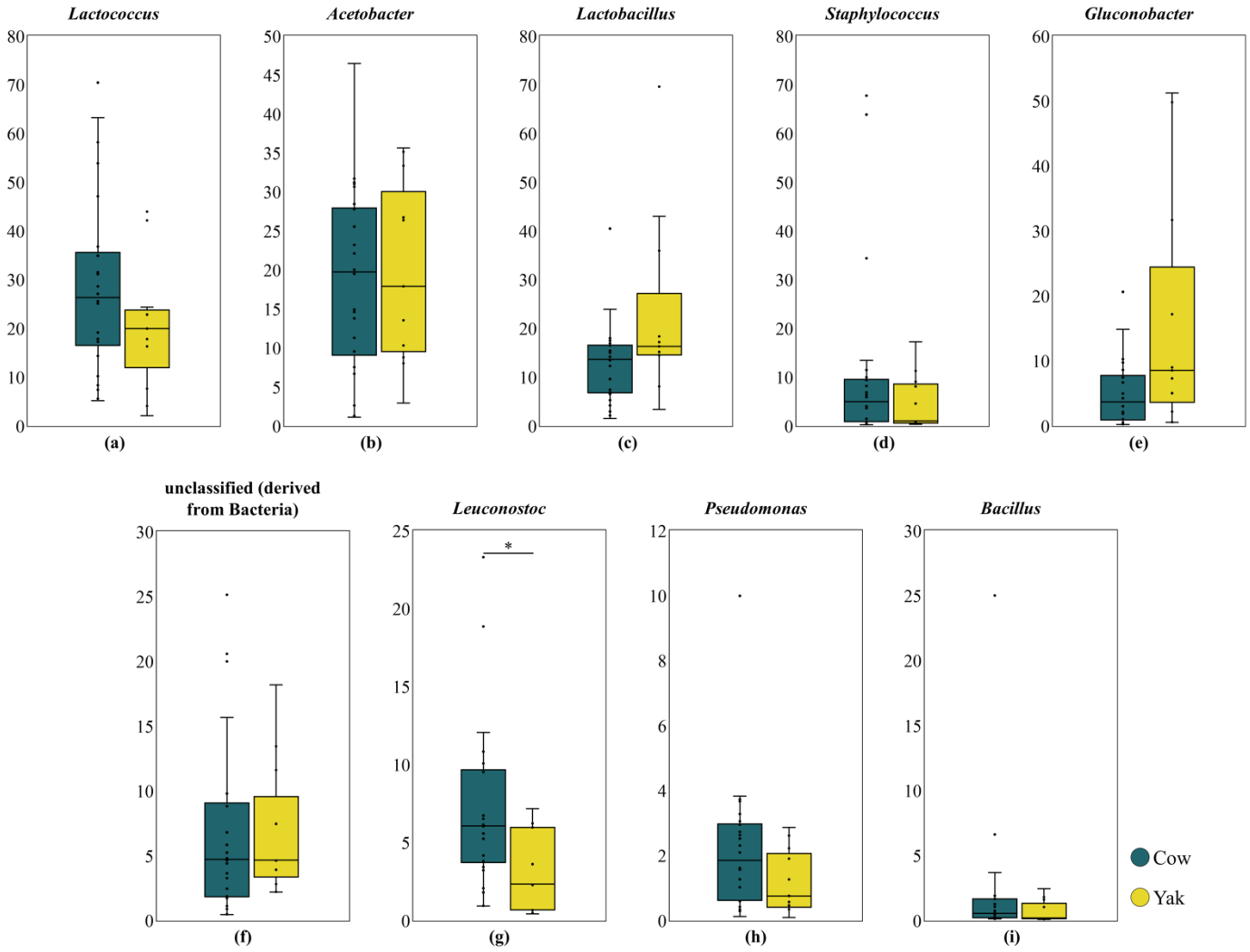


Figure 24: Pairwise comparison of bacterial genera- (a) *Lactococcus*, (b) *Acetobacter*, (c) *Lactobacillus*, (d) *Staphylococcus*, (e) *Gluconobacter*, (f) unclassified (derived from Bacteria), (g) *Leuconostoc*, (h) *Pseudomonas*, and (i) *Bacillus*, based on the animal milk source- cow and yak, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

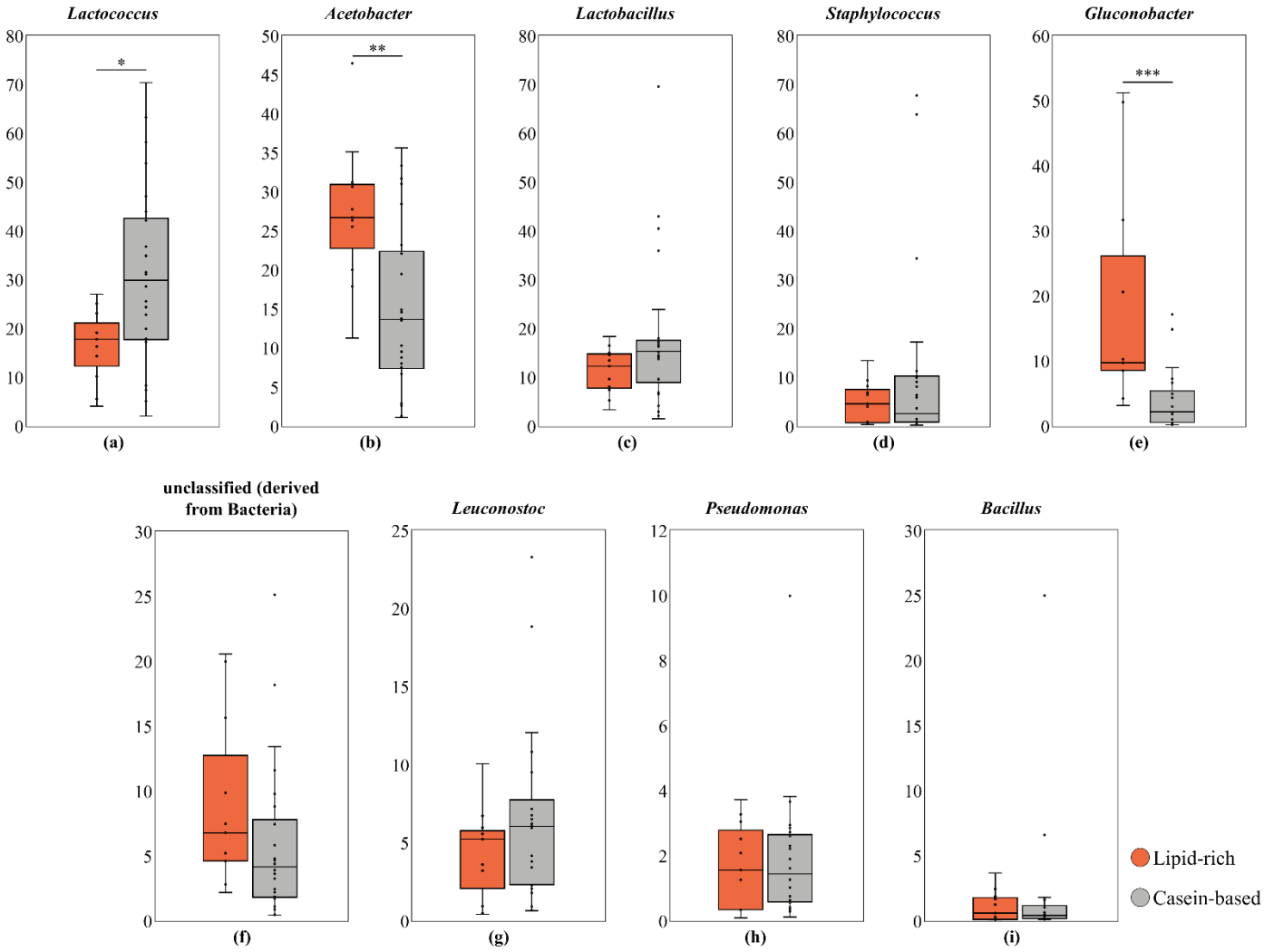


Figure 25: Pairwise comparison of bacterial genera- (a) *Lactococcus*, (b) *Acetobacter*, (c) *Lactobacillus*, (d) *Staphylococcus*, (e) *Gluconobacter*, (f) unclassified (derived from Bacteria), (g) *Leuconostoc*, (h) *Pseudomonas*, and (i) *Bacillus*, based on the nature of the products- lipid-rich and casein-based, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

Species level comparison

At the species level, *Lactococcus lactis* was significantly abundant in *chhurpi* in comparison to *mar* (p=0.00278). Similarly, *Lactobacillus helveticus* was also significantly abundant in *chhurpi* in comparison to *mar* (p=0.04444). On the other hand, *Gluconobacter oxydans* was found to be significantly abundant in *mar* in comparison to both *chhurpi* (p=0.01016) and *churkam* (p=0.00288). Uncultured bacterium was significantly abundant in *mar* (p=0.01278) and *chhurpi* (p=0.03236) in comparison to *churkam*. *Acetobacter lovaniensis* was significantly abundant in both *chhurpi* (p=0.04444) and *churkam* (p=0.01468) in comparison to *mar*. *Acetobacter syzygii* and *Acetobacter tropicalis* were significantly abundant in *mar* in comparison to *churkam* with *p*-values of 0.01278 and 0.04036, respectively. Additionally, *Lactococcus raffinolactis* was found to be significantly abundant in *chhurpi* in comparison to *mar* (p=0.02642). *Pseudomonas fluorescens* was significantly abundant in *chhurpi* in comparison to *churkam* (p=0.00544). Lastly, *Gluconobacter cerinus* was significantly abundant in both *mar* and *chhurpi* in comparison to *churkam* with *p*-values of 0.00012 and 0.01778, respectively (Fig. 26). There were no significant differences of all the bacterial species when comparing between the animal milk source- cow and yak (Fig. 27). *Lactococcus lactis* was observed to be significantly abundant in casein-based products in comparison to lipid-rich products (p=0.04444). Contrastingly, *Gluconobacter oxydans* was significantly abundant in lipid-rich products in comparison to casein-based products (p=0.00116). On the other hand, *Acetobacter lovaniensis* was significantly abundant in casein-based products (p=0.00906). Furthermore, *Acetobacter syzygii* (p=0.01208) and *Gluconobacter cerinus* (p=0.00046) were observed to be significantly abundant in lipid-rich products than in casein-based products (Fig. 28).

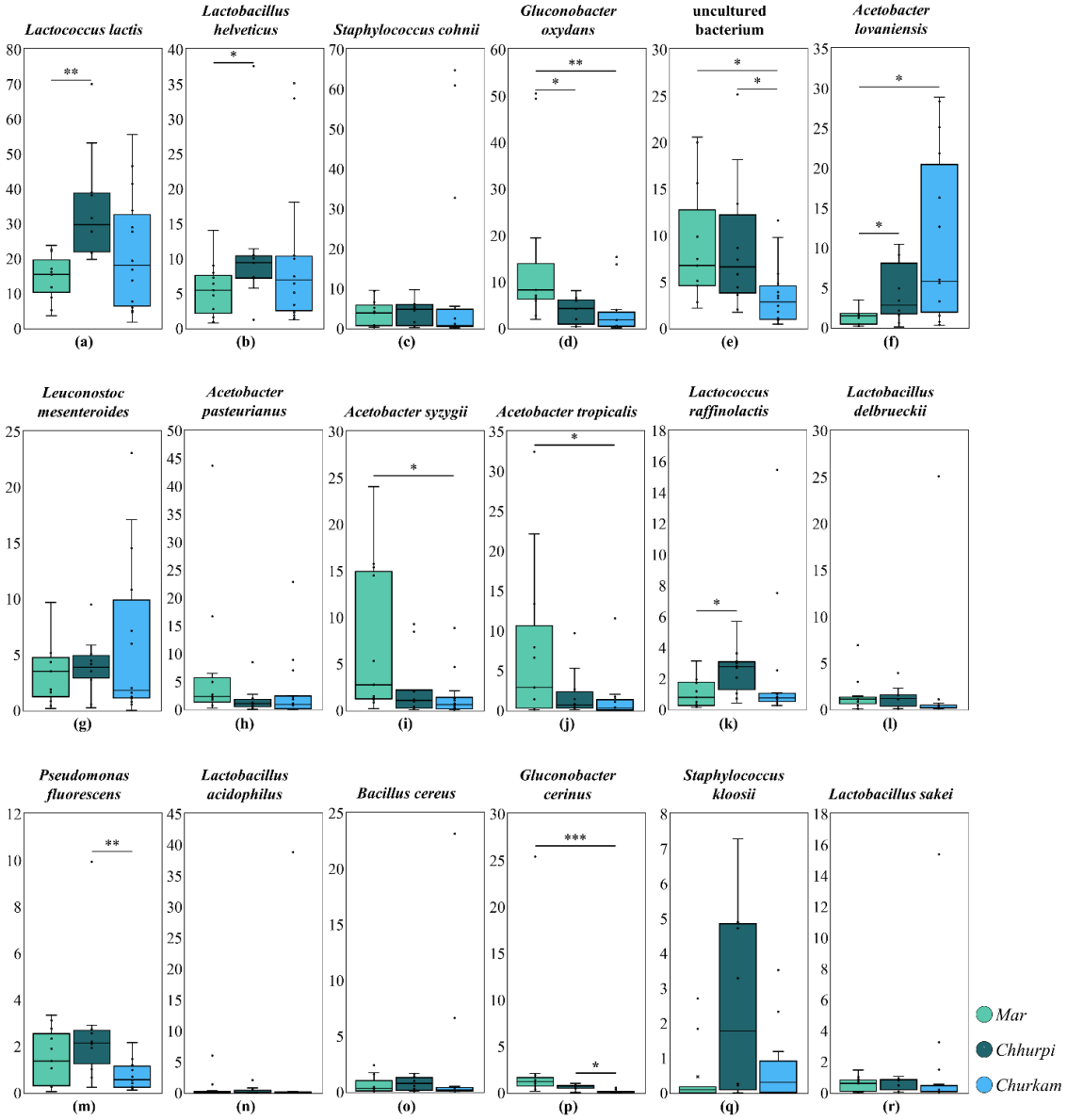


Figure 26: Pairwise comparison of bacterial species- (a) *Lactococcus lactis*, (b) *Lactobacillus helveticus*, (c) *Staphylococcus cohnii*, (d) *Gluconobacter oxydans*, (e) uncultured bacterium, (f) *Acetobacter lovaniensis*, (g) *Leuconostoc mesenteroides*, (h) *Acetobacter pasteurianus*, (i) *Acetobacter syzygii*, (j) *Acetobacter tropicalis*, (k) *Lactococcus raffinolactis*, (l) *Lactobacillus delbrueckii*, (m) *Pseudomonas fluorescens*, (n) *Lactobacillus acidophilus*, (o) *Bacillus cereus*, (p) *Gluconobacter cerinus*, (q) *Staphylococcus kloosii*, and (r) *Lactobacillus sakei*, based on the food types- *mar*, *chhurpi*, and *churkam*, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

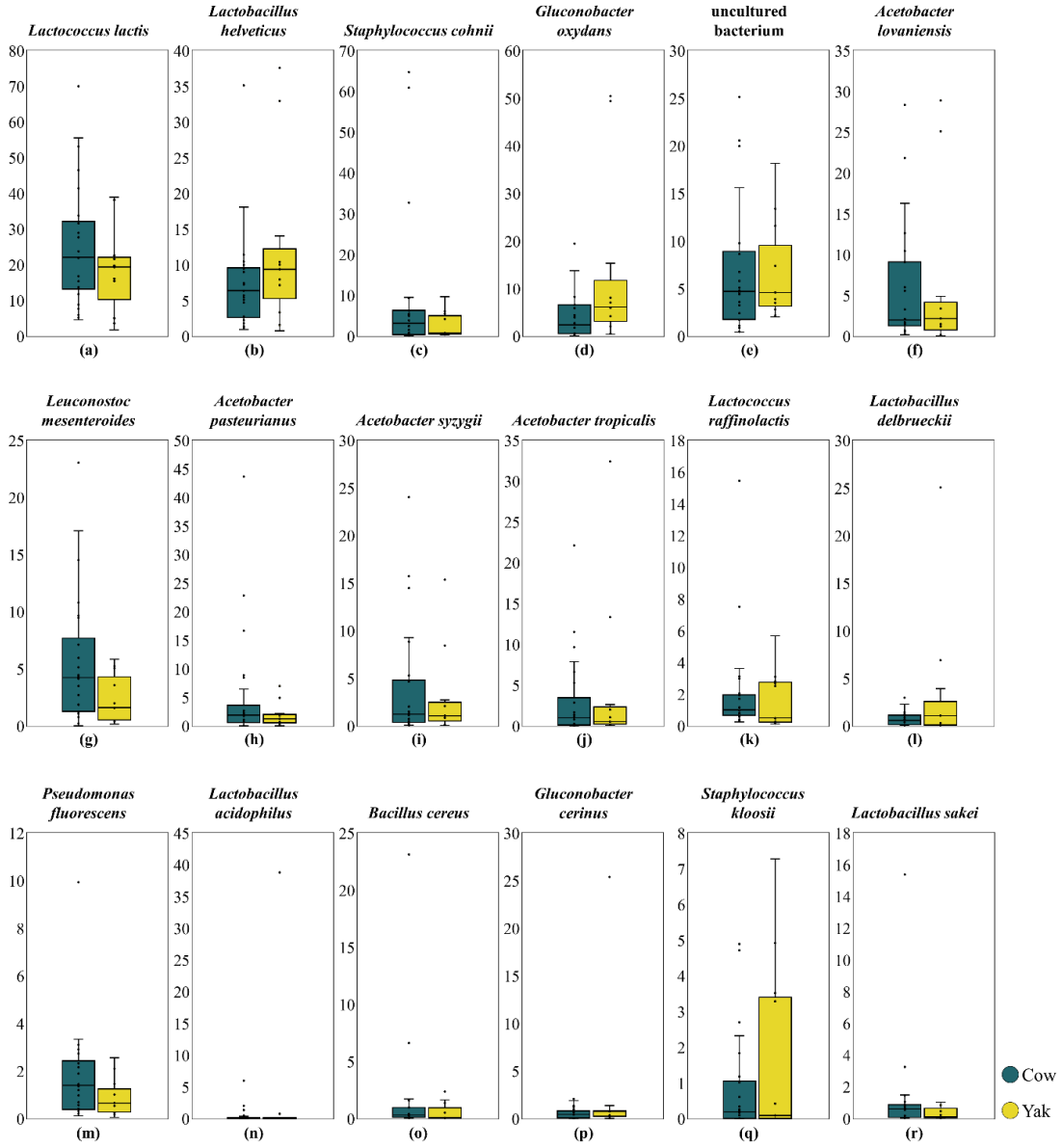


Figure 27: Pairwise comparison of bacterial species- (a) *Lactococcus lactis*, (b) *Lactobacillus helveticus*, (c) *Staphylococcus cohnii*, (d) *Gluconobacter oxydans*, (e) uncultured bacterium, (f) *Acetobacter lovaniensis*, (g) *Leuconostoc mesenteroides*, (h) *Acetobacter pasteurianus*, (i) *Acetobacter syzygii*, (j) *Acetobacter tropicalis*, (k) *Lactococcus raffinolactis*, (l) *Lactobacillus delbrueckii*, (m) *Pseudomonas fluorescens*, (n) *Lactobacillus acidophilus*, (o) *Bacillus cereus*, (p) *Gluconobacter cerinus*, (q) *Staphylococcus kloosii*, and (r) *Lactobacillus sakei*, based on the animal milk source-cow and yak, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

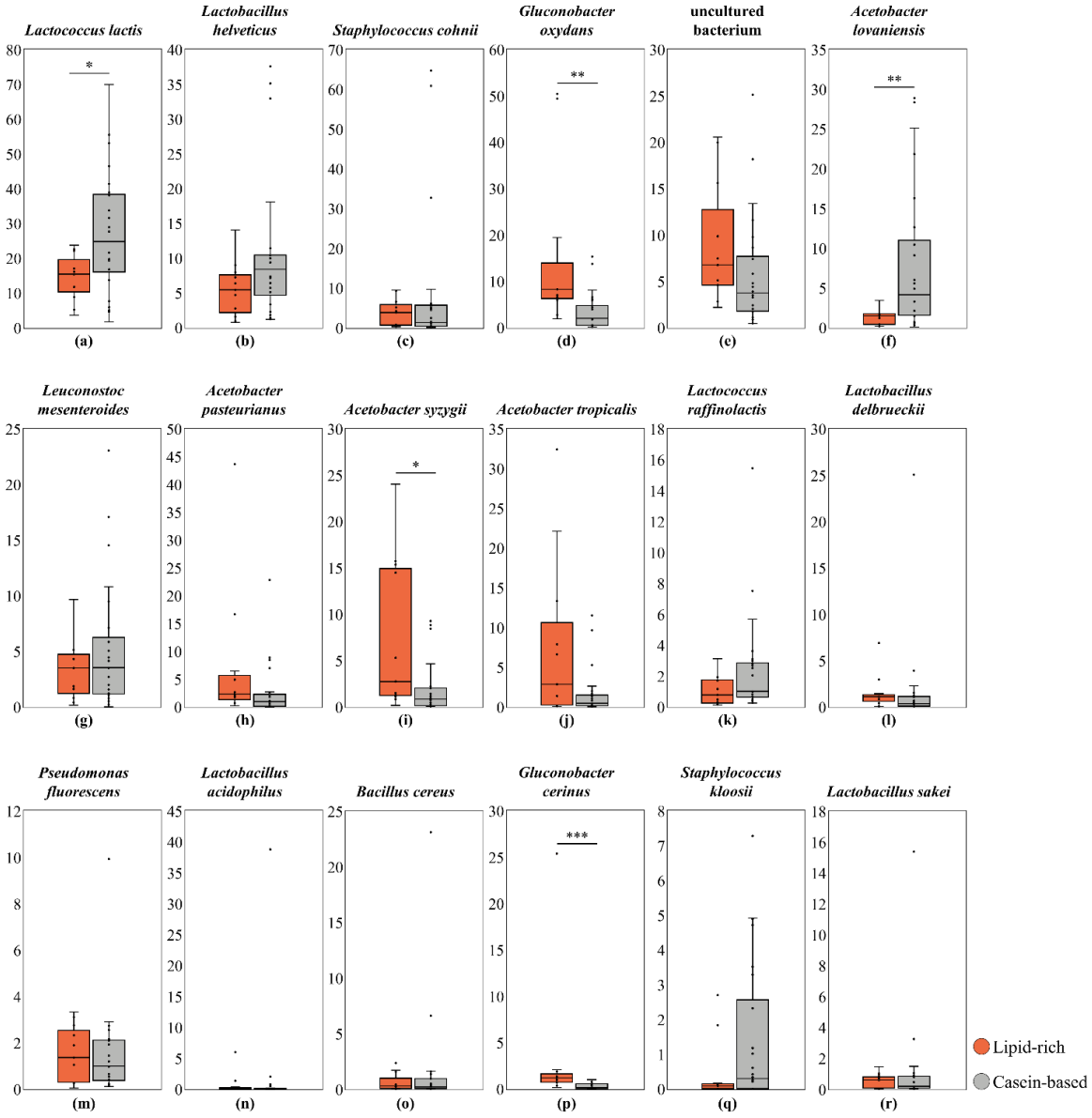


Figure 28: Pairwise comparison of bacterial species- (a) *Lactococcus lactis*, (b) *Lactobacillus helveticus*, (c) *Staphylococcus cohnii*, (d) *Gluconobacter oxydans*, (e) uncultured bacterium, (f) *Acetobacter lovaniensis*, (g) *Leuconostoc mesenteroides*, (h) *Acetobacter pasteurianus*, (i) *Acetobacter syzygii*, (j) *Acetobacter tropicalis*, (k) *Lactococcus raffinolactis*, (l) *Lactobacillus delbrueckii*, (m) *Pseudomonas fluorescens*, (n) *Lactobacillus acidophilus*, (o) *Bacillus cereus*, (p) *Gluconobacter cerinus*, (q) *Staphylococcus kloosii*, and (r) *Lactobacillus sakei*, based on the nature of the products- lipid-rich and casein-based, where significant differences are denoted by * ≤ 0.05; ** ≤ 0.01, and *** ≤ 0.001.

IN VITRO PROBIOTIC ATTRIBUTES OF THE ISOLATED LAB OF NFM OF ARUNACHAL PRADESH

To check the potential probiotic attributes of the 76 identified isolated LAB strains from NFM products of Arunachal Pradesh, several standard experimental tests were performed and analyzed using recommended literatures.

Acidification and coagulation

The ability of LAB strains to undergo acidification of milk and coagulation is important in dairy research. In the present analysis, all 76 identified LAB strains isolated from NFM products of Arunachal Pradesh, were able to undergo acidification and coagulation of the tested skim milk within 72 h with some strains being able to acidify and coagulate milk only after 24h (Table 20-22).

Table 20: Acidification and coagulation properties of the identified LAB isolated from *mar* of Arunachal Pradesh.

Animal source	Identified strains	Acidification	Coagulation		
			24h	48h	72h
Cow	*Levilactobacillus brevis (*AcMr06*)*	5.00±0.01	-ve	+ve	+ve
	Enterococcus durans (AcMr11)	4.88±0.03	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcMr18)	4.37±0.03	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcMr22)	4.31±0.02	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcMr25)	4.82±0.02	-ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcMr27)	4.38±0.02	+ve	+ve	+ve
	Enterococcus durans (AcMr34)	4.89±0.02	+ve	+ve	+ve
	Enterococcus durans (AcMr42)	4.91±0.02	+ve	+ve	+ve
	Enterococcus durans (AcMr53)	4.88±0.01	+ve	+ve	+ve
	Enterococcus durans (AcMr58)	4.91±0.02	+ve	+ve	+ve
	Enterococcus durans (AcMr60)	4.90±0.01	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcMr75)	4.22±0.01	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcMr82)	4.39±0.01	+ve	+ve	+ve
	Enterococcus durans (AcMr91)	4.90±0.01	+ve	+ve	+ve
	Enterococcus durans (AcMr94)	4.87±0.02	+ve	+ve	+ve
	Enterococcus durans (AcMr98)	4.92±0.02	+ve	+ve	+ve
Yak	*Enterococcus durans* (AyMr01)	4.87±0.03	+ve	+ve	+ve
	Enterococcus durans (AyMr03)	4.84±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyMr31)	4.20±0.01	+ve	+ve	+ve
	Enterococcus durans (AyMr38)	4.96±0.01	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AyMr44)	4.39±0.01	+ve	+ve	+ve
	Enterococcus durans (AyMr61)	4.89±0.03	+ve	+ve	+ve
	Enterococcus durans (AyMr65)	4.93±0.02	+ve	+ve	+ve
	Enterococcus durans (AyMr71)	4.95±0.01	+ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AyMr87)	4.09±0.02	+ve	+ve	+ve
Reference	*Lactiplantibacillus plantarum* subsp. *plantarum* (MCC2974)	3.99±0.02	+ve	+ve	+ve

Table 21: Acidification and coagulation properties of the identified LAB isolated from *chhurpi* of Arunachal Pradesh.

Animal source	Identified strains	Acidification	Coagulation		
			24h	48h	72h
Cow	*Lacticaseibacillus paracasei* subsp. *tolerans* (AcCh04)	4.04±0.03	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcCh06)	4.31±0.01	+ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AcCh11)	3.97±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCh14)	4.49±0.02	-ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AcCh17)	3.92±0.07	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcCh21)	4.35±0.01	+ve	+ve	+ve
	Lentilactobacillus parabuchneri (AcCh31)	5.70±0.02	-ve	-ve	+ve
	Levilactobacillus brevis (AcCh35)	5.12±0.02	-ve	-ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCh41)	4.47±0.02	-ve	+ve	+ve
	Loigolactobacillus coryniformis subsp. *torquens* (AcCh63)	4.87±0.02	-ve	-ve	+ve
	Lactococcus lactis subsp. *cremoris (*AcCh67*)*	5.20±0.03	-ve	-ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCh71)	4.52±0.01	-ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCh74)	4.51±0.03	-ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AcCh78)	3.93±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCh81)	4.50±0.01	-ve	+ve	+ve
	Levilactobacillus brevis (AcCh91)	5.04±0.01	-ve	+ve	+ve
Yak	*Leuconostoc mesenteroides* subsp. *mesenteroides* (AyCh01)	4.75±0.02	-ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AyCh28)	3.97±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh37)	4.19±0.02	-ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh45)	4.20±0.03	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh51)	4.81±0.04	-ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh55)	4.88±0.02	-ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh58)	4.89±0.01	-ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AyCh85)	4.39±0.02	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCh87)	4.91±0.01	-ve	+ve	+ve
	Lacticaseibacillus paracasei subsp. *tolerans* (AyCh94)	4.05±0.03	+ve	+ve	+ve
Reference	*Lactiplantibacillus plantarum* subsp. *plantarum* (MCC2974)	3.99±0.02	+ve	+ve	+ve

Table 22: Acidification and coagulation properties of the identified LAB isolated from *churkam* of Arunachal Pradesh

Animal source	Identified strains	Acidification	Coagulation		
			24h	48h	72h
Cow	*Lactococcus lactis* subsp. *lactis* (AcCk06)	4.31±0.02	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcCk11)	4.30±0.02	+ve	+ve	+ve
	Enterococcus durans (AcCk25)	4.82±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk35)	4.49±0.02	-ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk41)	4.52±0.02	-ve	+ve	+ve
	Lactococcus lactis subsp. *hordniae* (AcCk46)	5.09±0.01	-ve	-ve	+ve
	Enterococcus durans (AcCk51)	4.80±0.02	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk56)	4.71±0.02	-ve	+ve	+ve
	Lactococcus lactis subsp. *cremoris* (AcCk61)	5.09±0.03	-ve	-ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk64)	4.78±0.02	-ve	+ve	+ve
	Levilactobacillus brevis (AcCk67)	5.10±0.02	-ve	-ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk74)	4.75±0.03	-ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcCk75)	4.27±0.01	+ve	+ve	+ve
	Lactococcus lactis subsp. *lactis* (AcCk81)	4.31±0.02	+ve	+ve	+ve
	Enterococcus durans (AcCk83)	4.82±0.01	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AcCk91)	4.79±0.01	-ve	+ve	+ve
Yak	*Leuconostoc mesenteroides* subsp. *mesenteroides* (AyCk01)	4.86±0.02	-ve	+ve	+ve
	Enterococcus durans (AyCk04)	4.93±0.02	+ve	+ve	+ve
	Enterococcus durans (AyCk15)	4.91±0.01	+ve	+ve	+ve
	Lactococcus lactis subsp. *cremoris* (AyCk21)	5.04±0.01	-ve	+ve	+ve
	Enterococcus durans (AyCk28)	4.89±0.02	+ve	+ve	+ve
	Enterococcus durans (AyCk33)	4.79±0.02	+ve	+ve	+ve
	Lactococcus lactis subsp. *cremoris* (AyCk71)	5.08±0.02	-ve	-ve	+ve
	Enterococcus durans (AyCk84)	4.28±0.02	+ve	+ve	+ve
	Leuconostoc mesenteroides subsp. *mesenteroides* (AyCk93)	4.82±0.01	-ve	+ve	+ve
Reference	*Lactiplantibacillus plantarum* subsp. *plantarum* (MCC2974)	3.99±0.02	+ve	+ve	+ve

Acid tolerance

The ability of lactic acid bacteria to withstand or tolerate certain degrees of acid has been one of the important features for a potential probiotic strain to have, a condition of the gastrointestinal system where little as pH 2-3 is usually the case. A wide range of tolerance against acid was observed among the LAB strains. A good range of tolerance percentage was observed from 53.16% to 93.38% was observed, where *Levilactobacillus brevis* (AcCh91) showed the highest with 93.38±1.61%, followed by *Levilactobacillus brevis* AcCk67 (92.49±3.01%), *Levilactobacillus brevis* AcCh35 (90.72±0.88%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (88.08±4.78%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (87.76±6.44%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (87.38±0.92%), *Levilactobacillus brevis* AcMr06 (86.79±4.18%), *Lactococcus lactis* subsp. *cremoris* AyCk21 (86.69±2.6%), *Lactococcus lactis* subsp. *lactis* AcCk75 (86.42±2.51%), *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 (82.97±0.54%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (82.63±6.57%), *Enterococcus durans* AcCk25 (79.81±2.59%), *Lactococcus lactis* subsp. *lactis* AcMr75 (78.48±7.59%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (74.7±0.56%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (66.67±2.57%), *Enterococcus durans* AyCk84 (66.65±4.93%), *Enterococcus durans* AyMr03 (65.64±6.18%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 (62.31±2.63%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (57.67±0.45%), and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 (53.16±1.6%) (Fig. 29).

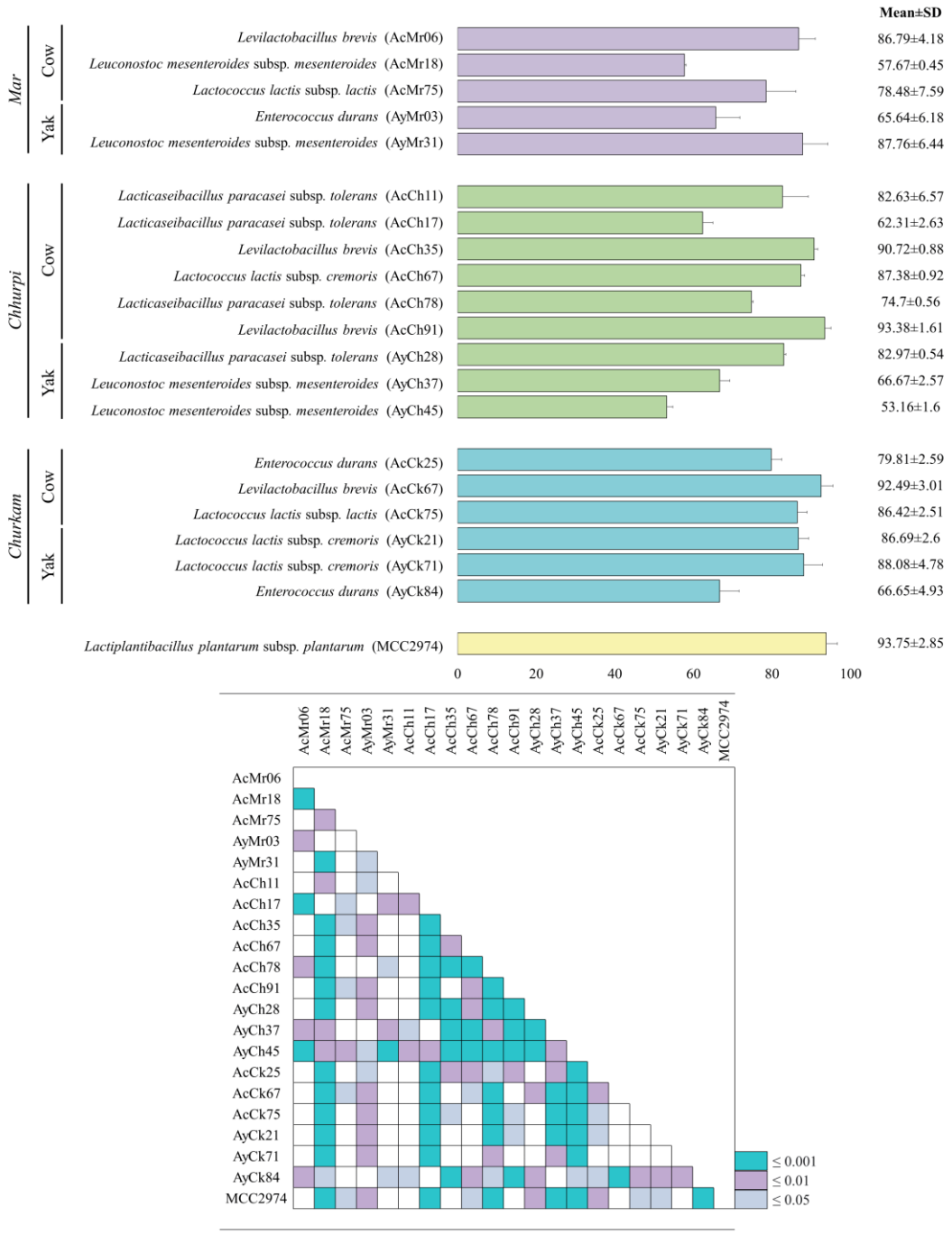


Figure 29: Acid tolerance (%) of the top LAB strains isolated from NFM of Arunachal Pradesh. Paired Student's t-test was performed among the strains and significant values ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) as shown in the heatmap representation.

Bile tolerance

A range of 53.18% to 86.68% tolerance of bile was observed amongst the LAB strains, with *Levilactobacillus brevis* (AcCh91) being the most tolerant strain with 86.68±2.69%, followed by *Lactococcus lactis* subsp. *cremoris* AyCk21 (84.83±1.7%), *Levilactobacillus brevis* AcCk67 (83.86±5.25%), *Leuconostoc mesenteroides* subsp. mesenteroides AyCh45 (83.06±5.81%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (82.77±6.33%), *Levilactobacillus brevis* AcCh35 (82.03±4.06%), *Levilactobacillus brevis* AcMr06 (80.87±9.39%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (76.54±2.71%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (76.4±11.16%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (75.89±1.68%), *Lactococcus lactis* subsp. *lactis* AcCk75 (75.55±9.82%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 (74.29±8.91%), *Enterococcus durans* AcCk25 (74.19±5.58%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (72.52±2.01%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (68.77±7.35%), *Enterococcus durans* AyCk84 (66.02±3.56%), *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 (61.31±4.08%), *Lactococcus lactis* subsp. *lactis* AcMr75 (54.98±0.6%), *Enterococcus durans* AyMr03 (54.45±5.11%), and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (53.18±4.39%) (Fig. 30).

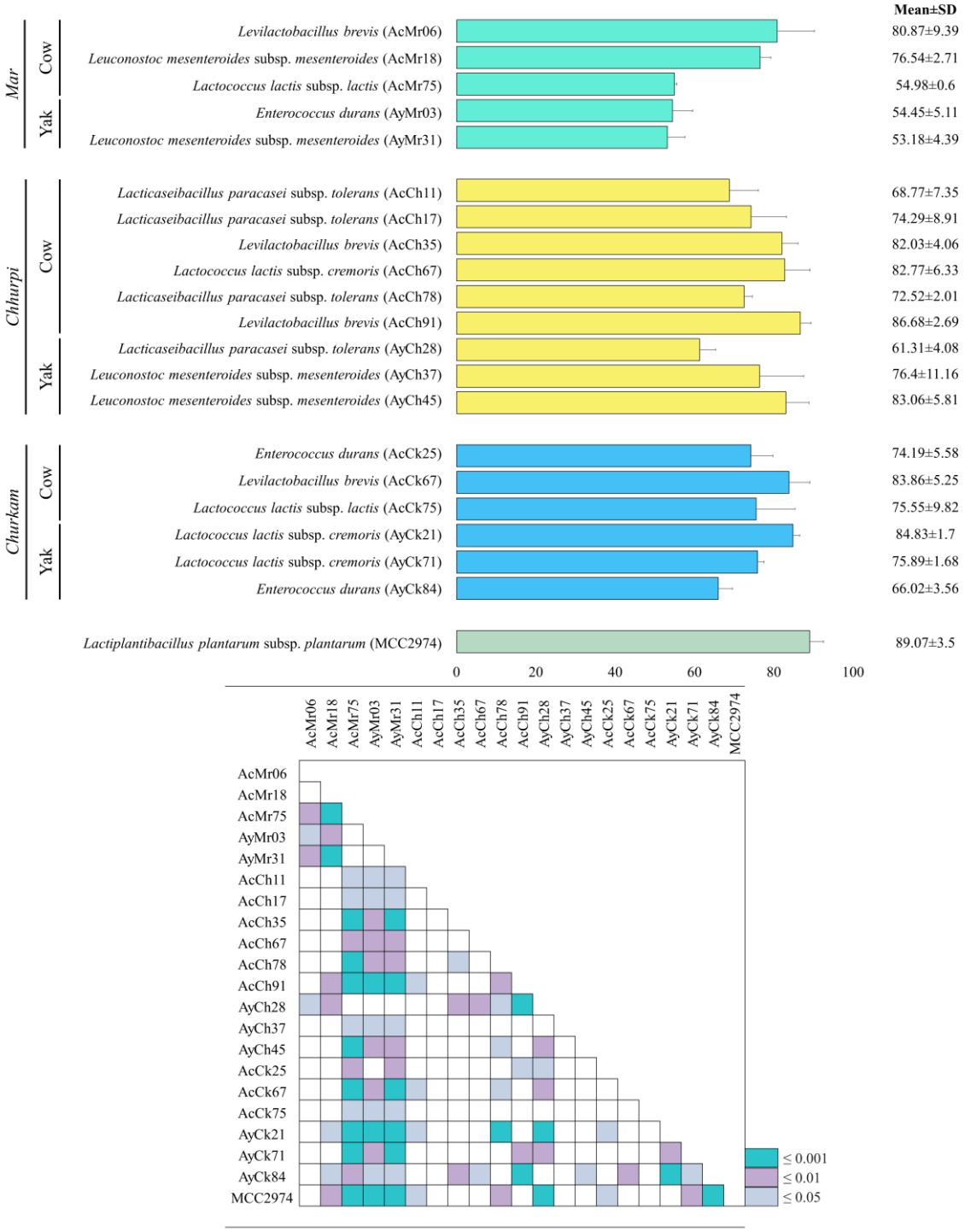


Figure 30: Bile tolerance (%) of the top LAB strains isolated from NFM of Arunachal Pradesh. Paired Student's t-test was performed among the strains and significant values ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) as shown in the heatmap representation.

Microbial attachments to hydrocarbons (MATH)

To check the cell surface hydrophobicity potential of the identified LAB strains, experimental tests were performed by measuring the adherence properties against hydrocarbons like chloroform, diethyl ether, n-hexadecane, toluene, and xylene. A good hydrophobicity range of 28.52% to 86.34% were observed where *Levilactobacillus brevis* AcCh91 showed the highest hydrophobicity average of 86.34±5.53%, followed by *Levilactobacillus brevis* AcMr06 (70.13±14.76%), *Lactococcus lactis* subsp. *cremoris* AyCk21 (69.96±13.78%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (69.69±18.54%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (69.66±12.66%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (68.99±16.92%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (67.58±14.84%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 (66.84±6.25%), *Enterococcus durans* AyCk84 (64.15±16.04%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (61.8±16.55%), *Lactococcus lactis* subsp. *lactis* AcMr75 (59.52±22.99%), *Levilactobacillus brevis* AcCh35 (59.26±20.97%), *Lactococcus lactis* subsp. *lactis* AcCk75 (58.8±16.14%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (56±29.42%), *Enterococcus durans* AcCk25 (55.02±15.74%), *Levilactobacillus brevis* AcCk67 (49.67±24.68%), *Enterococcus durans* AyMr03 (47.48±17.33%), *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 (47.34±13.96%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (43.46±24.95%), and *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 (28.52±17.2%) (Fig. 31).

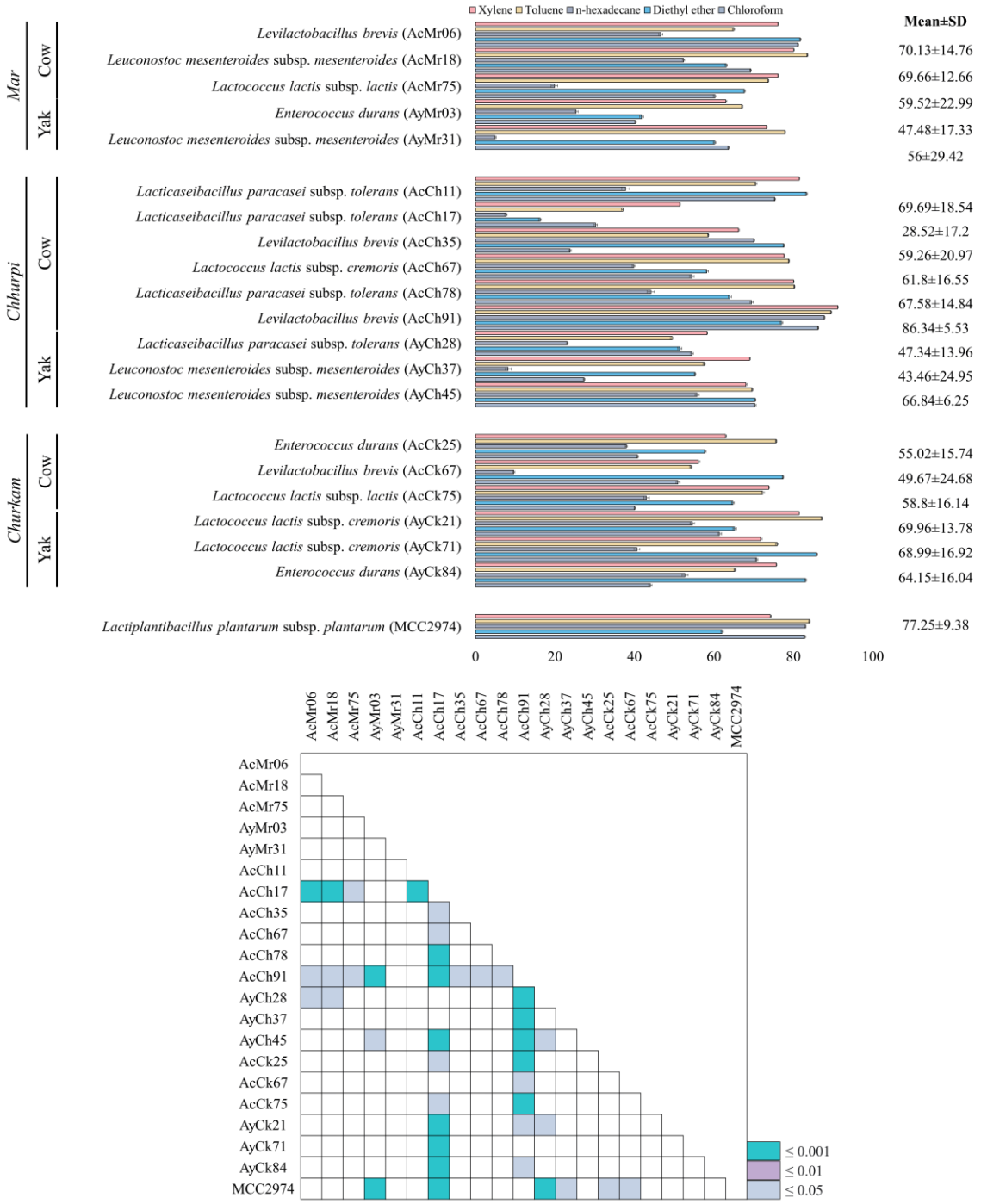


Figure 31: Microbial attachment to hydrocarbons, MATH (%) (using xylene, toluene, n-hexadecane, diethyl ether and chloroform) of the top LAB strains isolated from NFM of Arunachal Pradesh. Paired Student's t-test was performed among the strains and significant values ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) as shown in the heatmap representation.

Auto-aggregation

The ability of lactic acid bacteria to auto-aggregate determines another important feature relating to their cell surface properties. We observed a good range of 21.8% to 55.27% of auto-aggregation amongst the LAB strains. *Levilactobacillus brevis* AcCh91 (55.27±2.93%) showed the maximum percentage of auto-aggregation, followed by *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (48.27±2.53%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 (46.62±1.08%), *Lactococcus lactis* subsp. *lactis* AcMr75 (40.54±2.47%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (40.08±0.32%), *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 (38.76±2.26%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (38.52±1.03%), *Lactococcus lactis* subsp. *lactis* AcCk75 (38.23±1.61%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (35.71±1.9%), *Enterococcus durans* AcCk25 (35.21±1.84%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (35±2.12%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (32.52±2.42%), *Enterococcus durans* AyCk84 (29.75±1.33%), *Enterococcus durans* AyMr03 (28.43±0.79%), *Lactococcus lactis* subsp. *cremoris* AyCk21 (27.55±2.17%), *Levilactobacillus brevis* AcMr06 (26.84±2.64%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 (26.67±2.73%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (22.57±2.71%), and *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh94 (21.8±5.17%) (Fig. 32).

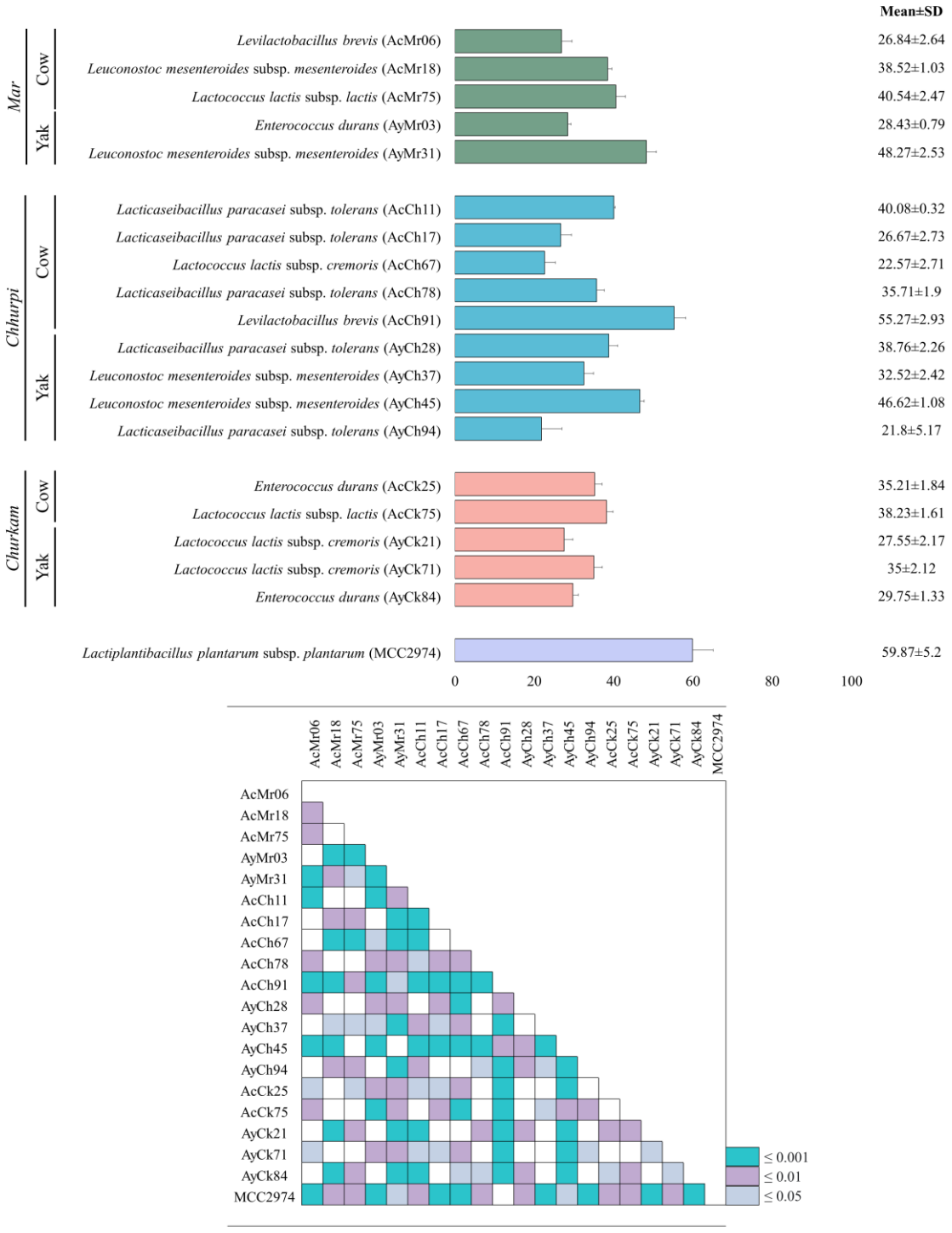


Figure 32: Auto-aggregation (%) of the top LAB strains isolated from NFM of Arunachal Pradesh. Paired Student's t-test was performed among the strains and significant values ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) as shown in the heatmap representation.

Co-aggregation

Another important features to determine the cell surface property is the ability of the LAB strains to adhere to other bacteria or aggregate and reduce their population. We observed a wide range from 21.28% to 83.3% amongst the tested LAB strains. Here, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 showed the maximum with 83.3±1.16%, followed by *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 (82.52±2%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (82.26±2.99%), *Levilactobacillus brevis* AcCh91 (82.25±2.69%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (81.4±3.57%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (80.5±4.78%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 (79.58±5.07%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (79.15±5.07%), *Levilactobacillus brevis* AcCh35 (78.26±6.04%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (77.23±6.39%), *Lactococcus lactis* subsp. *lactis* AcCk75 (76.53±5.84%), *Levilactobacillus brevis* AcCk67 (75.09±6.34%), *Lactococcus lactis* subsp. *lactis* AcMr75 (75.03±7.58%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (69.98±5.62%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (65.59±7.51%), *Enterococcus durans* AyCk84 (51.3±7.94%), *Lactococcus lactis* subsp. *cremoris* AyCk21 (49.32±8.09%), *Enterococcus durans* AcCk25 (47.26±8.68%), *Levilactobacillus brevis* AcMr06 (47.05±9.9%), *Enterococcus durans* AyMr03 (38.81±11.78%), and *Enterococcus durans* AcCk83 (21.28±8.48%) (Fig. 33).

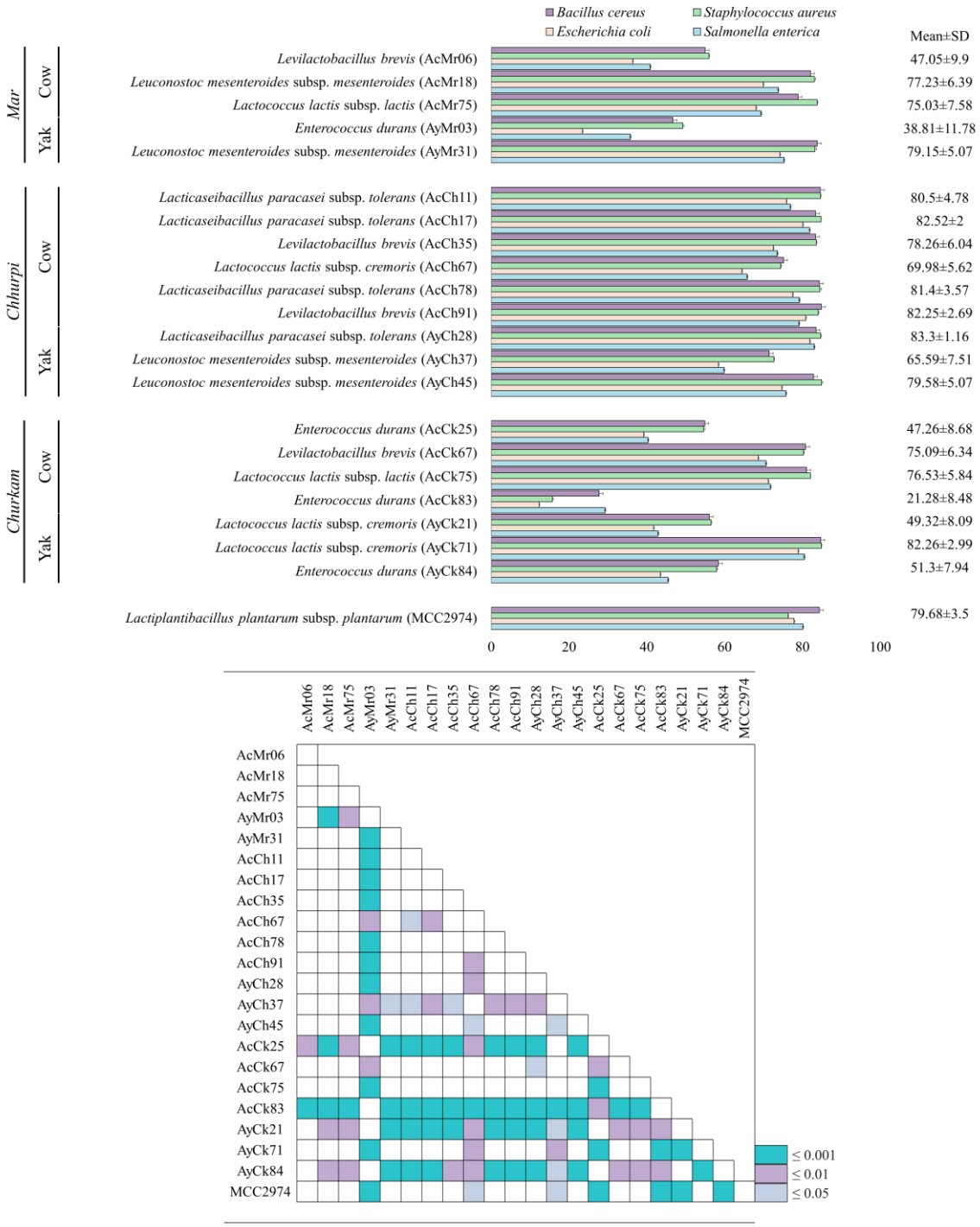


Figure 33: Co-aggregation (%) of the top LAB strains isolated from NFM of Arunachal Pradesh. Paired Student's t-test was performed among the strains and significant values ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) as shown in the heatmap representation.

Antimicrobial property against standard cultures of pathogenic strains

Testing the antimicrobial property of the LAB strains was performed against four pathogenic strains viz., *Bacillus cereus* MTCC1272, *Escherichia coli* MCC2413, *Salmonella enterica* MTCC3233, *Staphylococcus aureus* MTCC740 using *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974 as a positive control for activity which showed good antagonistic property against all the tested pathogenic strains. Only a handful strains showed positive activity against the tested strains. Strains *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, *Lactococcus lactis* subsp. *lactis* AcMr75, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lactococcus lactis* subsp. *lactis* AcCk75 showed positive antagonistic property against all the tested pathogenic strains. Strains *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17, *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 showed positive activity against all except *Salmonella enterica* MTCC3233. *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcMr06 showed activity only against three strains except *Staphylococcus aureus* MTCC740. *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 showed activity against all except *Escherichia coli* MCC2413. On the other hand, *Levilactobacillus brevis* AcCh35, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, *Lactococcus lactis* subsp. *cremoris* AcCh67, and *Enterococcus durans* AyMr03 showed positive activity only against *Bacillus cereus* MTCC1272, and *Escherichia coli* MCC2413. *Enterococcus durans* AcCk25, *Levilactobacillus brevis* AcCk67, and *Enterococcus durans* AyCk84 showed positive activity only against *Escherichia coli* MCC2413, and *Staphylococcus aureus* MTCC740. Lastly, *Lactococcus lactis* subsp. *cremoris* AyCk21, and *Lactococcus lactis* subsp. *cremoris* AyCk71 showed activity only against *Escherichia coli* MCC2413 (Table 23).

Table 23: Antimicrobial activity profiles of the identified LAB strains against four standard pathogenic strains.

Strains	Codes	*Bacillus cereus* MTCC1272	*Escherichia coli* MCC2413	*Salmonella enterica* MTCC3233	*Staphylococcus aureus* MTCC740
Levilactobacillus brevis	AcMr06	++	++	+	-
Levilactobacillus brevis	AcCh35	++	++	-	-
Levilactobacillus brevis	AcCh91	++	++	++	-
Levilactobacillus brevis	AcCk67	-	+	-	+
Lactococcus lactis subsp. *cremoris*	AcCh67	+	+	-	+
Lactococcus lactis subsp. *cremoris*	AyCk21	-	+	-	-
Lactococcus lactis subsp. *cremoris*	AyCk71	-	+	-	-
Lactococcus lactis subsp. *lactis*	AcCk75	++	++	+	++
Lacticaseibacillus paracasei subsp. *tolerans*	AyCh28	++	+++	++	+++
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh11	++	+++	+	+++
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh78	++	+++	+++	+++
Lactococcus lactis subsp. *lactis*	AcMr75	++	+++	+++	+++
Leuconostoc mesenteroides subsp. *mesenteroides*	AyMr31	++	+++	+++	+++
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh37	+	-	+	++
Enterococcus durans	AcCk25	-	-	+	++
Enterococcus durans	AyCk84	-	+	-	+
Enterococcus durans	AyMr03	++	+	-	-
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh45	++	++	-	-
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh17	+	+++	-	+++
Leuconostoc mesenteroides subsp. *mesenteroides*	AcMr18	+	+++	-	+++
Lactiplantibacillus plantarum subsp. *plantarum*	MCC2974	+++	+++	+++	+++

Note: "+++" > 18mm, "++" > 15mm, and "+" > 10mm, and "-" denotes negative activity.

Bile salt hydrolysis

All isolates did not hydrolyse cholic acid. However, only *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris* AcCh67, *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lactococcus lactis* subsp. *cremoris* AyCk71, *Lactococcus lactis* subsp. *lactis* AcCk75, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, *Lactococcus lactis* subsp. *lactis* AcMr75, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17, *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, and *Enterococcus durans* AcCk51 hydrolyse taurocholate and taurodeoxycholate acids, whereas all the isolates were found to be negative for bile salt hydrolysis.

Primary grouping based on the standard probiotic test

Based on the standard experimental tests viz., acid tolerance, bile tolerance, microbial attachments to hydrocarbons (MATH), auto-aggregation, co-aggregation, bile salt hydrolysis and antimicrobial activity, a primary grouping for selection of the most potential probiotic candidates was performed using two most used analysis using heatmap and biplot principal component analysis (PCA). For qualitative experiments, presence and absence were denoted by "1" or "0" respectively, and we used a log (x+1) transformation for normalizing all the quantitative values. Heatmap visualization analysis showed the presence of a clear clustering forming two main distinct clusters (A and B, Fig. 34). All strains falling in the cluster "A" are those strains which showed less activities towards all the tested experimental tests. However, the cluster "B" group are

of main interest as they show promising activities in the overall standard probiotic experimental tests. Similarly, we also observed a similar pattern using biplot principal component analysis (PCA), where strains with similar properties form visible clusters (Fig. 35). Through these two statistical and visualization analyses, we observed some potential strains with promising probiotic properties.

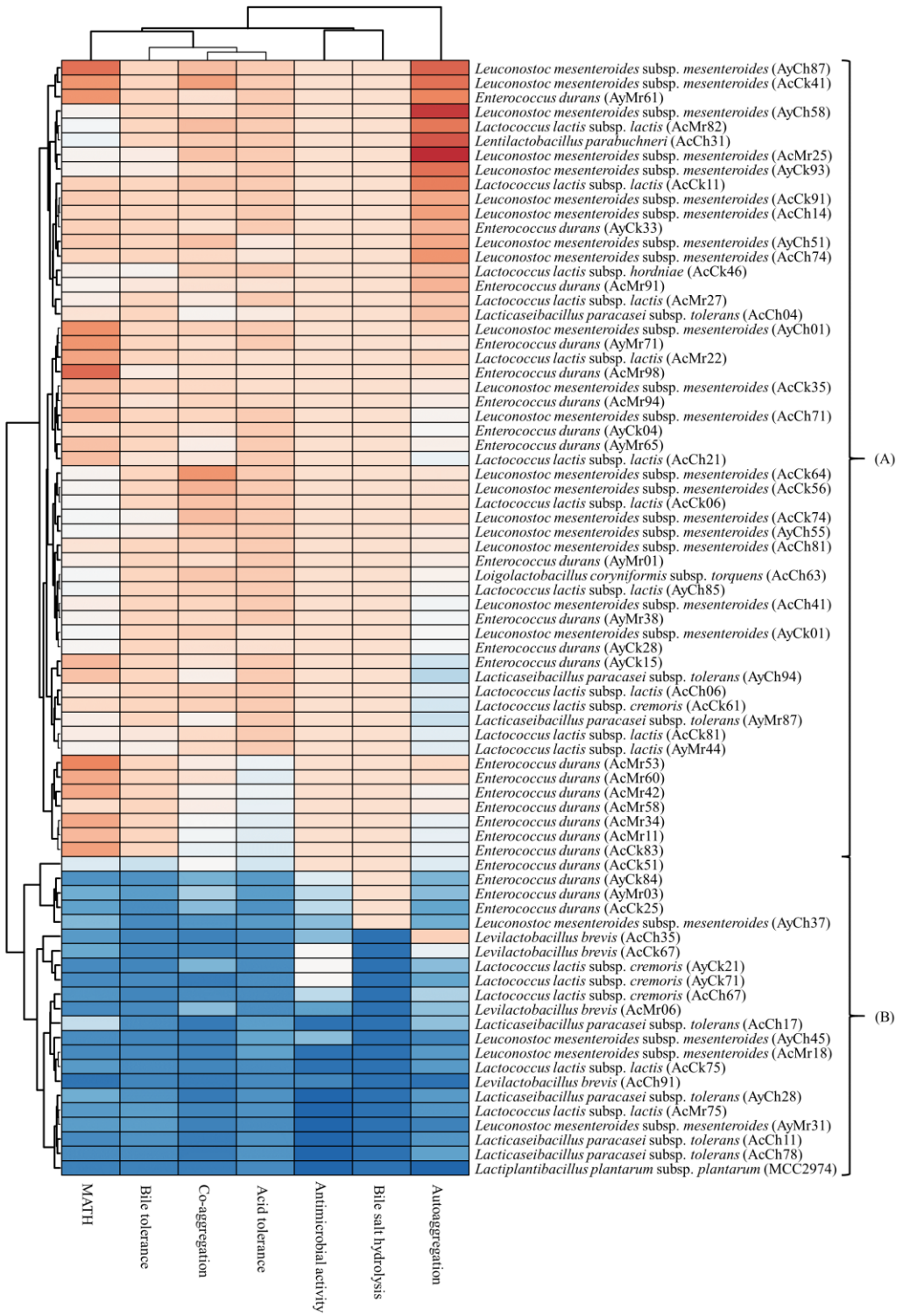


Figure 34: Heatmap visualization of the probiotic properties of the identified LAB isolated from NFM products of Arunachal Pradesh, which results into a group of two clusters (A) and (B). LAB isolates that form the B-cluster are found to be better potential probiotic bacteria compared to the A-cluster, which are also used for further tests.

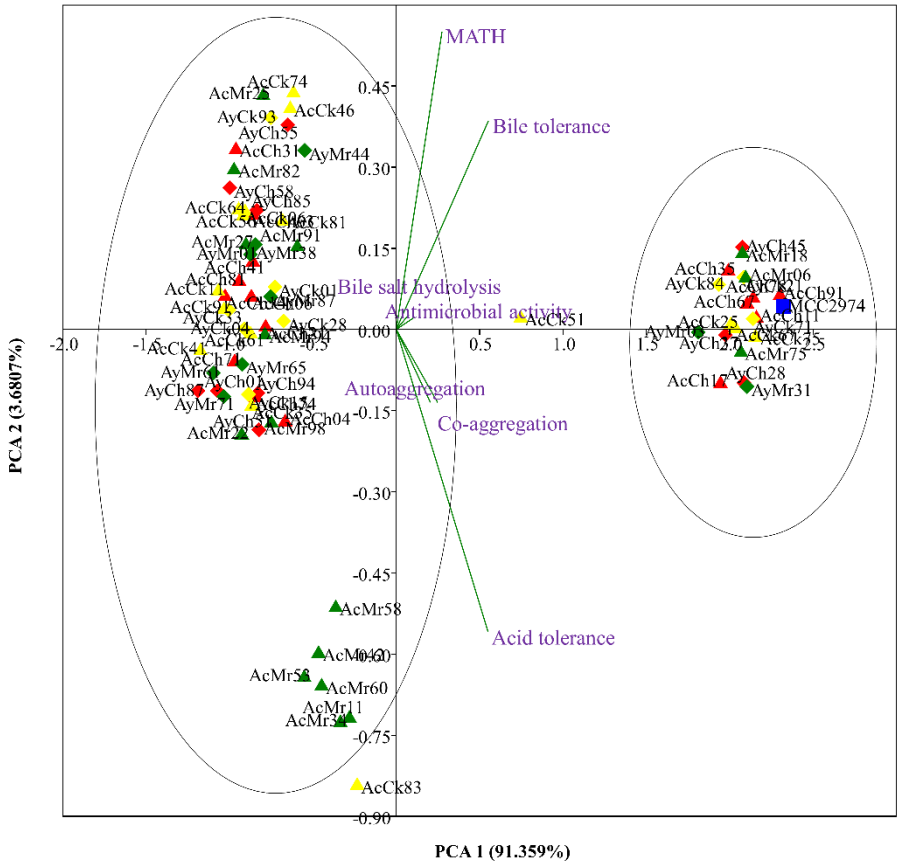


Figure 35: Biplot and principal component analysis (PCA) of the probiotic properties of the identified LAB isolated from NFM of Arunachal Pradesh. Strains with similar properties as per the selected experimental probiotic tests form clusters as clearly shown.

The LAB strains with potential promising probiotic properties were then selected for further characterization using some important tests of our interest including Beta-galactosidase, cholesterol reduction, exopolysaccharide, and Gamma amino butyric acid (GABA) production.

Beta-galactosidase

Three groups of groups were observed based on the production of beta-galactosidase where *Levilactobacillus brevis* AcMr06, *L. brevis* AcCh35, *L. brevis* AcCh91, *L. brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris* AcCh67, *Lactococcus lactis* subsp. *lactis*

AcMr75, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 are the best producers. *Lactococcus lactis* subsp. *cremoris* AyCk21, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 are intermediate producers, whereas *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *L. paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 are the least producers. *Lactococcus lactis* subsp. *lactis* AcCk75, *Enterococcus durans* AcCk25, *Enterococcus durans* AyCk84, *Enterococcus durans* AyMr03, and *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 are negative for beta-galactosidase enzyme production (Fig 36. Table 24).

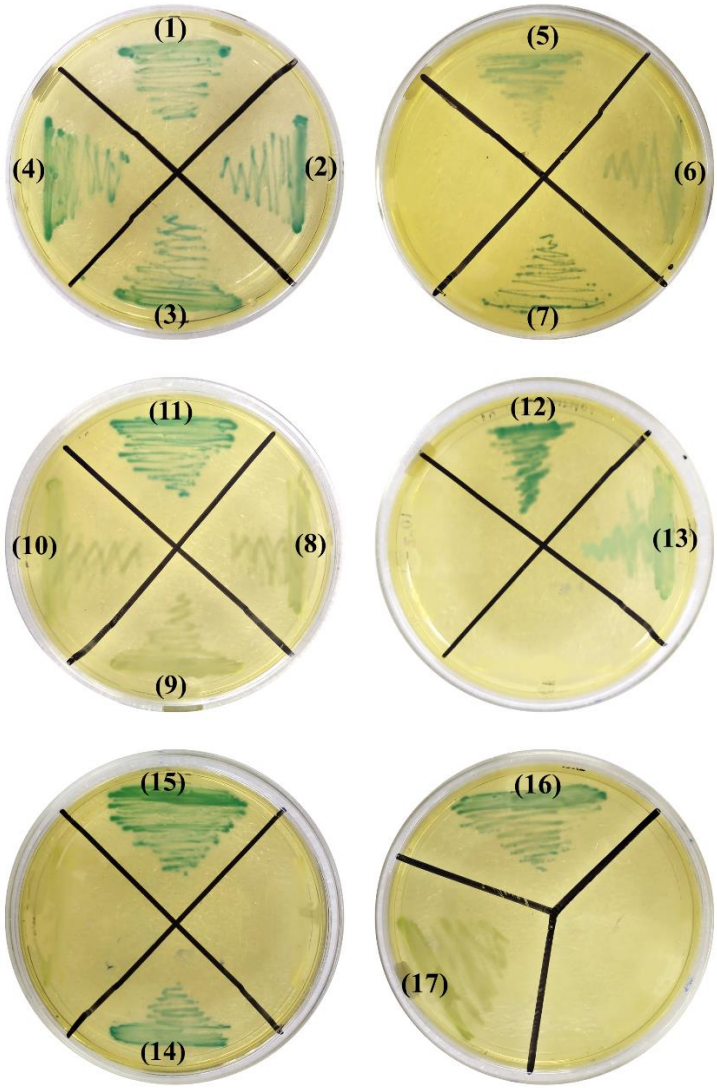


Figure 36: Detection of Beta-galactosidase production in LAB strains as indicated by bluish to green colony appearances: (1) *Levilactobacillus brevis* AcMr06, (2) *Levilactobacillus brevis* AcCh35, (3) *Levilactobacillus brevis* AcCh91, (4) *Levilactobacillus brevis* AcCk67, (5) *Lactococcus lactis* subsp. *cremoris* AcCh67, (6) *Lactococcus lactis* subsp. *cremoris* AyCk21, (7) *Lactococcus lactis* subsp. *cremoris* AyCk71, (8) *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, (9) *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, (10) *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, (11) *Lactococcus lactis* subsp. *lactis* AcMr75, (12) *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, (13) *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, (14) *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, (15) *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18. Positive controls (16) *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974 and (17) *Limosilactobacillus fermentum* MCC2760 were used.

Table 24: Beta-galactosidase activity of the LAB strains.

Strains	Codes	Beta-galactosidase
Levilactobacillus brevis	AcMr06	+++
Levilactobacillus brevis	AcCh35	+++
Levilactobacillus brevis	AcCh91	+++
Levilactobacillus brevis	AcCk67	+++
Lactococcus lactis subsp. *cremoris*	AcCh67	+++
Lactococcus lactis subsp. *cremoris*	AyCk21	++
Lactococcus lactis subsp. *cremoris*	AyCk71	+++
Lactococcus lactis subsp. lactis	AcCk75	-
Lacticaseibacillus paracasei subsp. *tolerans*	AyCh28	+
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh11	+
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh78	+
Lactococcus lactis subsp. *lactis*	AcMr75	+++
Leuconostoc mesenteroides subsp. *mesenteroides*	AyMr31	+++
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh37	++
Enterococcus durans	AcCk25	-
Enterococcus durans	AyCk84	-
Enterococcus durans	AyMr03	-
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh45	++
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh17	-
Leuconostoc mesenteroides subsp. *mesenteroides*	AcMr18	++
Lactiplantibacillus plantarum subsp. *plantarum*	MCC2974	+

Note: "+++" denotes best activity, "++" denotes medium activity, "+" denotes less activity observed, and "-" denotes negative activity.

Exopolysaccharide production

Based on the plate assay for exopolysaccharide production, only strains *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, *Enterococcus durans* AcCk25, *Enterococcus durans* AyCk84, *Enterococcus durans* AyMr03, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17, *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 were found to be positive, whereas strains *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris* AcCh67, *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lactococcus lactis* subsp. *cremoris* AyCk71, *Lactococcus lactis* subsp. *lactis* AcCk75, *Lactococcus lactis* subsp. *lactis* AcMr75, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 were found to be negative (Fig. 37, Table 25).

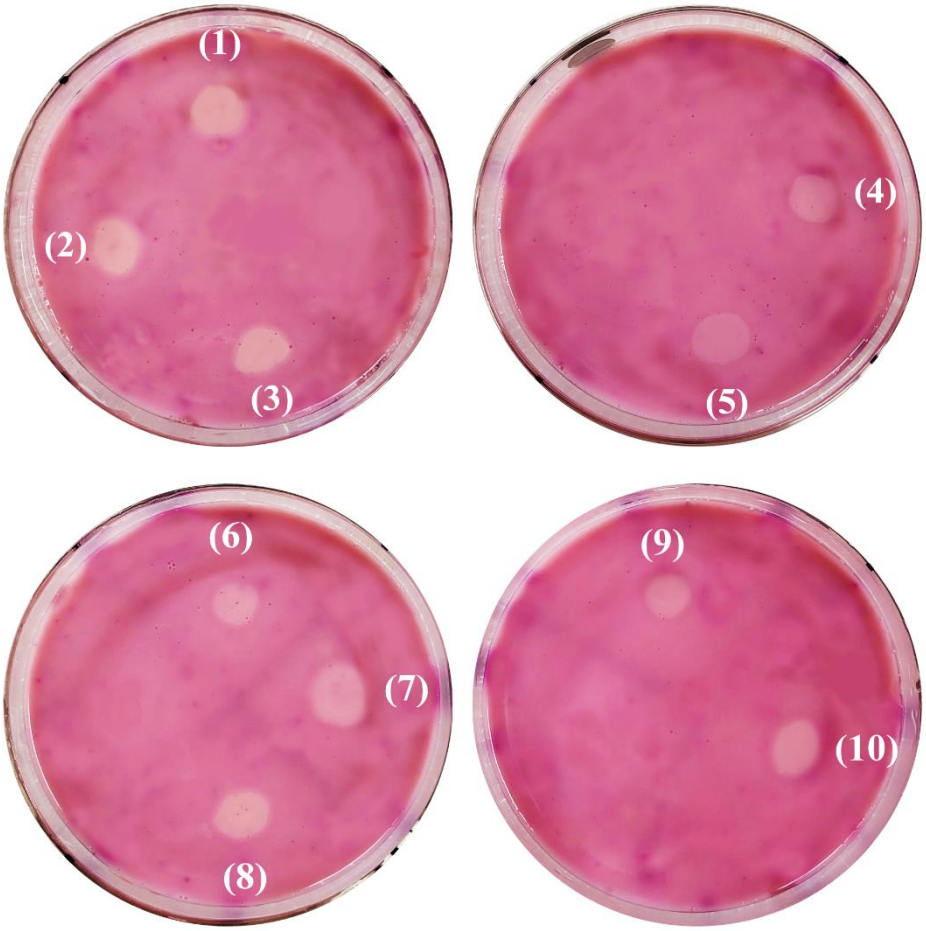


Figure 37: Detection of exopolysaccharide production (ropy colonies) in the presence of ruthenium red dye by some LAB strains: (1) *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, (2) *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, (3) *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, (4) *Enterococcus durans* AcCk25, (5) *Enterococcus durans* AyCk84, (6) *Enterococcus durans* AyMr03, (7) *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17, (8) *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18. Positive control (9) *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974, (10) *Levilactobacillus brevis* MCC2198T were used.

Table 25: Exopolysaccharide production profiles of the LAB strains.

Strains	Codes	Exopolysaccharide production
Lacticaseibacillus paracasei subsp. *tolerans*	AyCh28	+ve
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh11	+ve
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh78	+ve
Enterococcus durans	AcCk25	+ve
Enterococcus durans	AyCk84	+ve
Enterococcus durans	AyMr03	+ve
Lacticaseibacillus paracasei subsp. *tolerans*	AcCh17	+ve
Leuconostoc mesenteroides subsp. *mesenteroides*	AcMr18	+ve
Levilactobacillus brevis	AcMr06	-ve
Levilactobacillus brevis	AcCh35	-ve
Levilactobacillus brevis	AcCh91	-ve
Levilactobacillus brevis	AcCk67	-ve
Lactococcus lactis subsp. *cremoris*	AcCh67	-ve
Lactococcus lactis subsp. *cremoris*	AyCk21	-ve
Lactococcus lactis subsp. *cremoris*	AyCk71	-ve
Lactococcus lactis subsp. *lactis*	AcCk75	-ve
Lactococcus lactis subsp. *lactis*	AcMr75	-ve
Leuconostoc mesenteroides subsp. *mesenteroides*	AyMr31	-ve
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh37	-ve
Leuconostoc mesenteroides subsp. *mesenteroides*	AyCh45	-ve
Lactiplantibacillus plantarum subsp. *plantarum*	MCC2974	+ve
Levilactobacillus brevis	MCC2198T	+ve

Note: "+ve" denotes positive production and "-ve" denotes negative observation.

Cholesterol reduction

A range of 20.4 to 62.19% was observed among the LAB strains for their percentage ability to reduce cholesterol. We observed strain *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17 as the highest with 62.19±0.008%, followed by *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (61.19±0.005%), *Lactococcus lactis* subsp. *cremoris* AyCk21 (60.2±0.004%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 (59.2±0.003%), *Enterococcus durans* AcCk25 (57.21±0.004%), *Enterococcus durans* AyCk84 (54.23±0.006%), *Levilactobacillus brevis* AcCh91 (52.74±0.01%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (46.27±0.01%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37 (44.78±0.006%), *Lactococcus lactis* subsp. *lactis* AcCk75 (36.82±0.01%), *Lactococcus lactis* subsp. *lactis* AcMr75 (35.32±0.01%), *Lactococcus lactis* subsp. *cremoris* AyCk71 (33.83±0.01%), *Levilactobacillus brevis* AcMr06 (33.83±0.006%), *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 (29.35±0.006%), *Levilactobacillus brevis* AcCk67 (27.36±0.005%), *Lactococcus lactis* subsp. *cremoris* AcCh67 (25.37±0.016%), *Enterococcus durans* AyMr03 (25.37±0.004%), *Levilactobacillus brevis* AcCh35 (22.89±0.004%), *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11 (22.39±0.012%), and *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 (20.4±0.01%).

Gamma amino butyric acid (GABA) producers

Using thin layer chromatography, GABA was detected using the supernatant of the incubated media with the tested strains run against a GABA standard. Strains *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris*

AcCh67, *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lactococcus lactis* subsp. *cremoris* AyCk71, and *Lactococcus lactis* subsp. *lactis* AcCk75 (Fig. 38).

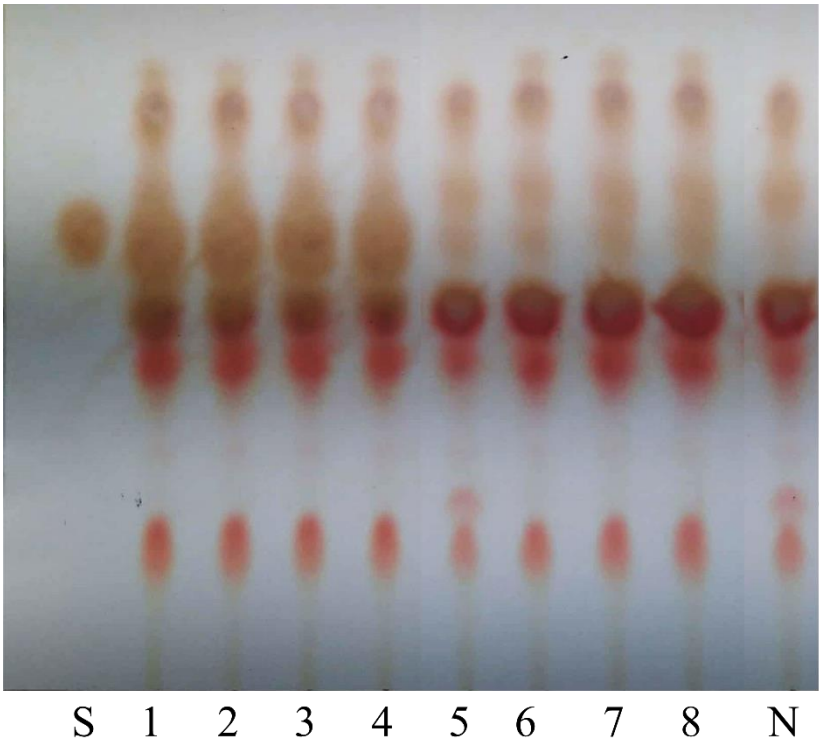


Figure 38: Thin layer chromatography (TLC) showing test positive for gamma(γ)-aminobutyric acid (GABA) production by LAB strains- (1) *Levilactobacillus brevis* AcMr06, (2) *Levilactobacillus brevis* AcCh35, (3) *Levilactobacillus brevis* AcCh91, (4) *Levilactobacillus brevis* AcCk67, (5) *Lactococcus lactis* subsp. *cremoris* AcCh67, (6) *Lactococcus lactis* subsp. *cremoris* AyCk21, (7) *Lactococcus lactis* subsp. *cremoris* AyCk71, and (8) *Lactococcus lactis* subsp. *lactis* AcCk75, run against GABA standard (S). N= negative control.

Safety evaluation of the LAB strains

All the LAB strains which passed the primary probiotic testing were also tested for their haemolytic ability using Sheep Blood Agar plate, all the 20 LAB strains showed no haemolysis, explaining the safety of these strains in probiotic applications.

Secondary grouping for selection of the most potential probiotic strains

Similarly, apart from the probiotic features, further grouping (selection) for final representative strains for whole-genome sequencing was carried out using the above four features of interest using heatmap and biplot PCA visualization. Transformation using log (x+1) and denotation by "1" and "0" for presence and absence was performed as previously mentioned. Through heatmap analysis, we observed five clusters (A, B, C, D, and E), where cluster A consisted of 7 strains (*Lactococcus lactis* subsp. *cremoris* AyCk21, *Levilactobacillus brevis* AcCh91, *Levilactobacillus* brevis AcMr06, *Lactococcus lactis* subsp. *cremoris* AyCk71, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris* AcCh67, and *Levilactobacillus brevis* AcCh35) with the highest mean activity, followed by cluster B that consists of *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Lactococcus lactis* subsp. *lactis* AcMr75, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37. Cluster C comprises of *Enterococcus durans* AyMr03, *Enterococcus durans* AyCk84, *Enterococcus durans* AcCk25, and *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17. Additionally, cluster D comprises of *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18. Strain with the least good activity form the cluster E that only comprises of *Lactococcus lactis* subsp. *lactis* AcCk75 (Fig. 39). Similarly, through biplot PCA analysis, a scattering plot was observed following a similar pattern of visible clusters, where strains with similar properties clusters together in respect to the experimental tests performed. Though, not all the strains show activity for all the tests, however, only few strains were promising for all the experiments including the probiotic tests (primary grouping). Therefore, one

strain was further selected as the best potential probiotic strain based on the overall tests (primary and secondary) viz., *Levilactobacillus brevis* AcCh91 (Fig. 40).

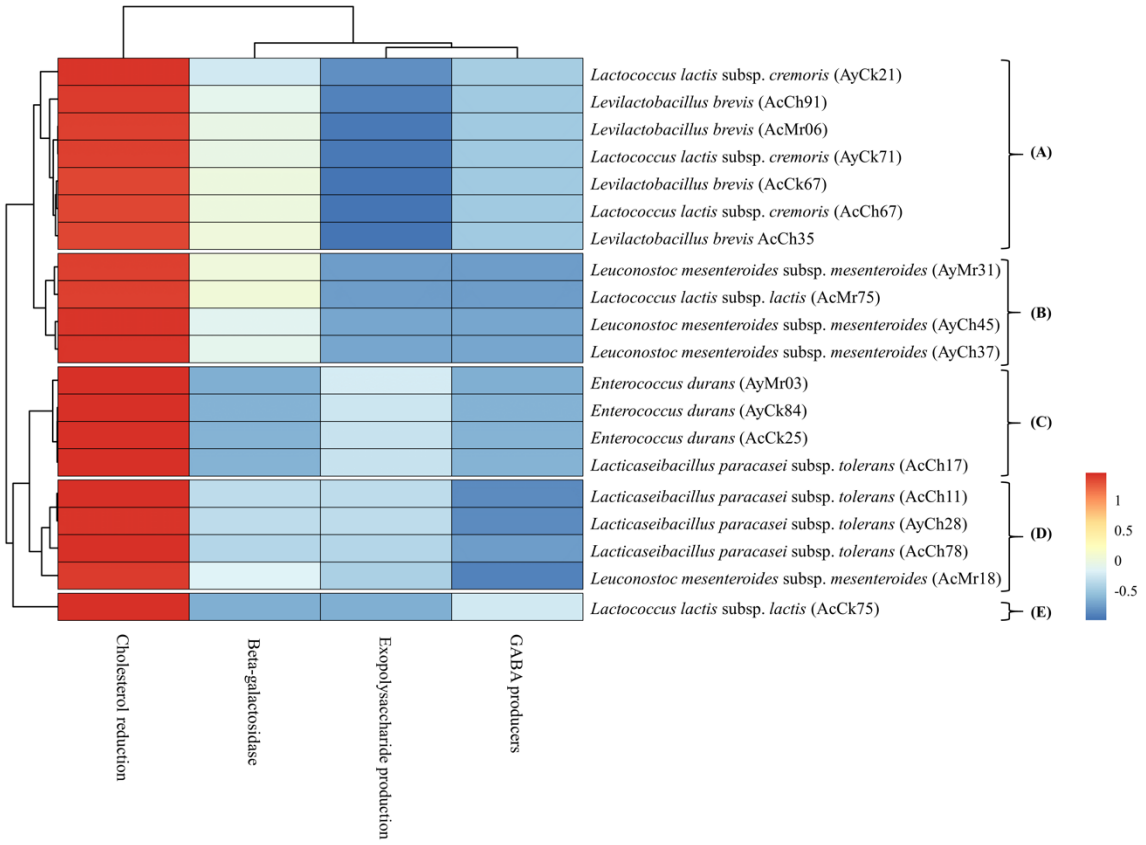


Figure 39: Heatmap visualization of the potential probiotic bacterial strains based on cholesterol reduction, beta-galactosidase, exopolysaccharide production and GABA production. Here, based on the above test of interest, a total of five clusters (A-E), out of which cluster A showed an overall higher mean activity in the above tests.

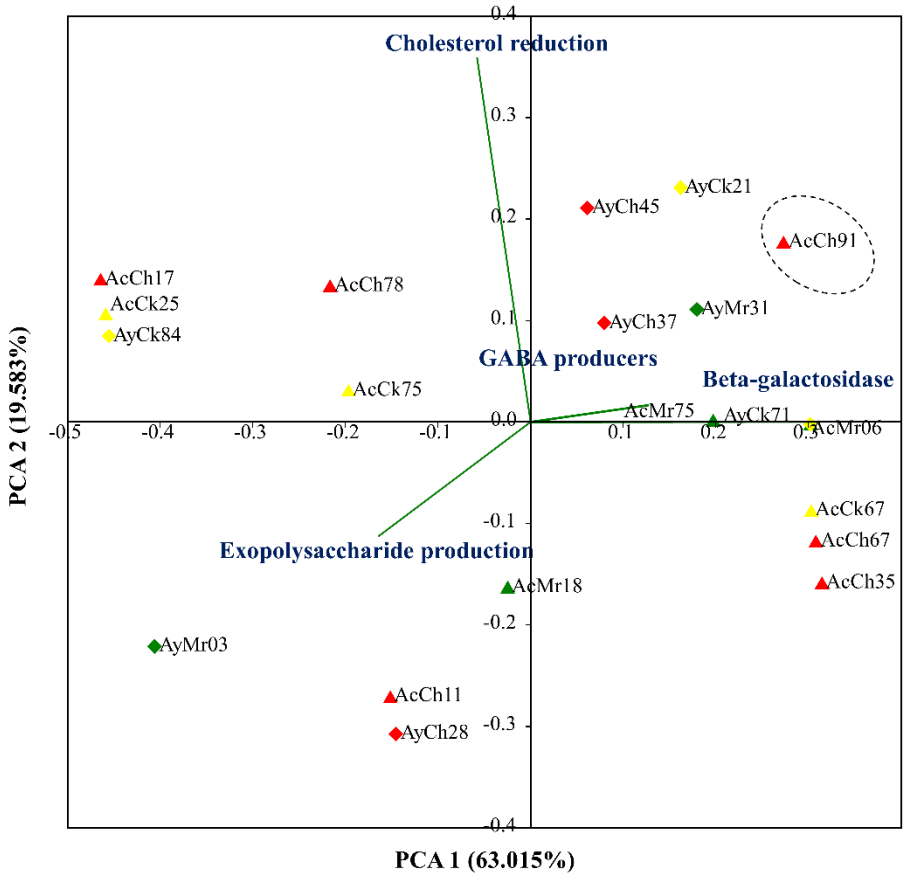


Figure 40: Biplot and principal component analysis (PCA) showing a scattering cluster of the isolated LAB strains. Overall, strain *Levilactobacillus brevis* (AcCh91) is the most promising strain.

Detection of few probiotic and functional genes using specific primers

Bile salt tolerance gene detection

Bacteria have different mechanisms to overcome bile salt tolerance and some of the predictive genes responsible for this phenomenon includes- *Ir0085* (Hypothetical protein), *LBA1679* (ABC transporter), *apf* (Aggregation-promoting factors), *LBA1432* (Hypothetical protein), *LBA0552* (Major facilitator superfamily permease), *LBA1429* (Major facilitator superfamily permease), *LBA1446* (Multidrug resistance protein), *Ir1584* (Major facilitator superfamily permease), and *Ir1516* (Putative esterase). No LAB strains were found to be positive for *Ir0085*, *LBA1679*, *LBA1432*, *LBA1429*, and

LBA1446. However, *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. *cremoris* AcCh67, *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lactococcus lactis* subsp. *cremoris* AyCk71, *Lactococcus lactis* subsp. *lactis* AcCk75, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, *Lactococcus lactis* subsp. *lactis* AcMr75, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 were positive for *apf*, Aggregation-promoting factors (Fig. 41)

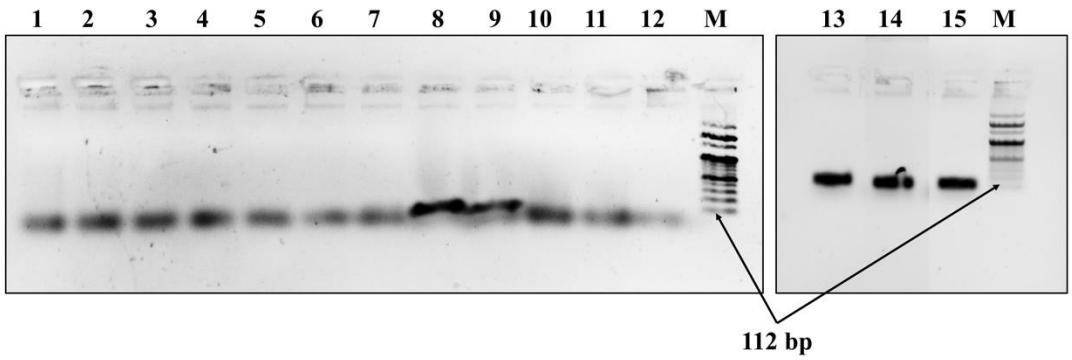


Figure 41: PCR detection of *apf* gene in LAB strains: 1- *Levilactobacillus brevis* AcMr06, 2- *Levilactobacillus brevis* AcCh35, 3- *Levilactobacillus brevis* AcCh91, 4- *Levilactobacillus brevis* AcCk67, 5- *Lactococcus lactis* subsp. *cremoris* AcCh67, 6- *Lactococcus lactis* subsp. *cremoris* AyCk21, 7- *Lactococcus lactis* subsp. *cremoris* AyCk71, 8- *Lactococcus lactis* subsp. *lactis* AcCk75, 9- *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, 10- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, 11- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, 12- *Lactococcus lactis* subsp. *lactis* AcMr75, and 13- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31. Positive controls used are 14- *Levilactobacillus brevis* MCC2198T, and 15- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

On the other hand, *Levilactobacillus brevis* AcCh91, *Levilactobacillus brevis* AcCk67, *Lactococcus lactis* subsp. cremoris AcCh67, *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, and *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 were positive for *LBA0552*, Major facilitator superfamily permease (Fig. 42).

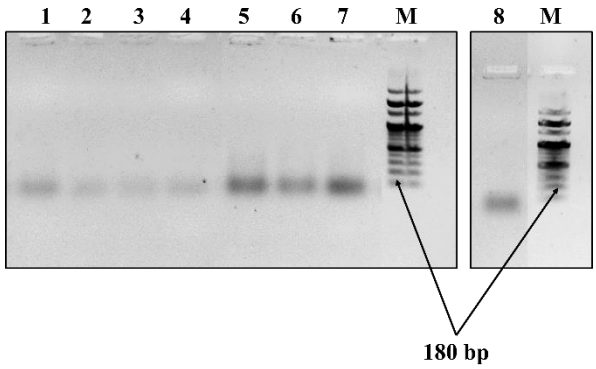


Figure 42: PCR detection of *LBA0552* gene in LAB strains: 1- *Levilactobacillus brevis* AcCh91, 2- *Levilactobacillus brevis* AcCk67, 3- *Lactococcus lactis* subsp. *cremoris* AcCh67, 4- *Lactococcus lactis* subsp. *cremoris* AyCk21, 5- *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, 6- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, 7- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78. Positive control used was 8- *Levilactobacillus brevis* MCC2198T. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Furthermore, amongst all the LAB strains, *Lactococcus lactis* subsp. *lactis* AcCk75 was the only strain to be positive for *LBA1429*- F (Fig. 43).

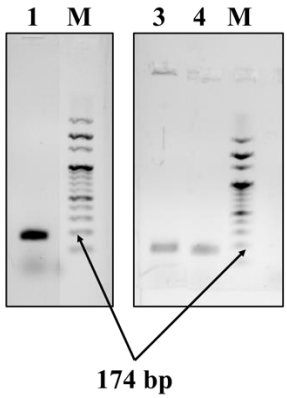


Figure 43: PCR detection of *LBA1429- F* gene was found to be observed in 1- *Lactococcus lactis* subsp. *lactis* AcCk75. Positive control used was 8- *Levilactobacillus*

brevis MCC2198T. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Only three LAB strains were detected for the presence of *Ir1584* gene- *Lactococcus lactis* subsp. *lactis* AcMr75, *Lactococcus lactis* subsp. *lactis* AcCk75 and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (Fig. 44).

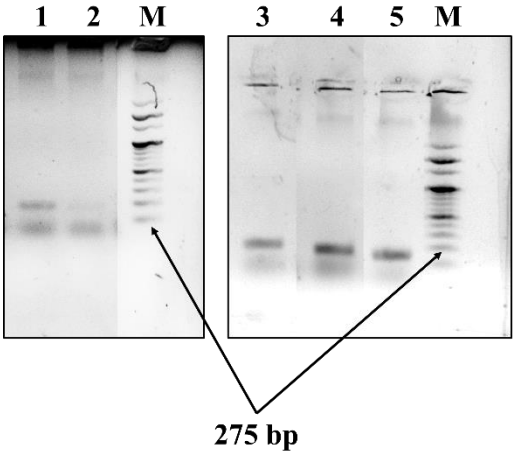


Figure 44: PCR detection of *Ir1584* gene in LAB strains: 1- *Lactococcus* lactis *subsp. lactis* AcMr75, 2- *Lactococcus lactis* subsp. *lactis* AcCk75 and 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31. Positive control used was 4- *Levilactobacillus brevis* MCC2198T and 5- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Similarly, *Lactococcus lactis* subsp. *lactis* AcMr75, *Lactococcus lactis* subsp. *lactis* AcCk75 and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 were also positive for *Ir1516* gene (Fig. 45).

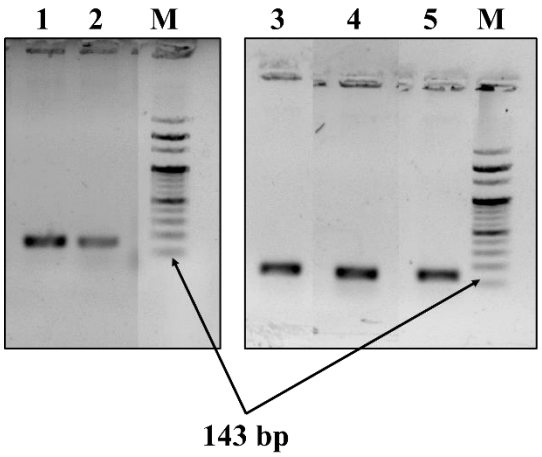


Figure 45: PCR detection of *Ir1516* gene in LAB strains: 1- *Lactococcus lactis* subsp. *lactis* AcMr75, 2- *Lactococcus lactis* subsp. *lactis* AcCk75 and 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31. Positive control used was 4- *Levilactobacillus brevis* MCC2198T and 5- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

pH tolerance gene detection

There are many possible mechanisms as to how LAB overcome pH tolerance. However, no LAB strains in this study were positive for *gtf*, *dltD*, *hdc*, *aguA*, *La995*, *LBA1272*-F1/R1, *odc*, and *tdc*. Three strains *Lactococcus lactis* subsp. lactis AcCk75, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 were found to be positive for *clpL* (Fig. 46).

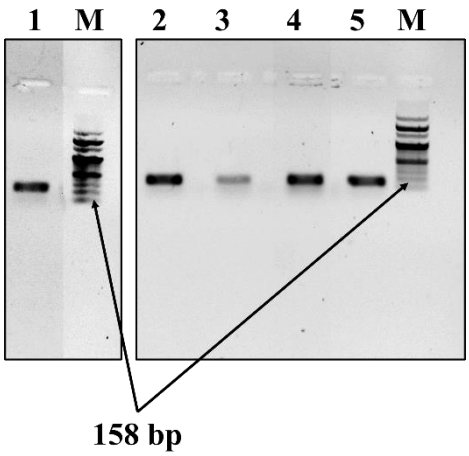


Figure 46: PCR detection of *clpL* gene in LAB strains: 1- *Lactococcus lactis* subsp. *lactis* (AcCk75), 2- *Leuconostoc* mesenteroides subsp. *mesenteroides* (AyMr31), and 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* (AyCh45). Positive control used was 4- *Levilactobacillus brevis* MCC2198T and 5- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Moreover, detection of *LBA1272*- F2/R2 gene was observed only in three strains- *Lactococcus lactis* subsp. lactis AcMr75, *Lactococcus lactis* subsp. *lactis* AcCk75, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 (Fig 47).

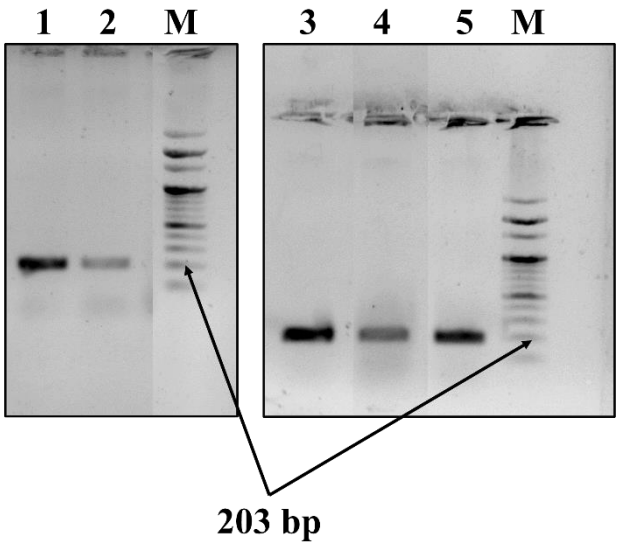


Figure 47: PCR detection of *LBA1272- F2/R2* gene in LAB strains: 1- *Lactococcus lactis* subsp. *lactis* AcMr75, 2- *Lactococcus lactis* subsp. *lactis* AcCk75, and 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31. Positive control used was 4- *Levilactobacillus brevis* MCC2198T and 5- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Interestingly, detection of *groEl* gene was only observed in three strains- *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, and *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78 (Fig. 48).

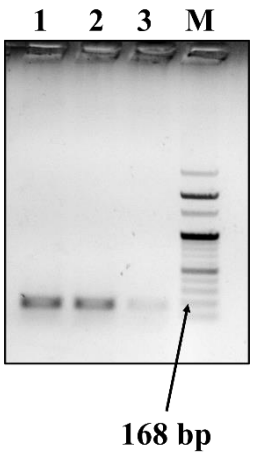


Figure 48: PCR detection of *groEl* gene in LAB strains: 1- *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, 2- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, and 3- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Bile salt hydrolase gene detection

Three different primer pairs were used to detect the bsh gene responsible for bile salt hydrolase. No bsh gene detection was observed for *bsh* F1, bsh F2 but however, *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus* brevis AcCh91, *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 were positive for *bsh* (Fig. 49).

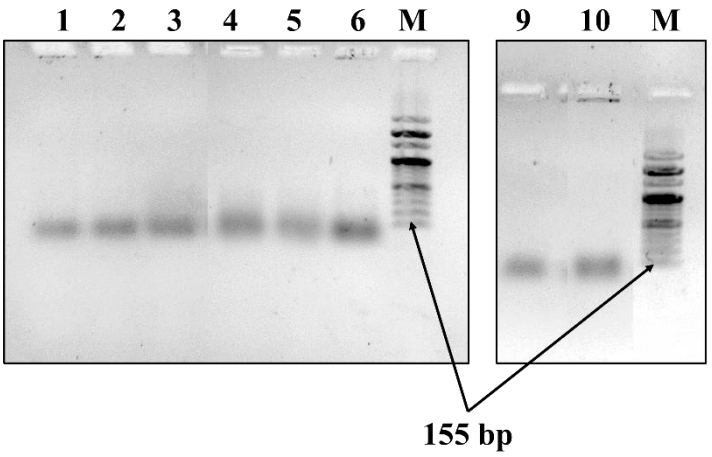


Figure 49: PCR detection of *bsh* gene in LAB strains: 1- *Levilactobacillus brevis* AcMr06, 2- *Levilactobacillus brevis* AcCh35, 3- *Levilactobacillus brevis* AcCh91, 4- *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, 5- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, 6- *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh78, and 7- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45. Positive control used was *Levilactobacillus brevis* MCC2198T. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Attachment/adherence gene detection

All LAB strains were negative *for sbp*, *sor*, *mub*, and *msa*. However, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, *and Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18 were positive for *fbp* gene (Fig. 50).

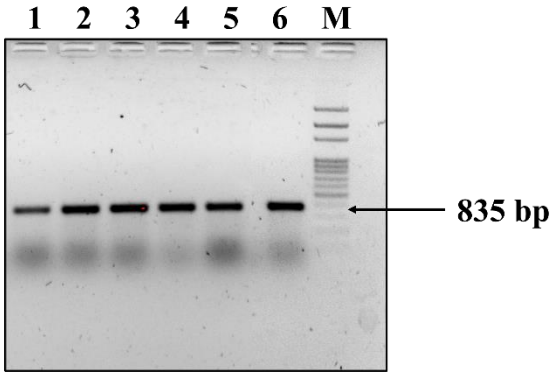


Figure 50: PCR detection of fbp gene in LAB strains: 1- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, 2- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, and 4- *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18. Positive control used was 5- *Limosilactobacillus fermentum* MCC2760, and 6- *Lactiplantibacillus plantarum* subsp. *plantarum* MCC2974. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Bacteriocin gene detection

Detection for leucocin A (*lcnA*), leucocin B (*lcnB*), leucocin K (*lcnK*), leucocin B (*mesB*), leucocin Y (*mesY*), lactococcin A, nisin (*nisR*), lacticin (*Lac 481*), and durancin (*durA*) was carried out in this present work. However, positive *lcnB* gene detection was only observed for *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, and *Leuconostoc mesenteroides* subsp. *mesenteroides* MTCC867 (Fig. 51).

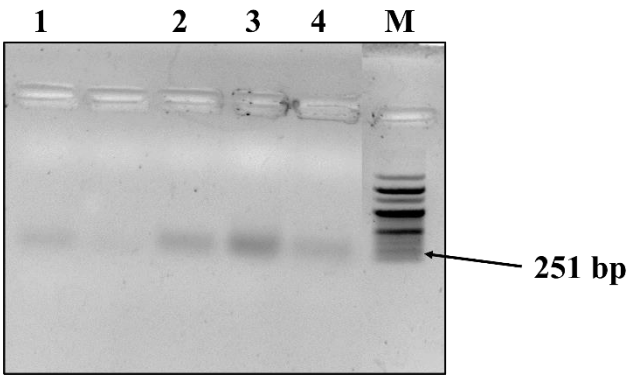


Figure 51: PCR detection of *lcnB* gene in LAB strains: 1- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, 2- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, and 3- *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18. Positive control used was 4- *Leuconostoc mesenteroides* subsp. *mesenteroides* MTCC867. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

GABA-production (*gad* gene) detection

Nine primer pairs were used to detect glutamate decarboxylase *viz*., *gadA4*, *gadA7*, *gadA8*, *gadB4*, *gadB6*, *gadB7*, *gadR2*, *gadR3*, and *gadR4*. Four strains were only positive for *gadR4 viz*., *Levilactobacillus brevis* AcMr06, *Levilactobacillus brevis* AcCh35, *Levilactobacillus brevis* AcCh91, and *Levilactobacillus brevis* AcCk67 (Fig. 52).

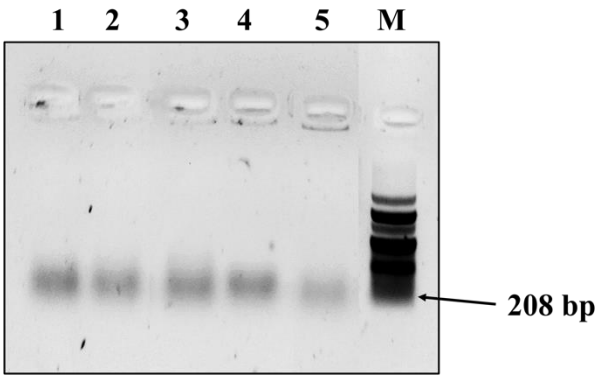


Figure 52: PCR detection of *gadR4* gene in LAB strains: 1- *Levilactobacillus brevis* AcMr06, 2- *Levilactobacillus brevis* AcCh35, 3- *Levilactobacillus brevis* AcCh91, 4- *Levilactobacillus brevis* AcCk67. Positive control used was 5- *Levilactobacillus brevis* MCC2198T. All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Vitamin synthesis gene detection

Two genes for vitamin synthesis were used for gene detection- *folP* (folate synthesis) and *ribA* (Riboflavin synthesis). All the LAB strains were negative for *folP*. Only *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh37, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45 were positive for *ribA* gene (Fig. 53).

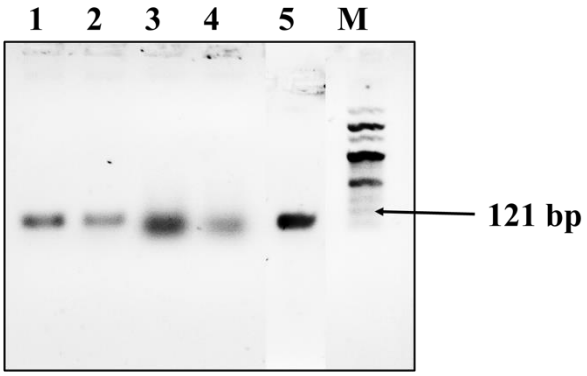


Figure 53: PCR detection of ribA gene in LAB strains: 1- *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, 2- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, 3- *Leuconostoc mesenteroides* subsp. mesenteroides AyCh37, and 4- *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45. Positive control was 5- *Lactiplantibacillus plantarum* subsp. *plantarum* (MCC2974). All product size is confirmed using 100 bp DNA Ladder (MBT049, HiMedia, India), run in 0.8% agarose gel electrophoresis and visualized using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

The overall genetic detection for both probiotic and functional attributes using specific PCR pairs are listed below in Table 26.

Table 26: Detected probiotic and functional marker genes in the LAB strains isoalted from NFM of Arunachal Pradesh.

#Identity	bile salt tolerance	pH tolerance	bile salt hydrolase	Attachment /Adherence	GABA	Vitamin production	Bacteriocin
Levilactobacillus brevis AcMr06	*apf*	-	*bsh*	-	*gadR*	-	-
Levilactobacillus brevis AcCh35	*apf*	-	*bsh*	-	*gadR*	-	-
Levilactobacillus brevis AcCh91	*apf, LBA552*	-	*bsh*	-	*gadR*	-	-
Levilactobacillus brevis AcCk67	*apf, LBA552*	-	-	-	*gadR*	-	-
Lactococcus lactis subsp. *cremoris* AcCh67	*apf, LBA552*	-	-	-	-	-	-
Lactococcus lactis subsp. *cremoris* AyCk21	apf, *LBA552*	-	-	-	-	-	-
Lactococcus lactis subsp. *cremoris* AyCk71	*apf*	-	-	-	-	-	-
Lactococcus lactis subsp. *lactis* AcCk75	*apf, LBA1429, Ir1584, Ir1516*	*clpL, LBA1272*	-	-	-	-	-
Lacticaseibacillus paracasei subsp. *tolerans* AyCh28	*apf, LBA552*	*groEl*	*bsh*	-	-	-	-
Lacticaseibacillus paracasei subsp. *tolerans* AcCh11	*apf, LBA552*	*groEl*	*bsh*	-	-	-	-
Lacticaseibacillus paracasei subsp. *tolerans* AcCh78	*apf, LBA552*	*groEl*	*bsh*	-	-	-	-
Lactococcus lactis subsp. *lactis* AcMr75	*apf, Ir1584, Ir1516*	*LBA1272*	-	-	-	-	-
Leuconostoc mesenteroides subsp. *mesenteroides* AyMr31	*apf, Ir1584*	*clpL, LBA1272*	-	*fbp*	-	*ribA*	*lcnB*
Leuconostoc mesenteroides subsp. *mesenteroides* AyCh37	-	-	-	*fbp*	-	*ribA*	-
Enterococcus durans AcCk25	-	-	-	-	-	-	-
Enterococcus durans AyCk84	-	-	-	-	-	-	-
Enterococcus durans AyMr03	-	-	-	-	-	-	-

Table 26: Detected probiotic and functional marker genes in the LAB strains isoalted from NFM of Arunachal Pradesh (contd.)

#Identity	bile salt tolerance	pH tolerance	bile salt hydrolase	Attachment /Adherence	GABA	Vitamin production	Bacteriocin
Leuconostoc mesenteroides subsp. *mesenteroides* AyCh45	-	*clpL*	*bsh*	*fbp*	-	*ribA*	*lcnB*
Lacticaseibacillus paracasei subsp. *tolerans* AcCh17	-	-	-	-	-	-	-
Leuconostoc mesenteroides subsp. *mesenteroides* AcMr18	-	-	-	*fbp*	-	*ribA*	*lcnB*
Levilactobacillus brevis MCC2198T	*apf, LBA552, LBA1429, Ir1584, Ir1516*	*clpL, LBA1272*	*bsh*	*fbp*	*gadR*	*ribA*	-
Limosilactobacillus fermentum MCC2760	-	-	-	*fbp*	-	-	-
Leuconostoc mesenteroides subsp. *mesenteroides* MTCC867	-	-	-	-	-	-	*lcnB*
Lactiplantibacillus plantarum subsp. *plantarum* MCC2974	*apf, LBA1429, Ir1584, Ir1516*	*clpL, LBA1272*	-	*fbp*	-	*ribA*	-

Microbial predictive gene functionality by PICRUSt2 and Piphillin tools

A total of 794 ASVs (amplicon sequence variants) were obtained using DADA2 denoising program and 214 clustered sequences using SILVA clustering method were used for the predictive functional analysis study. A total of 6520 and 5201 KO features resulted from PICRUSt2 and Piphillin predictive analyses respectively. All KO features from both predictive functionality analysis pipelines were then normalized using the MUSiCC algorithm and mapped to 178 and 157 KEGG pathways from PICRUSt2 and Piphillin analyses respectively. All the functional features from both the analyses were then categorized into their standard levels- category (level 1), super pathways (level 2) and pathways (level 3).

Predictive functional features

(a) PICRUSt2 analysis

At the category level (level 1), metabolism (86.05%) was the most abundant functional feature followed by environmental information processing (4.51%), human diseases (4.28%), cellular processes (2.77%), genetic information processing (1.55%), and organismal systems (0.84%) (Fig. 54). At the super pathway (level 2), amino acid metabolism (21.17%) was the abundant function, followed by metabolism of cofactors and vitamins (20.86%), carbohydrate metabolism (15.9%), energy metabolism (10.01%), nucleotide metabolism (8.34%), xenobiotics biodegradation and metabolism (6.04%), biosynthesis of other secondary metabolites (5.35%), metabolism of other amino acids (4.84%), lipid metabolism (4.5%), metabolism of terpenoids and polyketides (2.49%), and those functional features <1% were grouped as others (0.5%) (Fig. 55). The most abundant metabolic pathways (level 3) through PICRUSt2 analysis were observed to be purine metabolism (4.49%), followed pyrimidine metabolism

(3.84%), nicotinate and nicotinamide metabolism (3.83%), amino sugar and nucleotide sugar metabolism (3.68%), oxidative phosphorylation (3.48%), pantothenate and CoA biosynthesis (3.37%), porphyrin and chlorophyll metabolism (2.98%), glycine, serine and threonine metabolism (2.93%), glycerophospholipid metabolism (2.62%), phenylalanine, tyrosine and tryptophan biosynthesis (2.58%), cysteine and methionine metabolism (2.5%), histidine metabolism (2.49%), folate biosynthesis (2.46%), starch and sucrose metabolism (2.34%), arginine and proline metabolism (2.29%), ubiquinone and other terpenoid-quinone biosynthesis (2.08%), methane metabolism (1.83%), pentose phosphate pathway (1.73%), pyruvate metabolism (1.62%), monobactam biosynthesis (1.62%), carbon fixation pathways in prokaryotes (1.57%), alanine, aspartate and glutamate metabolism (1.51%), one carbon pool by folate (1.45%), glyoxylate and dicarboxylate metabolism (1.4%), thiamine metabolism (1.38%), nitrogen metabolism (1.37%), phenylalanine metabolism (1.34%), terpenoid backbone biosynthesis (1.33%), arginine biosynthesis (1.31%), pentose and glucuronate interconversions (1.27%), glutathione metabolism (1.24%), valine, leucine and isoleucine biosynthesis (1.16%), vitamin B6 metabolism (1.13%), selenocompound metabolism (1.1%), fructose and mannose metabolism (1.08%), phosphonate and phosphinate metabolism (1.05%), benzoate degradation (1.03%), sulfur metabolism (1%), and those <1% were grouped as others (22.52%) (Fig. 56).

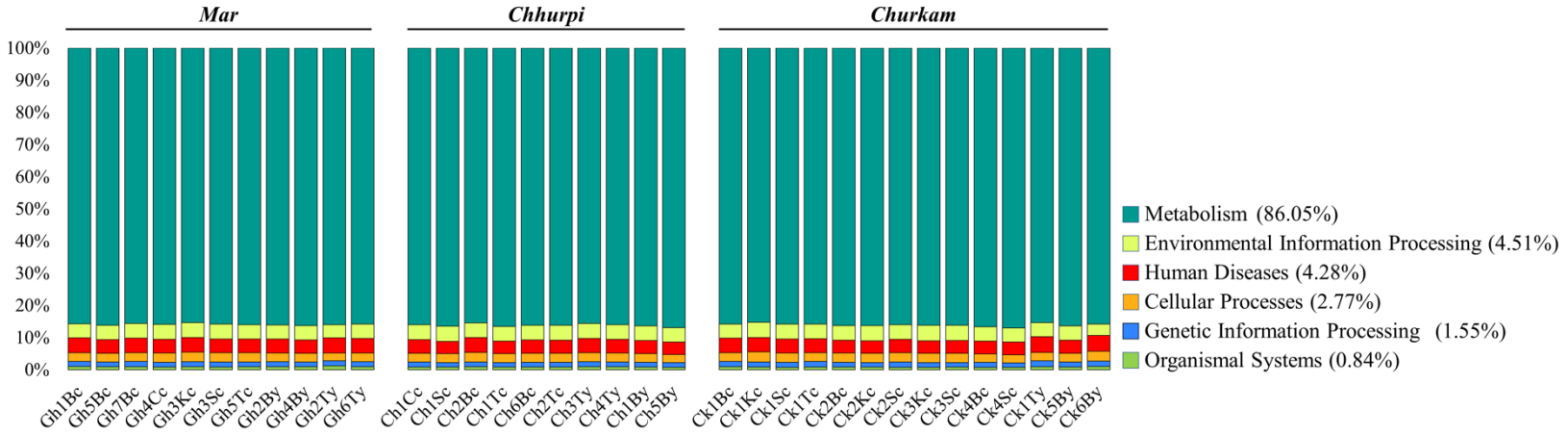


Figure 54: Category level (level 1) categorization of the functional features resulted from the PICRUSt2 predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

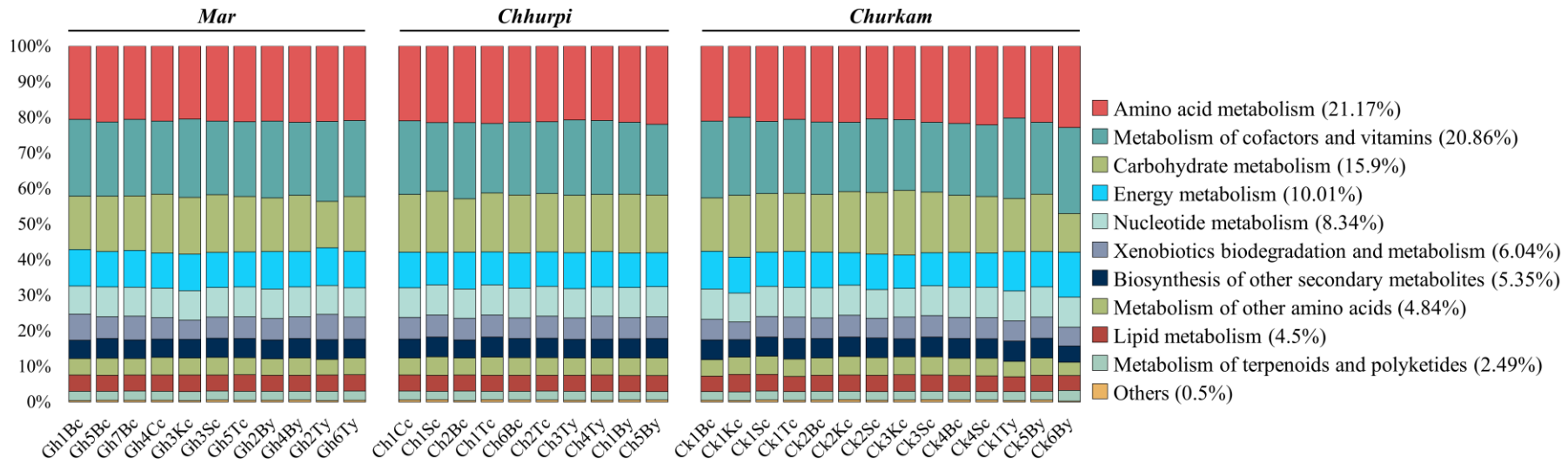


Figure 55: Super pathways (level 2) categorization of the functional features resulted from the PICRUSt2 predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

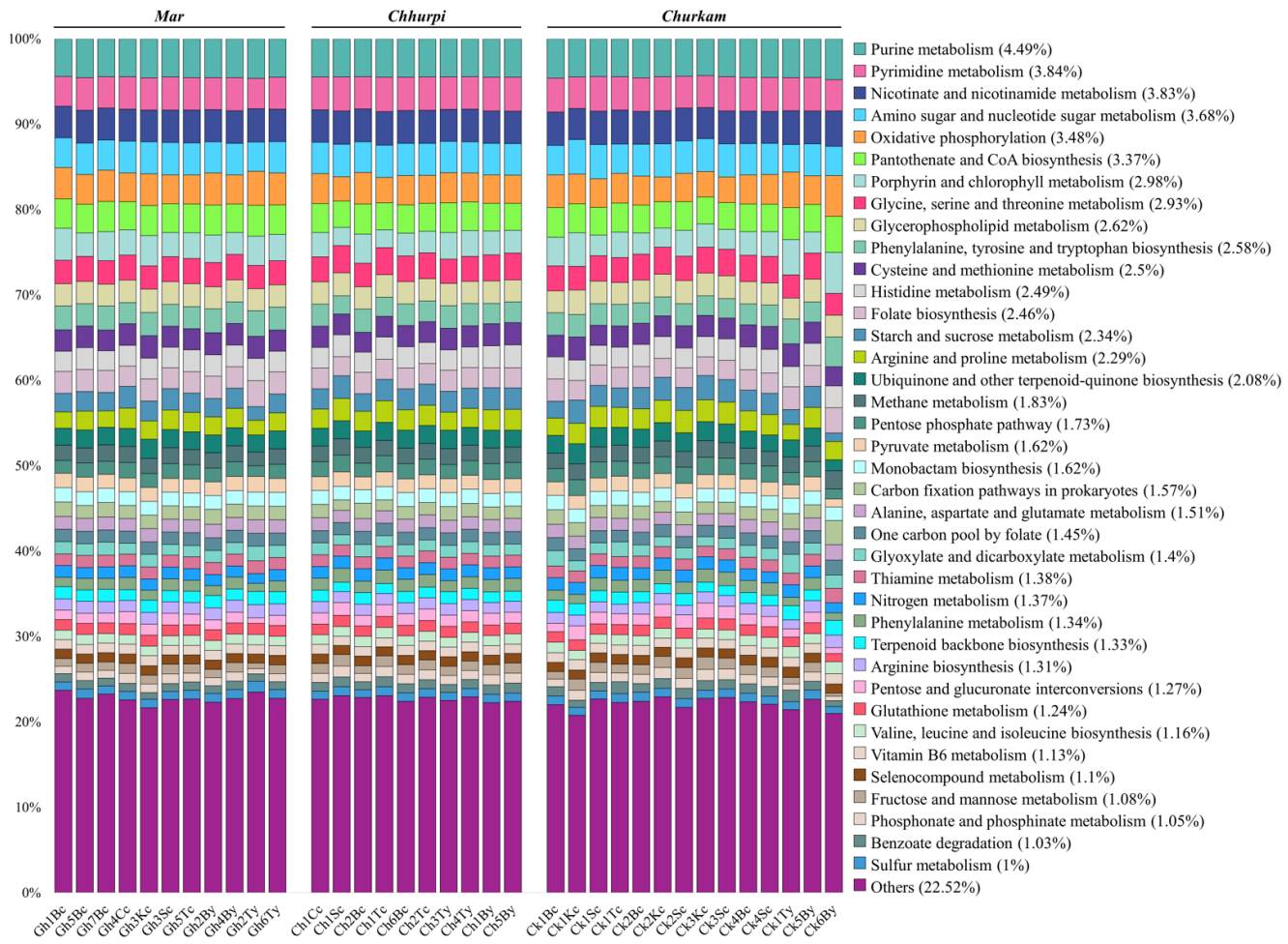


Figure 56: Metabolic pathways (level 3) categorization of the functional features resulted from the PICRUSt2 predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

(b) Piphillin analysis

On the other hand, a similar result was observed at the category level (level 1) with metabolism (84.53%) as the abundant category, followed by environmental information processing (5.88%), human diseases (5.05%), cellular processes (2.38%), organismal systems (1.16%), and genetic information processing (1%) (Fig. 57). Similarly, at the super pathway (level 2), amino acid metabolism (17.92%) was the abundant function, followed by metabolism of cofactors and vitamins (17.77%), carbohydrate metabolism (16.42%), nucleotide metabolism (13.34%), lipid metabolism (7.01%), metabolism of other amino acids (6.41%), energy metabolism (5.86%), xenobiotics biodegradation and metabolism (5.68%), biosynthesis of other secondary metabolites (4.55%), metabolism of terpenoids and polyketides (3.69%), glycan biosynthesis and metabolism (1.34%), and those <1% were grouped as others (0.01%) (Fig. 58). Interestingly, similar pathways were also observed through piphillin analysis which resulted in purine metabolism (7.67%) as the abundant function, followed by pyrimidine metabolism (5.67%), nicotinate and nicotinamide metabolism (4.83%), cysteine and methionine metabolism (4.67%), amino sugar and nucleotide sugar metabolism (4.66%), starch and sucrose metabolism (4.53%), glycerophospholipid metabolism (3.6%), pantothenate and CoA biosynthesis (3.28%), alanine, aspartate and glutamate metabolism (2.91%), taurine and hypotaurine metabolism (2.26%), monobactam biosynthesis (2.24%), terpenoid backbone biosynthesis (2.17%), glycine, serine and threonine metabolism (2.1%), one carbon pool by folate (2.07%), pentose phosphate pathway (1.95%), oxidative phosphorylation (1.91%), fatty acid biosynthesis (1.81%), pyruvate metabolism (1.78%), lysine biosynthesis (1.7%), drug metabolism - other enzymes (1.62%), carbon fixation in photosynthetic organisms (1.54%), selenocompound metabolism (1.53%), phenylalanine, tyrosine and tryptophan biosynthesis (1.52%), folate biosynthesis

(1.49%), glycerolipid metabolism (1.47%), glyoxylate and dicarboxylate metabolism (1.41%), riboflavin metabolism (1.37%), histidine metabolism (1.31%), porphyrin and chlorophyll metabolism (1.25%), glutathione metabolism (1.24%), thiamine metabolism (1.15%), non-ribosomal peptide structures (1.11%), methane metabolism (1.1%), aminobenzoate degradation (1.05%), arginine and proline metabolism (1%), and those <1% were grouped as others (17.02%) (Fig. 59).

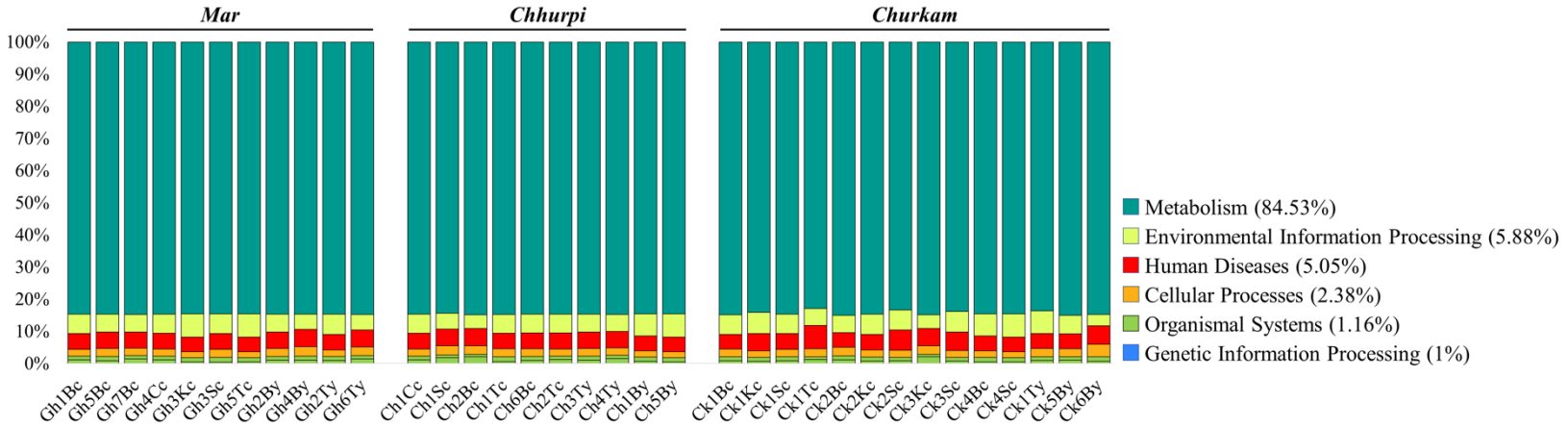


Figure 57: Category level (level 1) categorization of the functional features resulted from the Piphillin predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

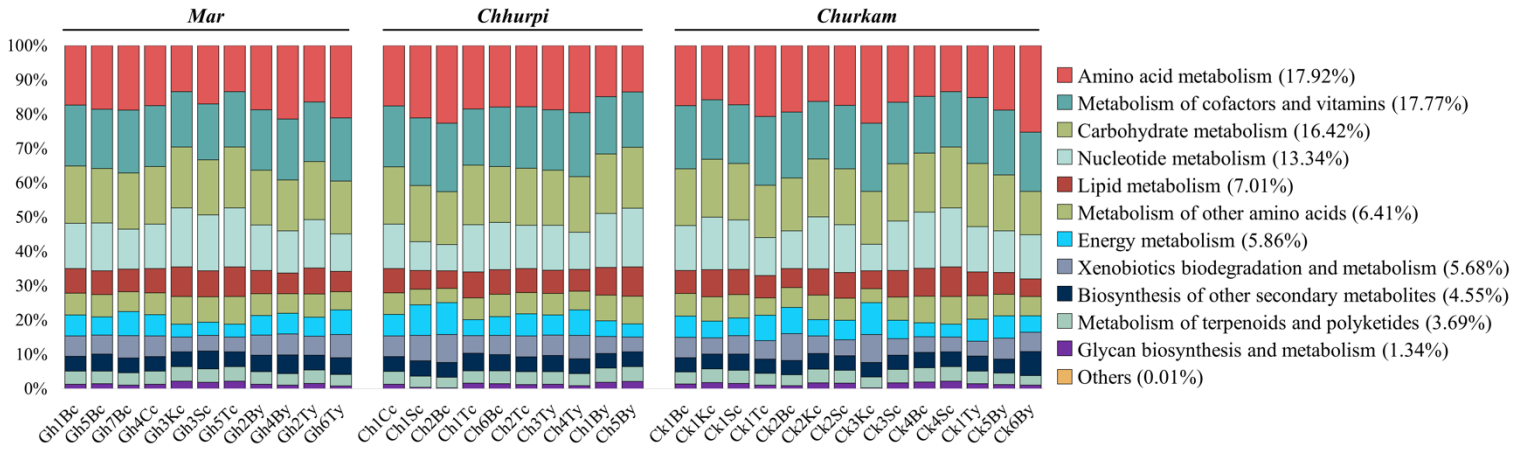


Figure 58: Super pathways (level 2) categorization of the functional features resulted from the Piphillin predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

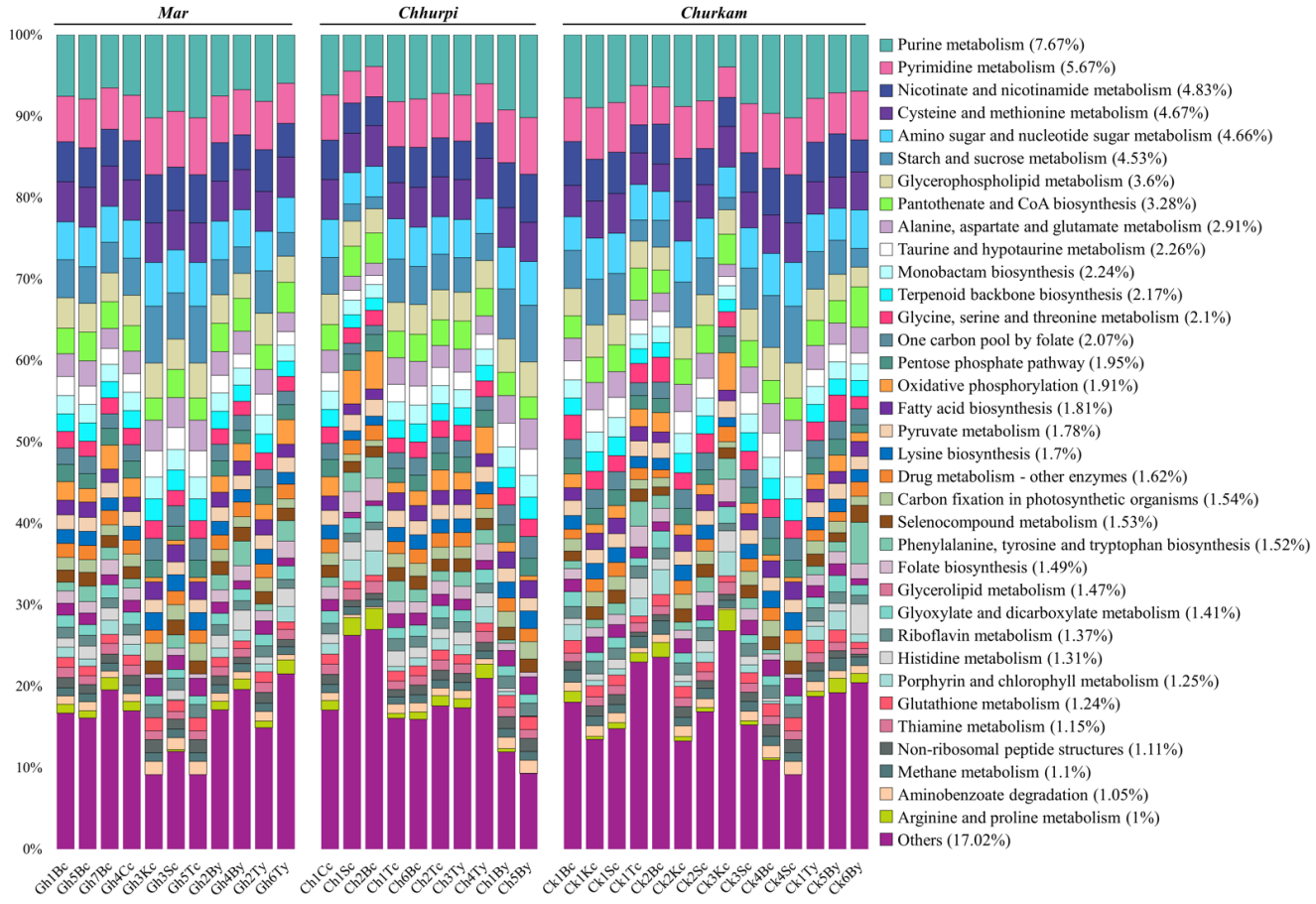


Figure 59: Metabolic pathways (level 3) categorization of the functional features resulted from the Piphillin predictive analysis using ASV error corrected sequences of NFM products of Arunachal Pradesh– *mar*, *chhurpi*, and *churkam*.

Comparison of KEGG functional features as predicted by PICRUSt2 and Piphillin

Prediction analysis showed variation from both the pipelines used in this study, and there were significant differences in the KEGG functional attributes predicted. Using STAMP, extendend error bar plot was plotted and significant differences were calculated using White's non-parametric t-test with Benjamini–Hochberg fasle discovery rate (FDR) error correction. At the categorical comparison (level 1), metabolism, genetic information processing and cellular processes were significantly higher through PICRSUt2 prediction and organismal systems, environmental information processing and human diseases were significantlsy higher through Piphillin prediction (Fig. 60). Similarly, in the super pathway (level 2), significant prediction from the two studied pipelines were observed. Amino acid metabolism, metabolism of cofactors and vitamins, energy metabolism and biosynthesis of other seconday metabolites were significantly higher through PICRUSt2 prediction, whereas, metabolism of other amino acids, lipid metabolism, metabolism of terpenoids and polyketides, nucleotide metabolism and carbohydrate metabolism were significantly higher through Piphillin prediction (Fig. 61). At the pathway level (level 3), phenylalanine, tyrosine and tryptophan biosynthesis, histidine metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism, porphyrin and chlorophyll metabolism, pantothenate and CoA biosynthesis, thiamine metabolism, oxidative phosphorylation, methane metabolism, folate biosynthesis, and glutathione metabolism were significantly higher through PICRUSt2 prediction method, whereas cysteine and methionine metabolism, one carbon pool by folate, terpenoid backbone biosynthesis, glycerophospholipid metabolism, starch and sucrose metabolism, nicotinate and nicotinamide metabolism, amino sugar and nucleotide sugar metabolism, pyrimidine metabolism, selenocompound metabolism, alanine, aspartate and glutamate

metabolism, monobactam biosynthesis, and purine metabolism were significantly higher through Piphillin prediction method (Fig. 62).

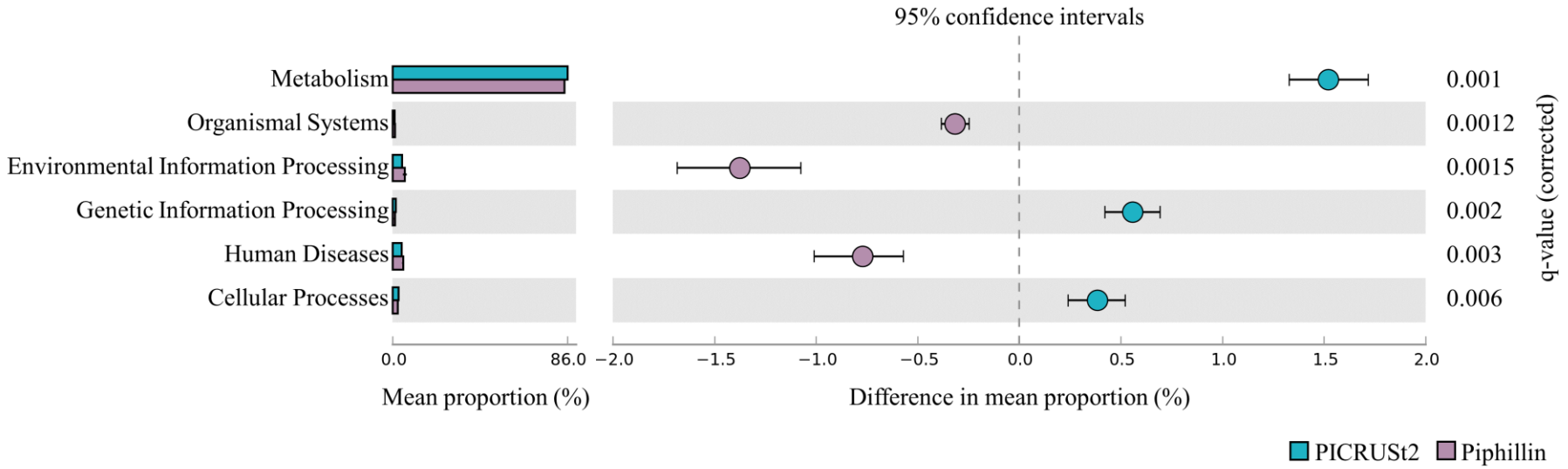


Figure 60: A categorical (level 1) comparison of KEGG functional features as predicted by PICRUSt2 and Piphillin, represented by an extended error bar chart. Total mean proportion of each prediction method is represented by a bar chart (left column) and the coloured circles (designated coded colour as shown in the figure) represents the 95% confidence intervals as calculated by White's non-parametric t-test with Benjamini–Hochberg fasle discovery rate (FDR) error correction method. Significant values (error-corrected *q*-values) are shown in the right side of the plot.

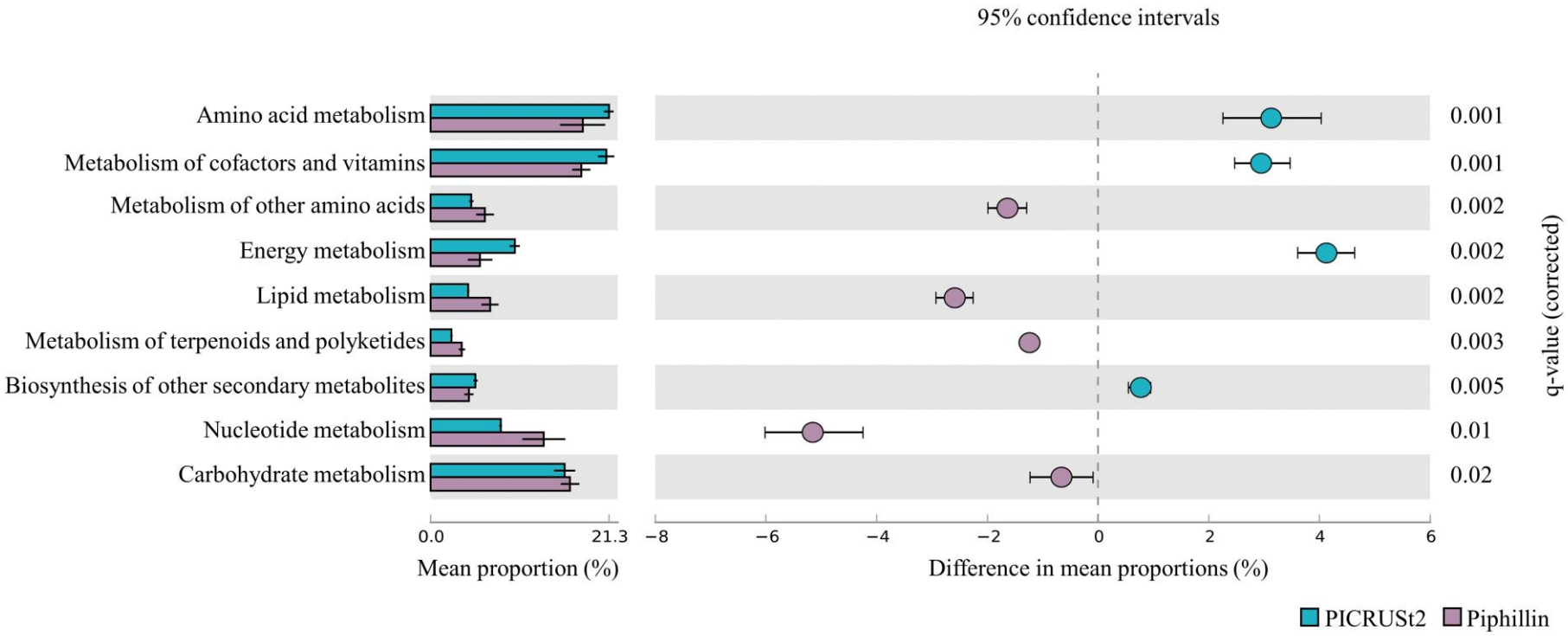


Figure 61: A super-pathway (level 2) comparison of KEGG functional features as predicted by PICRUSt2 and Piphillin, represented by an extended error bar chart. Total mean proportion of each prediction method is represented by a bar chart (left column) and the coloured circles (designated coded colour as shown in the figure) represents the 95% confidence intervals as calculated by White's non-parametric t-test with Benjamini–Hochberg fasle discovery rate (FDR) error correction method. Significant values (error-corrected *q*-values) are shown in the right side of the plot.

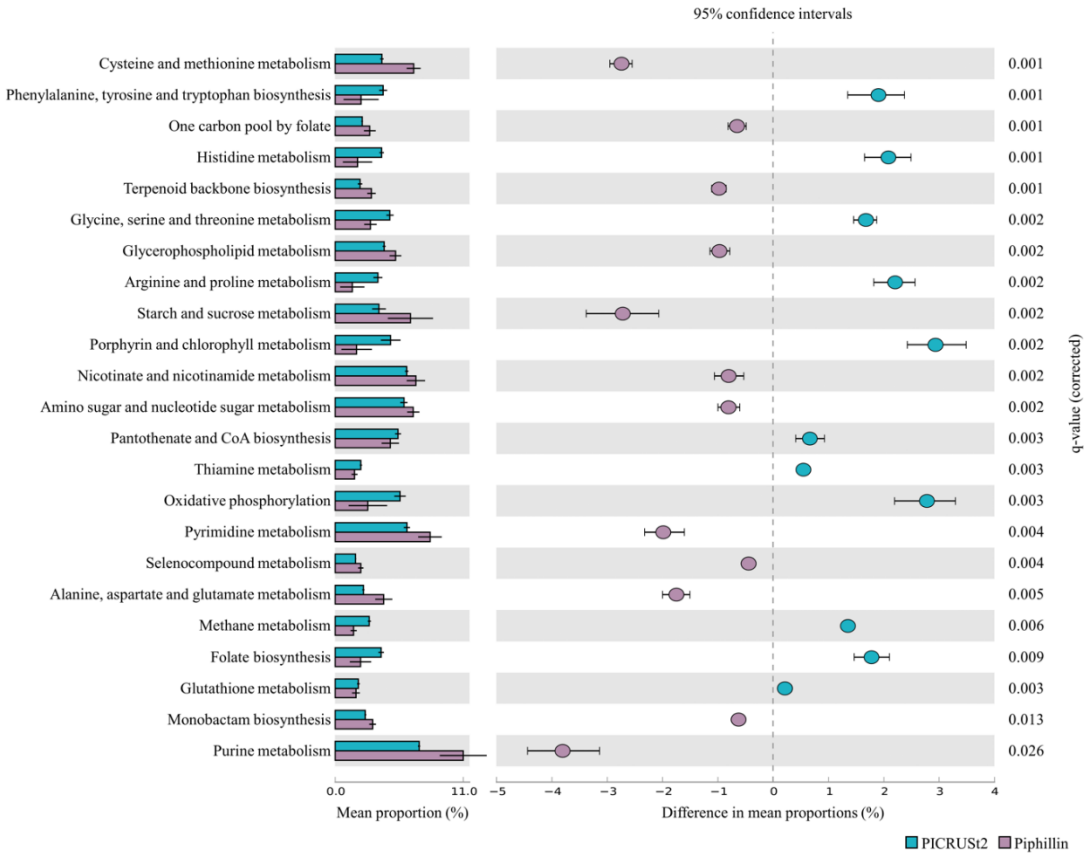


Figure 62: A pathway (level 3) comparison of KEGG functional features as predicted by PICRUSt2 and Piphillin, represented by an extended error bar chart. Total mean proportion of each prediction method is represented by a bar chart (left column) and the coloured circles (designated coded colour as shown in the figure) represents the 95% confidence intervals as calculated by White's non-parametric t-test with Benjamini–Hochberg fasle discovery rate (FDR) error correction method. Significant values (error-corrected *q*-values) are shown in the right side of the plot.

Non -parametric correlation (Spearman's)

Using SPSS, a correlation matrix between the abundant genera and functions was calculated by a non-parametric Spearman's correlation method. A complex interaction was observed between the abundant genera and the respective predictive functional features where either a positive or a negative correlation was noted. Significant positive correlation was observed between *Lactococcus* and *Leuconostoc* with phenylalanine metabolism whereas genera like *Lactobacillus*, *Acetobacter*, *Gluconobacter*, and Staphylococcus were observed to be negatively significant to the said function. *Acetobacter* was also observed to be positively and significantly correlated with glycine, serine, and threonine metabolism. *Bacillus* was also observed to be significantly and positively correlated to cysteine and methionine metabolism. Monobactam biosynthesis was shown to be negatively and significantly correlated to *Leuconostoc* but however showed significant positive correlation with *Gluconobacter*. There was also a positive significant correlation of pentose phosphate pathway with four abundant genera- *Lactococcus*, *Leuconostoc*, *Bacillus* and *Pseudomonas*. Pyruvate metabolism showed significant negative correlation to *Lactobacillus*. Pentose and glucuronate interconversions showed positive significant correlation with *Leuconostoc*, but however, showed negative significant correlation with *Lactobacillus*, *Acetobacter* and *Gluconobacter*. *Acetobacter*, *Gluconobacter* and *Staphylococcus* were observed to show positive significant correlation with carbon fixation pathways in prokaryotes, but negative significant correlation was observed with *Lactococcus*. *Bacillus* showed negative significant correlation to once carbon pool by folate. *Lactococcus* showed significant negative correlation with pantothenate and CoA biosynthesis and porphyrin and chlorophyll metabolism. We also observed a significant negative correlation of Staphylococcus with glutathione metabolism. Additionally, positive significant

correlation was observed between Gluconobacter with aminobenzoate degradation, and lastly, between drug metabolism - other enzymes and *Leuconostoc* and *Bacillus* (Fig. 63).

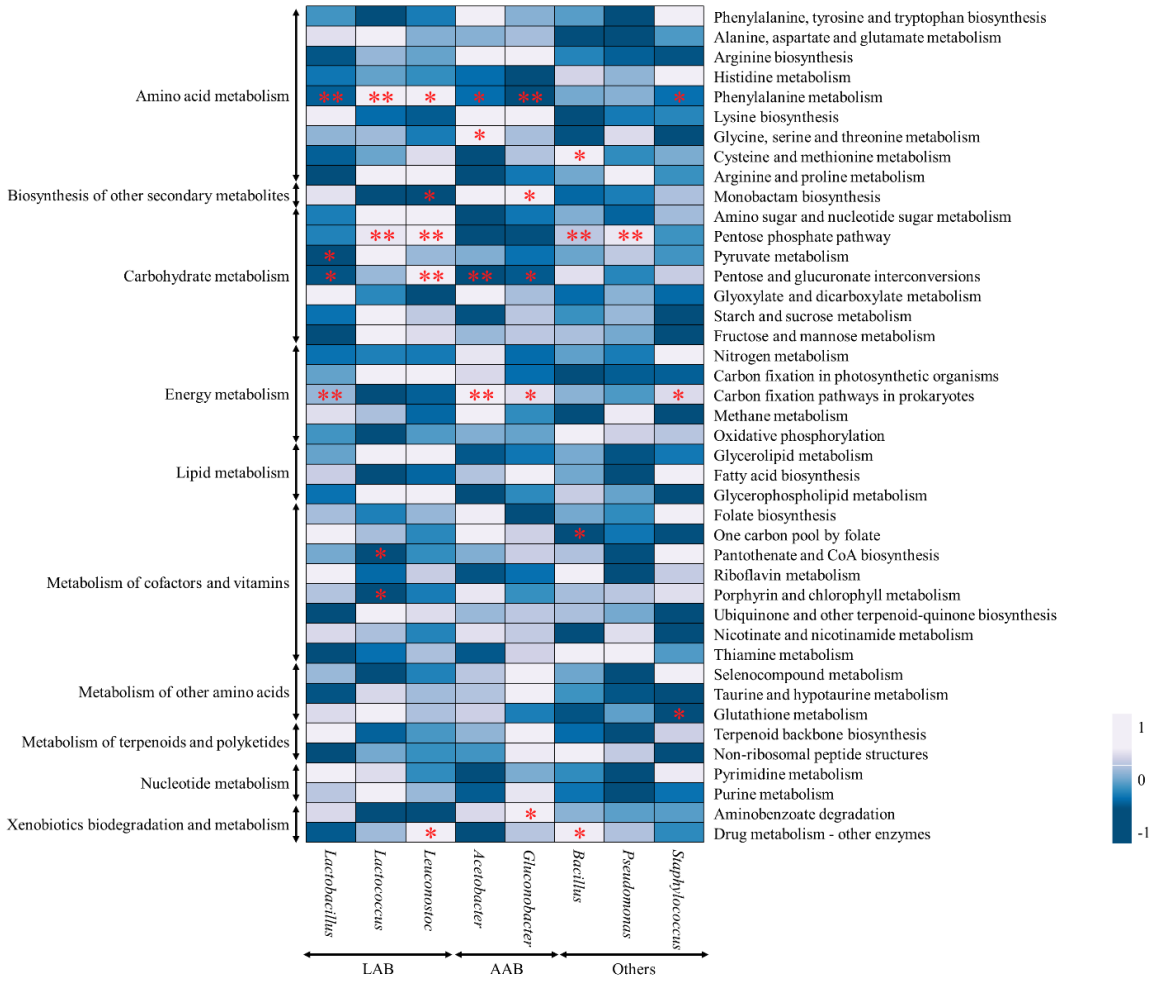


Figure 63: Correlation matrix between the abundant genera (LAB- lactic acid bacteria, AAB- acetic acid bacteria, and others- non-LAB/AAB) and functional features as calculated by a non-parametric Spearman's correlation method, where significant correlation was denoted by *<0.05 and **<0.01.

DISCUSSION

Indigenous knowledge of preparation of the NFM products

Drinking of cow and yak-milk and consumption of naturally fermented milk (NFM) products are the ancient food culture of the ethnic people of Arunachal Pradesh (Tamang et al., 2010, 2021). Traditional methods of preparation of various naturally fermented milk (NFM) products, both cow milk and yak milk, were predominated in two districts of Arunachal Pradesh viz. Tawang and West Kameng. As per ethnicity, preparation of various NFM products viz. *mar*, *chhurpi*, and *churkam* was confined only to *Brokpas* (sub-group of *Monpa*) the ethnic group. NFM products are one of the main dietary items in gastronomy of ethnic people of Arunachal Pradesh which are also sources of income for the local producers (Rai et al., 2016). NFM products, in these regions, are prepared only from both cow's and yak's milk; where cows are usually found in the lower regions while yaks reside in the higher regions closer to the snow-capped cold mountains (Shangpliang and Tamang, 2021). Due to pastoral systems in higher mountainous regions yaks (*Bos grannies*) are very important domesticated animals in Arunachal Pradesh (Wangchuk et al., 2013; Ingty, 2021). The method of traditional preparation of *mar*, *chhurpi*, and *churkam* of Arunachal Pradesh are similar to those of other Himalayan regions, which reflects the common culture, tradition and religions as they shared (Tamang et al., 2021). *Mar* is also produced in Sikkim, Darjeeling hills, Nepal, and Bhutan where it is known with different names- *ghee*/*gheu* (Nepali language) and *maa* (Lepcha dialect) (Dewan and Tamang, 2007). *Chhurpi* is common cottage cheese-like dairy product in Sikkim, Darjeeling hills, Ladakh, Nepal and Bhutan (Dewan and Tamang, 2007; Raj and Sharma, 2015; Rai et al., 2016; Panda et al., 2016) with the same name, however, some locals in Arunachal Pradesh called it *churapi* (Tiwari and Mahanta, 2007). On the other hand, *churkam*/*churkham* like product, called hard-*chhurpi* and *dudh-chhurpi* in produced in Sikkim and Darjeeling hill, Ladakh, Nepal and

Bhutan (Rai et al., 2016, Tamang et al., 2021). Though, the method of preparation of the NFM products seems to be simple but cattle rearing in these high-altitude regions is a difficult task. Most cattle herders stay outside the town; cow herders move to the lower altitude valleys while the yak herders move up towards the cold mountains. Most of the *Brokpas* do stay in the forests and the mountains, and they move from one place to another for 3-5 months in search of a suitable place for cattle grazing. People in this area do also use the NFM products as an exchange for other items or foods as a form of barter system. During the time of documentation and survey study, there was a lot of grievances regarding the production of the NFM products in these regions. With the development and modernization of societies, making of the NFM products and selling them becomes difficult, that even some of the *Brokpas* had turn to other businesses to make up their daily living. The nomad pastoralists do face a lot of challenges in this lifestyle where it is difficult to carry out tradition and livelihood in the future (Singh, 2009; Wangchuk et al., 2013), where not only livestock is decreasing but the market is also narrowed and localized. Till date, very few documental surveys have been reported on naturally fermented milk products of Arunachal radish (Singh et al., 2007; Tomar et al., 2009; Tiwari and Mahanta, 2007; Bora et al., 2014).

Microbiological Analysis

Culture-dependent analysis

Isolation, phenotypic and genotypic characterization of the predominant LAB

The NFM products (*mar*, *chhurpi*, and *churkam*) of Arunachal Pradesh are some of the rare exotic fermented foods in the Eastern Himalayas where their production and recognition are diminishing with time (Shangpliang and Tamang, 2021). Hence, isolation of the culturable LAB from these fermented foods is of significance. Based on

their nature, NFM products were categorized as lipid-rich (*mar*) and casein-based products (*chhurpi* and *churkam*). In general, casein-based products were found to be slightly acidic in nature than the lipid-rich products, regardless of the source of milk that they are prepared from, cow's or yak's milk. Till date, only one study has reported the isolation of LAB from *chhurpi* (Tomar et al., 2009), but not extensively, whereas other products have not yet been explored. The predominance of LAB in NFM products is a widely known fact which have been reported in various NFM products of India and other parts of the world (Dewan and Tamang, 2007; Tamang et al., 2000; Zhong et al., 2016; Macori and Cotter, 2018; Bayili et al., 2019; Wirawati et al., 2019; Tamang et al., 2020; Mallappa et al., 2020).

The overall LAB population was recorded to be in a range of 6.27±0.01 to 6.49±0.02 log cfu g^{-1}; a similar observation was also observed by Tomar et al. (2009) from *chhurpi* of Arunachal Pradesh. Firstly, using standard phenotypic tests, the isolated LAB from the NFM products were tentatively grouped into *Enterococcus*, *Lactococcus*, *Leuconostoc*, and lactobacilli, which were later confirmed by molecular identification tool using Sanger sequencing, targeting the 16S rRNA universal housekeeping gene. Sequencing of the 16S rRNA gene is the gold standard for any bacterial identification (Janda and Abbott, 2007; Pradhan and Tamang, 2019) which is supported by huge database like NCBI and EzTaxon, though there are several other housekeeping genes like *carB, clpX, dnaK, groeL, hsp60, murC, murE, mutL, pheS, pyrG, recA, rpoA, rpoB, rpoC, spxB, tuf, uvrC, yycH, dnaA,* and *dnaJ* are sometimes used for discriminating different LAB species (Ouadghiri et al., 2009; Bao et al., 2016; Li et al., 2018; Huang et al., 2018; Ricciardi et al., 2020; Chen et al., 2021). Recently, lactic acid bacterial members belonging to the genus *Lactobacillus* were re-classified under the new nomenclature (Zheng et al., 2020). Hence, *Lactobacillus paracasei* subsp. *tolerans* was

reclassified as *Lacticaseibacillus paracasei* subsp. *tolerans*; *Lactobacillus brevis* as *Levilactobacillus brevis*; *Lactobacillus coryniformis* subsp. *torquens* as *Loigolactobacillus coryniformis* subsp. *torquens*; and *Lactobacillus parabuchneri* as *Lentilactobacillus parabuchneri* (Zheng et al., 2020). Through 16S rRNA gene sequencing analysis, 7 genera were identified *viz*., *Enterococcus*, *Lacticaseibacillus*, *Lactococcus*, *Lentilactobacillus*, *Leuconostoc*, *Levilactobacillus*, and *Loigolactobacillus*, comprising of 9 species from cow-milk products and 5 species from yak-milk products, respectively. *Enterococcus durans* was the predominant species but was only detected in samples of *mar* and *churkam*, except *chhurpi*. Due to the low abundance of *Enterococcus durans* in *chhurpi*, which may be the reason that colonies failed to appear in the used MRS culture media. The usage of only one type of culture media may also bring about less bacterial diversity in this present culture-dependent study (Delbès et al., 2007; Perin et al., 2017).

Enterococci are mostly associated with human gastrointestinal tract (Graham et al., 2020), however, also reported to be highly associated with various traditional food products like dry traditional dry smoked fermented meat sausages (Santos et al., 2017), traditional fermented dough (Li et al., 2016), dry fermented sausage (Demirgül and Tuncer, 2017), fermented meat products (Fuka et al., 2020), fermented milks (Cissé et al., 2019; Mo et al., 2019; Terzić-Vidojević et al., 2020). Though enterococci are one of the main LAB found in fermented foods, their presence is still of a concern as these bacteria are known as antimicrobial-resistant pathogens containing virulent genes (Castro et al., 2016; Fugl et al., 2017; Chajęcka-Wierzchowska et al., 2017; İspirli et al., 2017). *Enterococcus durans* has been isolated and identified from various fermented milk products (Shangpliang et al., 2017). It has also been reported as predominant species from yak products of China (Chen et al., 2010; Ao et al., 2012). Though the

occurrence of *E. durans* in milk products is still controversial. However, studies have shown that they may be responsible for ripening of cheese and development of flavour compounds that contributes to sensory properties (Castro et al., 2016; Nami et al., 2019). *Enterococcus durans* isolated from milk and milk products has some probiotic properties (Pieniz et al., 2014; Shangpliang et al., 2017; Li et al., 2018; Popović et al., 2018; Yerlikaya and Akbulut, 2020; Mercha et al., 2020; Akpinar et al., 2020; Comerlato et al., 2020; Bindu and Lakshmidevi, 2021). It is also well known to produce bacteriocins which acts against pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Perin et al., 2016; Castro et al., 2016; Silva et al., 2018). However, species belonging to the genus *Enterococcus* are yet to be recommended for the *qualified presumption of safety* (*QPS*) list and nor is it included in the Generally Regarded as Safe (GRAS) list (Hanchi et al., 2018; Graham et al., 2020).

Leuconostoc mesenteroides subsp. *mesenteroides* was detected in all samples of NFM products. It is commonly isolated from fermented products (Morita et al., 2016; Kaur et al., 2017; Vasiee et al., 2018; Li et al., 2018). Its technological properties have also been reported to have potential probiotic traits, which includes- the ability to produce antimicrobial properties (Giles-Gómez et al., 2016; Liu et al., 2017), reduces growth of *Listeria monocytogenes* in fermented cream (Borges et al., 2019), bacteriocin production (Arakawa et al., 2016). It has also been showed to produce antioxidant activities and cholesterol-lowering effects (Macori and Cotter, 2018; Lee and Kim, 2019) and anti-obesity (Castro-Rodríguez et al., 2020). Additionally, *Leuconostoc mesenteroides* is reported to contribute aroma development in the dairy products (van Mastrigt et al., 2019; Özcan et al., 2019).

Species belonging to the genus *Lactobacillus* are perhaps the most important members among all the LAB members with several health-promoting properties (Goel et al.,

2020). *Levilactobacillus brevis* [Basonym: *Lactobacillus brevis*; (Zheng et al., 2020)] has been previously reported from *chhu*, a NFM product of Sikkim (Dewan and Tamang, 2006). It is one of the LAB species to have been granted a Qualified Presumption of Safety (QPS) status (Feyereisen et al., 2019). Most strains belonging to this species are known as a good producer of γ-aminobutyric acid, GABA (Wu and Shah, 2017; Wu et al., 2017; Sokovic Bajic et al., 2019; Yu et al., 2020). Potential probiotic and antioxidant properties of *Levilactobacillus brevis* have been reported from fermented fish products (Aarti et al., 2017). It is known for bacteriocin activity (Iseppi et al., 2019), and has shown antimicrobial and antidiabetic agents (Kumari et al., 2016; Xu et al., 2016; Son et al., 2017; Abdelazez et al., 2018; Vasiee et al., 2018; Jang et al., 2019; Hojjati et al., 2020), anti-listerial activity (Campagnollo et al., 2018). *Levilactobacillus brevis* isolated from goat dairy products showed inhibition against *Salmonella typhi* (da Silva Ferrari et al., 2016). Interestingly, it is also reported to impart sensory properties in the final dairy products (Castro et al., 2016; Park et al., 2017).

Lentilactobacillus parabuchneri (Basonym: *Lactobacillus parabuchneri*) is another species isolated from *kefir*, a fermented milk-kefir grains product of Russia (Magalhaes et al., 2010). *Lentilactobacillus parabuchneri* is one of the main non- LAB starters used for production of many fermented dairy products (Van Hoorde et al., 2008; Magalhaes et al., 2010; Sohier et al., 2012; Nalbantoglu et al., 2014; Perin et al., 2017; Terzić-Vidojević et al., 2020). It mainly contributes to eye formation during cheese ripening (Fröhlich-Wyder et al., 2013). Due to its heat tolerance (Wechsler et al., 2021), *Lentilactobacillus parabuchneri* produces biogenic amines (*e.g*., histamines), a common problem of histamine-poisoning in cheeses (Diaz et al., 2016; Møller et al., 2020). The use of this species as probiotics has not been reported extensively, however, it has been

screened to have promising technological probiotic properties from Brazilian milk products (Agostini et al., 2018).

Lacticaseibacillus paracasei subsp. *tolerans* (Basonym: *Lactobacillus paracasei* subsp. *tolerans*), another *Lactobacillus* sp. which has also been reported from *kefir* (Magalhaes et al., 2010). Probiotic *Lacticaseibacillus paracasei* has been isolated from traditional Greek dairy products, artisanal goat cheeses, Egyptian raw milk from camel, sheep, goat, buffalo, and cow (Zoumpopoulou et al., 2018; Meng et al., 2018; Darwish et al., 2018; Mulaw et al., 2019). It is known to have several probiotic traits including bacteriocins production (de Almeida Júnior et al., 2015); cholesterol-lowering properties (Albano et al., 2018); inhibiting adherence of *Escherichia coli* and *Salmonella* typhimurium, *Yersinia enterocolitica* (Maragkoudakis et al., 2006; Damodharan et al., 2020); anti-fungal activity (Hassan and Bullerman, 2008), ability to produce GABA (Ribeiro et al., 2018). Additionally, it has also been study for its ability to produce biosurfactants (Hippolyte et al., 2018).

Lactococcus lactis is another LAB member which has been associated with many milk products such as Brazilian cheeses (Perin et al., 2015; de Freitas Martins et al., 2020), *karish* cheese of Egypt and Arab (Allam et al., 2017). It has also been reported from NFM products of Bhutan (Shangpliang et al., 2017). It is a well-known species for its probiotic traits and has shown to produce antibacterial properties (Bougherra et al., 2017), and anti-listerial substances (Dygico et al., 2019). It is known to produce bacteriocin production especially nisin and lactolisterin (El-Ghaish et al., 2017; Hwanhlem et al., 2017; Mirkovic et al., 2020; Khelissa et al., 2021) and has ability to reduce cholesterol level (Shehata et al., 2019). Strains of *Lactococcus lactis* produce aroma compounds that contribute to flavour in many fermented foods (Roncal et al., 2017; Fusieger et al., 2020). We also observed the higher species diversity in casein-

based products (*chhurpi* and *churkam*) in comparison to lipid-based products (*mar*), indicating casein to be a good media for bacterial proliferation in comparison to lipids (Zhang et al., 2011).

Culture-independent analysis

High-throughput analysis using next-generation Illumina MiSeq sequencing

Culture-dependent methods have several disadvantages for profiling the microbial diversity in the samples, such as media selectivity, growth conditions, failure to recover less abundant microorganisms, appearance, or growth of only culturable microorganisms, etc. (Fiore et al., 2020; Van Reckem et al., 2020). With the advancement of technology in the field of sequencing, next generation sequencing (NGS) method is one of the commonly used culture-independent method for extensive microbial diversity study in fermented foods (Mayo et al., 2021), where a holistic microbial community structure can be profiled (Tamang et al., 2021). The NGS method profiles the maximum microbial community present in samples, especially those which failed to be grown in culture media (Zapka et al., 2017). The NGS analysis of the NFM products of Arunachal Pradesh (*mar*, *chhurpi* and *churkam*) resulted in a huge diversity of microbial species, where *Firmicutes* is the dominant phylum, followed by *Proteobacteria*, which are commonly found to be associated with fermented milks (Moonga et al., 2020; Chi et al., 2021). Predominant (>1% relative abundance) *Firmicutes*-associated species consisted of LAB (*Lactococcus lactis*, *Lactobacillus helveticus*, *Leuconostoc mesenteroides*, *Lactococcus raffinolactis*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, and *Latilactobacillus sakei*) and non-LAB (*Staphylococcus cohnii*, *Bacillus cereus*, and *Staphylococcus kloosii*). On the other hand, predominant (>1% relative abundance) *Proteobacteria*-associated species

included members of the acetic acid bacterial (AAB) group *Gluconobacter oxydans*, *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Acetobacter syzygii*, *Acetobacter tropicalis*, *Pseudomonas fluorescens*, and *Gluconobacter cerinus*. OTU-based analysis, in this present study, could not detect *Companilactobacillus farciminis* (Basonym: *Lactobacillus farciminis*), *Loigolactobacillus bifermentans* (Basonym: *Lactobacillus bifermentans*), *Lentilactobacillus hilgardii* (Basonym: *Lactobacillus hilgardii*), which were reported earlier in NFM products (*dahi*, *chhu*, and *chhurpi*) of Sikkim through culture-dependent study (Tamang et al., 2000; Dewan and Tamang, 2006; 2007). The top three predominant species- *Lactococcus lactis* (Streptococcaceae), *Lactobacillus helveticus* (Lactobacillaceae), and *Leuconostoc mesenteroides* (Lactobacillaceae, formerly Leuconostocaceae) are the three most observed species in fermented milk products which are also known as the primary cultures in milk fermentation (Tamang et al., 2016). Metagenomics-based studies of fermented milk products around the world have also reported the predominance of these species (Yao et al., 2017; Yasir et al., 2020; Kazou et al., 2021). Apart from the lactic acid bacterial group, *Proteobacteria*-associated acetic acid bacteria belonging to the family Acetobacteraceae were also detected at a higher percentage in these NFM products, particularly *mar* samples. Acetobacteraceae, particularly *Acetobacter* and *Gluconobacter* have also been reported as one of the predominant members in many fermented milk products including traditional milk products, *ghee*, cheeses, kefir etc (Ongol and Asano, 2009; Keisam et al., 2016; Motato et al., 2017; Joishy et al., 2019; Gao and Zhang, 2019).

All the LAB species detected in culturable method were also detected through culture-independent method (Shangpliang et al., 2018). The study of AAB in fermented foods is very limited through plating methods as the cultivation, isolation, and identification

is cumbersome though they are usually reported through high-throughput sequencing studies (De Roos and De Vuyst, 2018). Apart from bacteria, yeasts are also another groups of microorganisms which have been reported to be associated with traditional fermented foods (Tofalo et al., 2020). Yeasts have also been used as potential probiotics, and several research have claimed their potential in food applications and in health-promoting aspects (Agarbati et al., 2020). Exploration of these groups of microorganisms apart from LAB from NFM products is also of great importance.

Predominance of detected operational taxonomical units (OTUs) was hypothetically tested using relevant non-parametric Mann-Whitney U-test for each taxonomical level (phylum, family, genus, and species) and for each concerned group based on the products (*mar*, *chhurpi*, and *churkam*), animal milk source (cow and yak), and nature of the products (casein-based and lipid-rich), as already discussed in the previous section. *Firmicutes* was significantly higher in *chhurpi* and *churkam* (casein-based) products while on the other hand, *Proteobacteria* was significantly higher in *mar* (lipid-rich) products. The difference in the nature of the products may have favoured growth of different groups of bacteria, where casein-based samples favoured the LAB while the lipid-rich samples favoured the acetic acid bacteria (Shangpliang et al., 2018; Li et al., 2020, 2021). However, there was no significant differences at the phyla level based on the animal milk source. Similarly, at the family level, Acetobacteraceae (*Acetobacter* and *Gluconobacter*) was significantly higher in *mar* (lipid-rich) samples in comparison to both *chhurpi* and *churkam* (casein-based) samples. However, Streptococcaceae was only significantly higher in *chhurpi* compared to *mar*, whereas in *churkam* the distribution of Streptococcaceae varied throughout the samples. *Chhurpi*, being semi-solid, may have been a favourable media for Streptococcaceae, whereas *churkam*, being fully dried, may have been a selective medium in case of this family (Bonnet et al.,

2019). On the other hand, Pseudomonadaceae (genus: *Pseudomonas* and species: *Pseudomonas fluorescens*) was significantly higher in *chhurpi* compared to *churkam*, since the former is more nutritious that the latter and which explains that there is a higher chance of unwanted bacterial contamination in *chhurpi* rather than in *churkam*. Interestingly, Leuconostocaceae (*Leuconostoc*) was significantly higher in cow-based products in comparison to yak-based products. The distribution of *Lactococcus* in *chhurpi* samples showed uniformity and was significantly higher in number than *mar*. However, even though its population in *churkam* is abundant, it showed diverse distribution and was not significant to either of the former products. *Churkam* is a dried product which is sun-dried for a longer time (Shangpliang and Tamang, 2021), and due to this process, it may be not a suitable medium for proper distribution of bacteria (Bonnet et al., 2019). Alpha diversity (Chao1) showed the highest in *chhurpi*, indicating its microbial richness (Deka et al., 2021). On the other hand, Shannon's and Simpson's diversity indices showed the highest in lipid-samples (*mar*) in comparison to casein products (*chhurpi* and *churkam*), indicating a representative of a diverse and equally distributed community in the samples (Moonga et al., 2020).

Overall, the predominance of *Proteobacteria*-associated AAB in lipid samples (*mar*) may be associated to the traditional method of preparation, where it is usually obtained simply by churning of the collected milk (Shangpliang et al., 2018). However, for obtaining *chhurpi* and *churkam*, the collected buttermilk (*dhara*) is subjected to heating (Shangpliang and Tamang, 2021). Although the presence of Acetobacteraceae members were still present in the post-heated samples, their relative abundances reduced while members of the lactic acid bacteria group increased drastically, where Streptococcaceae (*Lactococcus* sp.) is predominant in *chhurpi* samples and Lactobacillaceae

(*Lactobacillus* sp.) is subsequently predominant in *churkam* samples (Shangpliang et al., 2018).

Enterococcus sp. is one of the commonly found LAB in NFM products (Shangpliang et al., 2017; Dapkevicius et al., 2021), however, by high-throughput sequencing analysis, *Enterococcus* was recorded in very low abundance in this NFM products of Arunachal Pradesh. On the contrary, through culture-dependent analysis, *Enterococcus durans* was predominant in *mar* and *churkam* samples. Unwanted bacterial contamination is a major concern in NFM products where presence of contaminants in milk fermentation does pose a threat to consumers and food quality (Amenu et al., 2019; Akinyemi et al., 2021). Presence of Staphylococcaceae, Bacillaceae, Pseudomonadaceae and Clostridiaceae has been often reported in several fermented milk products (Motato et al., 2017; Joishy et al., 2019; Diaz et al., 2019), however, these families were present in low abundances in the NFM of Arunachal Pradesh. Pseudomonadaceae (*Pseudomonas* sp.) is a very common psychrotolerant contaminant in raw milk and milk products which is frequently associated with cold storage (Wiedmann et al., 2000; Quigley et al., 2013). Additionally, the presence of Clostridiaceae (*Clostridium* sp.) is another concerned contamination which has been associated with spoilage, causing late blowing defect in cheese (Panelli et al., 2013; Bassi et al., 2015; Levante et al., 2017). Since *mar*, *chhurpi* and *churkam* are spontaneously fermented with no standardized scientific methods but rather by traditional knowledge practiced from time to time, bacterial contaminants may have been transferred by improper handling of the items used in the processing like water, containers, and the surroundings (Ringø et al., 2014; Ssajjakambwe et al., 2017). The presence of abundant uncultured bacteria (unclassified sequences) in the NFM products of Arunachal Pradesh is noteworthy, reflecting the probability of many other unknown native microorganisms being unidentified which may have important role in the

processing of these products. Unculturable microorganisms are usually detected in many NGS-related studies. However, the context of being "uncultured" is often misunderstood, when it is just that no present knowledge of their metabolism is known for them to be grown under laboratory conditions (He et al., 2017). The detection of unclassified sequences is well-known in culture-independent studies (Fagbemigun et al., 2021) and were also reported in fermented milk products (Biswal et al., 2021), suggesting the presence uncultivable microorganisms (de Melo Pereira et al., 2020). One of the main reasons is due to the fact that most databases used (SILVA, Greengenes, RDP etc) in taxonomic classification are based only on cultured microbes (Breitwieser et al., 2019), and those microbes which have never been cultured before remains unknown or simply classified as unclassified sequences of uncultured microbes (Konstantinidis et al., 2017).

Technological properties of the isolated LAB strains

Evaluation of the technological properties of the 76 identified LAB strains, isolated from NFM products of Arunachal Pradesh was performed using some standard experimental tests. According to ICMR-DBT guidelines (Ganguly et al., 2011), acid resistance, bile resistance, antimicrobial activity, ability to reduce pathogens and bile salt hydrolase activity are the standard methodology recommended for in vitro screening of putative probiotic strains (Pradhan and Tamang, 2021). Additionally, few other tests were also tested based on some related literatures which includes cholesterol reduction, beta-galactosidase activity, exopolysaccharide, and gamma butyric acid (GABA) production (Angmo et al., 2016). In this present work, selection of the best strains from the total 76 identified LAB strains was achieved by a two-step grouping method using principal component analysis (PCA) and heatmap analysis. Firstly, focus was made on the

standard in vitro mentioned above as per (Ganguly et al., 2011). Subsequently in the second step, using the same grouping method, the best few strains from the previous standard in vitro experiments were then grouped as per the extra properties of interest.

All the LAB isolates isolated from the NFM products of Arunachal Pradesh were able to coagulate skim milk with decrease in the pH level. The ability of the LAB strains to undergo milk acidification caused coagulation which add to their potential ability to be used as milk adjuvants to produce milk products and prevention of undesirable bacterial growth (Dewan and Tamang, 2007; Yi et al., 2011). Tolerance to acidic pH, a basic *in vitro* simulation of the human stomach (Mackie et al., 2020), is one of the main properties for which a putative probiotic strain should have (Plaza-Diaz et al., 2019). Four strains of *Levilactobacillus brevis*- AcCh91, AcCk67, AcCh35, AcMr06 showed a good acid tolerance with strain AcCh91 as the highest. Strains of *Levilactobacillus brevis* isolated from fermented foods have been shown to have high tolerance against acid (Angmo et al., 2016; Aarti et al., 2017; Hojjati et al., 2020). Similarly, five strains of *Lactococcus lactis*: AyCk71, AcCh67, AyCk21, AcCk75, AcMr75 also showed good tolerance against acid. Previously, *Lactococcus lactis* isolated from *dahi* and *datshi*, (NFM of Bhutan) have been reported to have good tolerance to acidic pH (Shangpliang et al., 2017). Another group of LAB strains which showed good tolerance to low pH includes *Lacticaseibacillus paracasei* subsp. *tolerans*, strains: AyCh28, AcCh11, AcCh78, AcCh17. Many reports of strains isolated from fermented milks have also shown the ability of this species to tolerate low pH (Ye et al., 2017; Mantzourani et al., 2018; Plessas et al., 2020). Lastly, strains of *Enterococcus durans*- AcCk25, AyCk84, AyMr03 also showed good range of tolerance to low pH. Strains of *Enterococcus durans* isolated from milk products have tolerate to low pH (Pieniz et al., 2014; Albayrak and Duran, 2021). *Leuconostoc mesenteroides* (strains: AyMr31, AyCh37, AcMr18, AyCh45) were

also observed to be able to tolerate low pH, and some strains isolated from milk products have been reported to have good tolerance to pH (Haghshenas et al., 2017).

Tolerance to bile salts is important aspect to screen for putative probiotic strains, which not only is it the important component of intestinal bile but also do possess antimicrobial property (Hu et al., 2018). A good probiotic candidate must have good tolerance to 0.3% bile salts as a simulation to human intestinal bile content (Zhang et al., 2016). A good range of 53.18% to 86.68% was observed, with *Levilactobacillus brevis* AcCh91 as the highest, which includes other strains also- AcCk67, AcCh35, AcMr06. Many reports have shown the ability of strains, isolated from milk products, belonging to this species that could tolerate bile salts (Sharifi Yazdi et al., 2017; Zhang et al., 2020). Several other strains showed good tolerance to bile salts which included *Lactococcus lactis* subsp. *cremoris* AyCk21, AcCh67, AyCk71; *Lactococcus lactis* subsp. *lactis* AcCk75, AcMr75; *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh17, AcCh78, AcCh11, AyCh28; *Leuconostoc mesenteroides* subsp. *mesenteroides* AyCh45, AcMr18, AyCh37, AyMr31; and *Enterococcus durans* AcCk25, AyCk84, AyMr03.

The ability of putative probiotic strains to reduce pathogenic bacterial population is often explained by their mode of adherence property (Plaza-Diaz et al., 2019). Usually, this is indirectly determined by their cell surface properties, which includes attachment to hydrocarbons, auto-aggregation, and co-aggregation (Tuo et al., 2013; de Melo Pereira et al., 2018). Five different hydrocarbons were used in this present work for determining the cell surface hydrophobicity, which included chloroform, diethyl ether, n-hexadecane, toluene, and xylene. Most strains showed difference in their hydrophobic nature towards the tested hydrocarbons (De Paula et al., 2015). Usually, hydrophobicity index >70% is considered as hydrophobic (Nostro et al., 2004). Strains of *Levilactobacillus brevis* AcCh91 and AcMr06 showed the highest hydrophobic index.

Levilactobacillus brevis isolated from fermented foods have good hydrophobicity (Ramos et al., 2013; Angmo et al., 2016; Chait et al., 2021). Other strains which showed good hydrophobicity included *Lactococcus lactis* subsp. *cremoris* AyCk21, *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, and *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, while others having a hydrophobicity index of <70%. *Lactococcus lactis* subsp. *cremoris* isolated from NFM products of Sikkim showed hydrophobicity of >80% (Rai, 2020). Strains of *Lacticaseibacillus paracasei* with good hydrophobic nature have been reported in milk and milk products (Dewan and Tamang, 2007; Reuben et al., 2020). Similarly, strains belonging to *Leuconostoc mesenteroides* isolated from cheese have been reported to show good hydrophobic nature too (De Paula et al., 2015).

Cell surface properties are also evaluated by the ability of the LAB strains to undergo auto-aggregation and co-aggregation (adherence to other bacterial cells preferably pathogens) as one of the many mechanisms of reducing their population (De Paula et al., 2015). Auto-aggregation and co-aggregation are very necessary for putative probiotic bacteria since these properties can help understand their ability to adhere to the intestinal epithelial cells and forming a barrier that can prevent pathogenic colonization (Grujović et al., 2019). Grujović et al., (2019) further demonstrated the importance of these properties in evaluating a potential probiotic bacteria isolated from fermented dairy products, where they have reported some strains of *Levilactobacillus brevis* to be showing the best cell surface properties based on auto-aggregation and co-aggregation. In our present work, *Levilactobacillus brevis* AcCh91 showed the highest among the other strains. On the other hand, strains *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28 showed the highest for co-aggregation against the test pathogenic strains. Many reports from fermented milk products have shown the aggregation index

of >90% of *Lacticaseibacillus paracasei* (Solieri et al., 2014; Reuben et al., 2020) against tested pathogenic strains. Other strains which are promising for aggregation properties includes *Leuconostoc mesenteroides* subsp. *mesenteroides, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris* etc, which are common studied in many fermented milk products (Solieri et al., 2014; De Paula et al., 2015; de Oliveira Coelho et al., 2019).

Lacticaseibacillus paracasei is one of lactic acid bacterial species to have been widely used as a probiotic strain (Luz et al., 2021). One of its major properties is ability to produce antimicrobial substances against pathogens and helps in immune system modulation (Silva et al., 2020; Luz et al., 2021). Three strains of *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, AcCh11, AcCh78 in our present study showed good antimicrobial property against all the tested pathogenic strains. Similarly, *Lactococcus lactis* subsp. *lactis* AcMr75 and *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31 also showed antimicrobial against all the pathogens. Strains of *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* have been isolated from other dairy products with their antimicrobial properties (Yerlikaya, 2019; Silva et al., 2020). Among the *Levilactobacillus brevis* strains, only strain AcCh91 showed antimicrobial property against 3 out of 4 tested pathogens. *Levilactobacillus brevis* isolated from camel milk showed antimicrobial property (Rahmeh et al., 2019; Singh et al., 2020), which is associated to their ability to produce bacteriocins (Sharma et al. 2021).

Bile salt hydrolysis is the catalysis of conjugated bile salts into free bile salts by the production of bile salt hydrolases, which is also an attribution to cholesterol-lowering effect (de Melo Pereira et al., 2018; Gil-Rodríguez and Beresford, 2021). Most of the strains showed hydrolysis activity against the conjugated bile salt which included

Levilactobacillus brevis AcMr06, AcCh35, AcCh91, and AcCk67; *Lactococcus lactis* subsp. *cremoris* AcCh67, AyCk21, and AyCk71; *Lactococcus lactis* subsp. *lactis* AcCk75 and AcMr75; *Lacticaseibacillus paracasei* subsp. *tolerans* AyCh28, AcCh11, AcCh78, and AcCh17; *Leuconostoc mesenteroides* subsp. *mesenteroides* AyMr31, AyCh37, and AcMr18; and *Enterococcus durans* AcCk51. The ability of producing hydrolases enzymes, which can catalyse bile salts have been reported from many LAB isolates isolated from milk and milk products which are also assumed for their good attributes to their potential probiotic status (Sharma et al., 2021).

Based on the above standard experiments for probiotic evaluation (Ganguly et al., 2011), all the records from the tests were then grouped using in silico analyses- multivariate statistics, heatmap visualization and principal component analysis (PCA) (Vijayalakshmi et al., 2020; Mallappa et al., 2020), to scale down the isolated strains with the best probiotic attributes. A total number of 20 top strains with the overall best properties were obtained, which included *Enterococcus durans* AcCk25, AyCk84, and AyMr03; *Lacticaseibacillus paracasei* subsp. *tolerans* AcCh11, AcCh17, AcCh78, and AyCh28; *Lactococcus lactis* subsp. *cremoris* AcCh67, AyCk21, and AyCk71; *Lactococcus lactis* subsp. *lactis* AcCk75, and AcMr75; *Leuconostoc mesenteroides* subsp. *mesenteroides* AcMr18, AyCh37, AyCh45, and AyMr31; *Levilactobacillus brevis* AcCh35, AcCh91, AcCk67, and AcMr06.

To further determining the best strains with probiotic potential, the LAB strains were tested for our experiments of interest, which included beta-galactosidase, cholesterol reduction, exopolysaccharide, and gamma amino butyric acid (GABA) production. Strains of *Levilactobacillus brevis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides* showed the best beta-galactosidase activity. Strains of *Levilactobacillus brevis* isolated from Vietnamese

traditional fermented foods reported the ability of production of beta-galactosidase (Le et al., 2015), and fermented foods and beverages of Ladakh (Angmo et al., 2016). Kondrotiene et al. (2020) studied 169 *Lactococcus lactis* strains isolated from fermented milk and products and reported some strains to be able to show good beta-galactosidase activity. Positive activity for beta-galactosidase enzyme was also reported from *Lactococcus lactis* isolated from NFM of Sikkim (Rai, 2020). In our present study, not all *L. Lactis* were positive for this enzyme. Hence, there may be strain-specificity for enzyme production (De Paula et al., 2015).

LAB are also known to produce secondary metabolites like exopolysaccharides (EPS) which are necessary for food quality and shelf life of the product (De Paula et al., 2015). In this present work, strains of *Lacticaseibacillus paracasei* subsp. *tolerans*, *Enterococcus durans*, and *Leuconostoc mesenteroides* subsp. *mesenteroides* were detected for their ability to produce exopolysaccharide using plate assay. Exopolysaccharides produced by *Lacticaseibacillus paracasei* can potentially benefitting the gastrointestinal tract which can help in gut microbiota modulation (Bengoa et al., 2020). In some case, EPS are known to have antioxidant properties (Almalki, 2020). *Enterococcus durans* strains isolated from Iranian fermented milk, *kishk*, also were reported to producing EPS which have antioxidant and antibacterial activity (Rahnama Vosough et al., 2021). Recently, positive strains of *Leuconostoc mesenteroides* for EPS production was also reported from *kefir* with applications like bio-thickeners and bio-stabilisers (Wang et al., 2021).

The ability of LAB to solubilize cholesterol suggests the implications of them being able to prevent heart-related diseases and reduce its uptake from the gut (de Melo Pereira et al., 2018). Two strains of *Lacticaseibacillus paracasei* subsp. *tolerans* (AcCh17 and AcCh78) showed the highest percentage of reduction of cholesterol level in broth

analysis. This species has been reported to have potential in cholesterol lowering which can be used for dairy applications (Albano et al., 2018). Among other stains, *Lactococcus lactis* and *Leuconostoc mesenteroides* isolated from camel milk and kimchi also have been reported to exhibit remarkable cholesterol lowering abilities (Abushelaibi et al., 2017; Lee and Kim, 2019). It has also been reported that *Levilactobacillus brevis* isolated from yoghurt and cheese showed cholesterol-lowering properties (Nami et al., 2018). Additionally, *Enterococcus durans* isolated from naturally fermented cream of China also have been shown to have potential cholesterol-lowering properties (Nami et al., 2018).

Furthermore, in this present work, screening for production of GABA (gamma-aminobutyric acid, or γ-aminobutyric acid) was carried out. GABA, commonly produced by LAB, is an important amino acid that acts as a neurotransmitter which helps in relaxation and reduce anxiety (Diez-Gutiérrez et al., 2020; Yu et al., 2020). Since LAB are mostly associated with fermented foods, therefore, GABA is likely to be enriched in such natural products (Yogeswara et al., 2020). *Levilactobacillus brevis* AcMr06, AcCh35, AcCh91, and AcCk67 were shown to be active in GABA production, while detection of its production was also observed in *Lactococcus lactis* subsp. *cremoris* AcCh67, AyCk21, and AyCk71, and *Lactococcus lactis* subsp. *lactis* AcCk75. The species *Levilactobacillus brevis* is a well-known producer of GABA, and it has been reported from various fermented dairy products (Sokovic Bajic et al., 2019; Santos-Espinosa et al., 2020).

Gene detection for probiotic and functional properties

In this thesis, few probiotic and functional genes were screened using specific-primer pairs under defined PCR conditions and specific target amplicon length (Kim et al.

2020). Many LAB species have different mechanisms to undergo different probiotic functions. For example, survival under low pH has been reported to be associated with the production of some proteins (encoding genes) like histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tdc*), ornithine decarboxylase (*odc*), agmatine deiminase (*aguA*), heat shock protein 60 (*groEL*), cyclopropane FA synthase (*LBA1272*), D-alanine transfer protein (*dltD*), amino acid permease (*La995*), and amino acid antiporter (*La57*) (Turpin et al., 2011). Furthermore, pH and bile salt tolerance has also been associated with glucan synthase (*gtf*), ATPase (*clpL*) and putative esterase (*lr1516*) (Turpin et al., 2011). The presence apf gene is very common in many lactobacilli (Turpin et al., 2012), not only that they may aid bacteria to tolerate bile, stress but is also associated with other properties like adhesion and aggregation (Saito et al., 2019). In this present study, *apf* gene was the most distributed gene among the other genes in the LAB strains. The presence of *apf* gene in *Levilactobacillus brevis* is not very common, however, recent transcriptomic study reported the expression of genes related to aggregation promoting factors which aid in stress tolerance (Banerjee et al., 2021). The presence of aggregation-promoting factor has also been reported in *Lacticaseibacillus paracasei* (Lozo et al., 2007). Recently, the detection of *apf* and *Ir1584* gene in *Lactococcus lactis* strains was also reported from NFM products of Sikkim (Rai, 2020). Tolerance to bile has also been observed to be associated with the expression of transporter proteins, permeases (gene: *LBA0552*), which also acts as a multidrug resistance (MDR) transporter, protecting the cell from several toxic substances (Pfeiler and Klaenhammer, 2009). Furthermore, genes *Ir1584* and *Ir1516* was also reported from *Lactococcus lactis* (Rai, 2020). Though expression of genes like *hdc*, *tdc* and *odc* which have also been reported to be aid some LAB species in withstand low pH, however, these genes also do involve in the biogenic amine synthesis. The absence of these genes

in the LAB strains, in this present work, may also explains the safety of these strains (Pumriw et al., 2021). For bile salt tolerance, proteins (encoding genes) like conjugated bile salt acid hydrolase, hypothetical protein (*lr0085*, *LBA1432*), major facilitator superfamily permease (*lr1584*, *LBA0552*, *LBA1429*), multidrug resistance protein (*LBA1446*), ABC transporter (*LBA1679*), and aggregation-promoting factors (*apf*) have been explained to be predictively involved in this process (Turpin et al., 2011). The presence of *clpL* and *LBA1272* genes in *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* has also been reported in many findings (Huang et al., 1993; Turpin et al., 2011; Jana and Biswas, 2020), and recently in LAB strains isolated from NFM of Sikkim (Rai, 2020). Interestingly, in this present work, gene *groEl* was only detected in three species of *Lacticaseibacillus paracasei* subsp. *tolerans*. Gene *groEl* has been reported from *Lacticaseibacillus casei* [Basonym: *Lactobacillus casei*; (Zheng et al., 2020)] group which includes *Lacticaseibacillus paracasei* (Koirala et al., 2015). Recently, whole genome analysis of *Lacticaseibacillus paracasei* detected the presence of the gene *groEl* (Qureshi et al., 2020). Gene *bsh* was also detected from several LAB strains of NFM of Arunachal Pradesh which includes- *Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei* and *Levilactobacillus brevis*. The presence of this gene in the above species has also been reported from several different studies (Widodo et al., 2020; Gil-Rodríguez and Beresford, 2021), and from NFM of Sikkim (Rai, 2020). Adhesion, (or cell attachment) and aggregation of LAB species have been demonstrated to be associated with different mechanisms like the presence of specific cell receptor proteins like fibronectin-binding protein, ATP binding-substrate protein, sortase, mucin-binding protein, mannose-specific adhesin (Archer and Halami, 2015). Detection of genes *fbp*, *sbp*, *sor*, *mub*, and *msa* was carried out using specific primer pairs (Archer and Halami, 2015). Only *Leuconostoc mesenteroides*

subsp. *mesenteroides* was detected for *fbp* gene, as also reported by (Rai, 2020). On the contrary, *apf* gene (codes for aggregation-promoting factors) have also been reported to aid LAB species in cell adhesion and aggregation. This may explain the possible mechanism of the LAB in this present work for cell adhesion and aggregation as most species were not positive for genes like *fbp*, *sbp*, *sor*, *mub*, and *msa* but were positive for *apf* gene (Rai, 2020). The presence of *apf* gene have also been reported to enhance aggregation in LAB species isolated from dairy products of China (Jin et al., 2021). For vitamin synthesis, two genes were used- *folP* and *ribA*. However, only *ribA* was detected in few strains of *Leuconostoc mesenteroides* subsp. *mesenteroides*. The presence of *ribA* gene in some strains of *Leuconostoc mesenteroides* is known (Turpin et al., 2011). Few bacteriocin-encoded genes were also used in this study. However, only gene *lcnB* was detected in strains of *Leuconostoc mesenteroides* subsp. *mesenteroides*. Additionally, detection of *gad* (glutamate decarboxylase) was also detected only in strains of *Levilactobacillus brevis* using *gadR* specific primers (Mancini et al., 2019). This may also probably explain the detection of GABA, gamma(γ)-aminobutyric acid, production as reflected in the thin-layer chromatography (TLC) plate.

Limited genes were used for genetic screening in this study. Though some genes were detected which have some relationship to the phenotypic properties with respect to the *in vitro* tests. However, not all mechanisms were able to be explained since there are still a lot of gaps in this area. It is also important to note that different bacteria have different possible mechanisms for different probiotic and functional attributes (Turpin et al., 2011; Turpin et al., 2012). The use of gene detection with specific primer pairs is a rapid way to detect the presence of some gene of interest. However, genomic content does not necessarily translate to gene expression (Jagadeesan et al., 2019). In-depth

genomic analysis and gene expression can only be achieved by using whole genome sequencing (Goel et al., 2020) and proteomics/transcriptomics (Li et al., 2021).

Lastly, using the principle mentioned above for primary grouping based on the preliminary probiotic tests, secondary grouping was applied for the above-mentioned extra properties of interest by applying multivariate statistics, heatmap visualization and PCA. Based on these parameters, *Levilactobacillus brevis* (AcCh91) is selected as the best potential probiotic candidate, which may be developed as starter culture for milk fermentation (Bintsis, 2018). However, more studies must be done to further understand their mechanisms and other important applications to food.

Microbial gene prediction using PICRUSt2 and Piphillin

In this present study, predictive metabolic pathways of OTUs, generated through high-throughput sequence analysis of NFM products of Arunachal Pradesh were inferred by using pipelines such as PICRUSt2 (Douglas et al., 2020) and Piphillin (Narayan et al., 2020). Inferences of indirect gene function showed the predominance of metabolism, which implies a very active metabolic activity of microbes in these products, as reported of their huge diversity (Shangpliang et al., 2018). These findings were very similar to other fermented milk products (Zhang et al., 2017; Zhu et al., 2018; Chen et al., 2020; Choi, et al., 2020). The detection of various metabolic pathways such as the metabolism of amino acids, carbohydrates, vitamins, lipids, cofactors, and many secondary metabolites etc indicates a complex bacteria-bacteria interaction. Fermented milk products of the Eastern Himalayas are rich resource of microbial communities with lactic acid bacteria and acetic bacteria as the predominant groups (Tamang et al. 2000; Dewan and Tamang 2006, 2007; Shangpliang et al. 2018; Ghosh et al. 2019; Tamang et al., 2021). Non-parametric Spearman's correlation analysis of the predominant genera

and the predictive functionalities resulted in a complex microbial-function interactions; and, showed important and significant correlation. Various interacting features were observed from the analysis with both negative and positive significant correlation, which is expected from such complex and diverse microbiota. Similar findings on this complex correlation were reported on study of cheese microbiome (Yang et al., 2020). Metabolic activity of amino acid metabolism has been reported to correspond to flavour development in cheese (Yvon and Rijnen, 2001), whereas high metabolism of carbohydrate also contributes to flavour and aroma development in milk fermentation (Pan et al., 2014). The pre-dominance of functional pathways related to metabolism of amino acids, lipid, energy, and carbohydrates were earlier reported in fermented milk and milk products (Zhang et al. 2017; Ramezani et al. 2017; Zhu et al. 2018; Yasir et al. 2020; Chen et al. 2020). Additionally, the presence of functional features like metabolism of cofactors and vitamins- ubiquinone and other terpenoid-quinone biosynthesis and lipoic acid metabolism have also been linked to their importance for other microbial metabolism (Yao et al., 2021). A high correlation of functional properties and LAB have also been reported in cheeses (Yang et al. 2020) since LAB are the most predominant microorganisms in fermented milk products (Rezac et al. 2018; Chen et al. 2020). We observed a positive correlation of *Staphylococcus* with the predictive metabolic features of these NFM products, and interestingly, *Staphylococcus* is metabolically active in dairy products playing functional activities such as amino acid metabolism, carbohydrate metabolism, lipid metabolism and nitrogen metabolism (Leroy et al. 2020). Additionally, acetic acid bacteria (AAB) have also been reported to contribute to the functional features of fermented foods like the production of secondary metabolites and volatile compounds (Illeghems et al., 2015). Recently, it has also been

reported that the presence of AAB in fermented foods cause a high yield in the essential amino acids in the fermentation of sourdough (Li et al., 2021).

In this present study, predictive profiles from both PICRSUt2 and Piphillin was normalized using MUSiCC (Manor and Borenstein, 2015). MUSiCC is a gene marker marker-gene based method which uses a single-copy genes for biasness correction of genes abundances (Noecker et al., 2017). Using MUSiCC normalization has been proven necessary for metabolic gene functional study (Vincent et al., 2017), where it corrects biases by rescaling the predominant predicted KOs to the actual gene copy number (Manor and Borenstein, 2017). Piphillin is mostly used for clinical samples, while PICRUSt2 is generally used for environmental and human gut (Narayan et al., 2020; Douglas et al., 2020). However, these two pipelines have also been applied in fermented dairy products (Choi, et al., 2020a;b). Therefore, from the present analysis, a consolidated profile from both the outcomes of the two pipelines were combined, with the expectation of one prediction compensating the other for a better understanding of the microbial gene function. Additionally, though microbial gene prediction was only speculations using bioinformatics tools, a general outlook into the possible function and complex interaction was studied and observed. Therefore, at present, in the absence of shotgun metagenomics studies of the NFM products of Arunachal Pradesh, using pipelines like PICRUSt2 and Piphillin does give us an insight into the predictive gene function which is reliable at present.

CONCLUSION

In this present work, exploration of the traditional knowledge and practices of the exotic NFM products of Arunachal Pradesh was carried out through field survey, documentation using structured questionnaire. Different types of the indigenous NFM products were studied and examined for their physiological and microbiological contents. With the aid of culture-dependent and culture-independent techniques which involves phenotypic and genotypic methods, bacterial diversity was explored and thoroughly studied. This work also serves as the first in-depth microbiological study of these NFM products of the state using high-throughput next-generation sequencing (Illumina MiSeq) approach. The predominance of lactic acid bacteria (LAB) in these products infers a good source of health-promoting microbes in these products. Though, a small number of unwanted microbes were also detected, however, these are usually associated with the traditional methods of preparation, handling, and transportation of the products. Since, till present, production and processing of these products are with minimal hygienic practices, therefore, a chance of unwanted microbial contamination is still a possibility. Nevertheless, a maximum number of predominant bacteria belong to LAB with GRAS status, and some of them showed promising probiotic features. A total of 20 LAB strains were selected as representatives, out of which 1 strain *Levilactobacillus brevis* AcCh91, was selected as the best probiotic strain. The presence of acetic acid bacteria is also of interest for future research studies. Additionally, selective, and optimized cultivation of predominant species for industrialization and product development with health-promoting probiotic strains is another gap to be filled.

SUMMARY

The practice of milk fermentation is a part of tradition and culture of *Monpa* (*Brokpa*) community, one of the ethnic groups of Arunachal Pradesh, India. Till date, there is very limited documentation on the naturally fermented milk (NFM) products of Arunachal Pradesh, therefore, this present Thesis was aimed to explore the traditional practices of milk fermentation by the ethnic people with emphasis on the microbial diversity and isolation of the native lactic acid bacteria having probiotic properties. In Tawang and West Kameng of Arunachal Pradesh, the preparation of NFM products is only associated mainly with *Brokpa* community, the cattle-herders residing in the high-altitude mountains of the western part of the state. Yaks and cows are the main cattle where NFM products from whose milk is used for fermentation, where the former is usually found near the snow-capped mountains and the latter towards the lower regions. Depending on the cattle type the local people rear, milk products can be prepared from either cow or yak's milk. Different NFM products which includes- *mar* (artisanal butter), *chhurpi* (soft cottage cheese) and *churkam* (hard cottage cheese), prepared from both cow and yak's milk were well studied and documented in this present study. All samples were collected from the production centers aseptically in sterile containers as were transported to the laboratory the earliest with utmost precautions and care. The pH of all the samples were mild acidic in nature (5.32 ± 0.01 to 6.62 ± 0.01), with viable LAB count of 6.27 ± 0.01 to 6.49 ± 0.02 log cfu g^{-1}. Lactic acid bacteria (LAB) were the predominant microorganisms in the samples, and they were thoroughly studied through culture-dependent and culture-independent studies. A total of 307 LAB were isolated and further grouped randomly to 76 based on standard phenotypic classification depending on the biochemical, physiological tests, cell morphology, etc, which tentatively identify as Enterococcus, Lactococcus, Leuconostoc and lactobacilli. Genetic identification based on 16S rRNA gene sequencing revealed the identity of 7 genera

viz., *Enterococcus*, *Lacticaseibacillus*, *Lactococcus*, *Lentilactobacillus*, *Leuconostoc*, *Levilactobacillus* and *Loigolactobacillus*, with 9 species in samples of naturally fermented cow-milk products and 5 species in samples of naturally fermented yak-milk products, respectively. *Enterococcus durans* was the predominant isolated LAB followed by *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Levilactobacillus brevis*, *Lactococcus lactis* subsp. *cremoris*, *Loigolactobacillus coryniformis* subsp. *torquens*, Lentilactobacillus *parabuchneri*, and *Lactococcus lactis* subsp. *hordniae*. In terms of species diversity analysis, casein-based products (*chhurpi* and *churkam*) showed a high diversity in comparison to lipid-rich product (*mar*), where *churkam* is slightly higher than that of *chhurpi*, indicating casein as a good medium for LAB proliferation in comparison to high lipid content.

Bacterial diversity was further studied using culture-independent, high-throughput Illumina MiSeq-based sequencing. *Firmicutes* was the most abundant phylum detected followed by *Proteobacteria*. At the family level, the most predominant families belong to Acetobacteraceae, Streptococcaceae, Lactobacillaceae, Staphylococcaceae, unclassified (derived from Bacteria), Leuconostocaceae, Bacillaceae, and Pseudomonadaceae, among those >1% of the total relative abundance. *Lactococcus* was the predominant genera detected followed by *Acetobacter*, *Lactobacillus*, *Staphylococcus*, *Gluconobacter*, unclassified (derived from Bacteria), *Leuconostoc*, *Pseudomonas*, *Bacillus*, among those >1% of the total relative abundance. Additionally, at the species level, *Lactococcus lactis* was the predominant species, followed by, *Lactobacillus helveticus*, *Staphylococcus cohnii*, *Gluconobacter oxydans*, uncultured bacterium, *Acetobacter lovaniensis*, *Leuconostoc mesenteroides*, *Acetobacter pasteurianus*, *Acetobacter syzygii*, *Acetobacter tropicalis*, Lactococcus *raffinolactis*,

Lactobacillus delbrueckii, *Pseudomonas fluorescens*, *Lactobacillus acidophilus*, *Bacillus cereus*, *Gluconobacter cerinus*, *Staphylococcus kloosii*, and *Latilactobacillus sakei* (Basonym: *Lactobacillus sakei*), among those >1% of the total relative abundance. It is very interesting to have detected acetic acid bacteria (*Acetobacter* and *Gluconobacter*) which are groups of fermenters which are rarely studied in fermented foods. Additionally, the presence of some unwanted contaminants explains the need of safety precautions during the preparation of the NFM products. Since all the traditional NFM products are prepared locally in small scale at household level, there is a high chance of contamination by unwanted bacteria which may cause spoilage and brings about unwanted characteristics in the final products.

Furthermore, another part of this present study was to isolate and screen potential LAB with probiotic properties. Using standard in-vitro experimental tests as per ICMR-DBT guidelines and available literatures, all the isolated and identified LAB were characterized for their probiotic properties. Two-part of experiments were carried out to screen and evaluate the identified LAB for probiotic activity. Firstly, all LAB were subjected to primary test evaluation which includes- acid tolerance, bile tolerance, microbial attachments to hydrocarbons (MATH), auto-aggregation, co-aggregation, bile salt hydrolysis and antimicrobial activity; and secondly, secondary tests were employed to further characterize these LAB for few extra features of interest which includes cholesterol reduction, beta-galactosidase, exopolysaccharide production and GABA production. Furthermore, based on the available literature, few limited probiotic genes were also studied using PCR-based detection. Target genes used include (a) bile salt tolerance- *Ir0085*, *LBA1679*, *apf*, *LBA1432*, *LBA0552*, *LBA1429*, *LBA1429- F1*/*R1*, *LBA1446*, *Ir1584*, and *Ir1516*; (b) pH tolerance- *gtf*, *dltD*, *hdc*, *aguA*, *La995*, *clpL*, *LBA1272- F1*/*R1*, *LBA1272- F2*/*R2*, *groEl*, *odc*, and *tdc*; (c) bile salt hydrolase- *bsh*; (d)

attachment/adherence- *fbp*, *sbp*, *sor*, *mub*, and *msa*; (e) bacteriocin- *lcnA*, *lcnB*, *lcnK*, *mesB*, *mesY*, *Lactococcin A*, *nisR*, *Lac481*, and *durA*; (f) GABA- *gadA*, *gadB*, and *gadR*. Lastly, using standard statistical and in-silico analysis- PCA and heatmap visualization, grouping was achieved using primary and secondary tests. Based on these parameters above, *Levilactobacillus brevis* AcCh91 was selected as the best potential probiotic candidate, which may be developed as starter culture for milk fermentation.

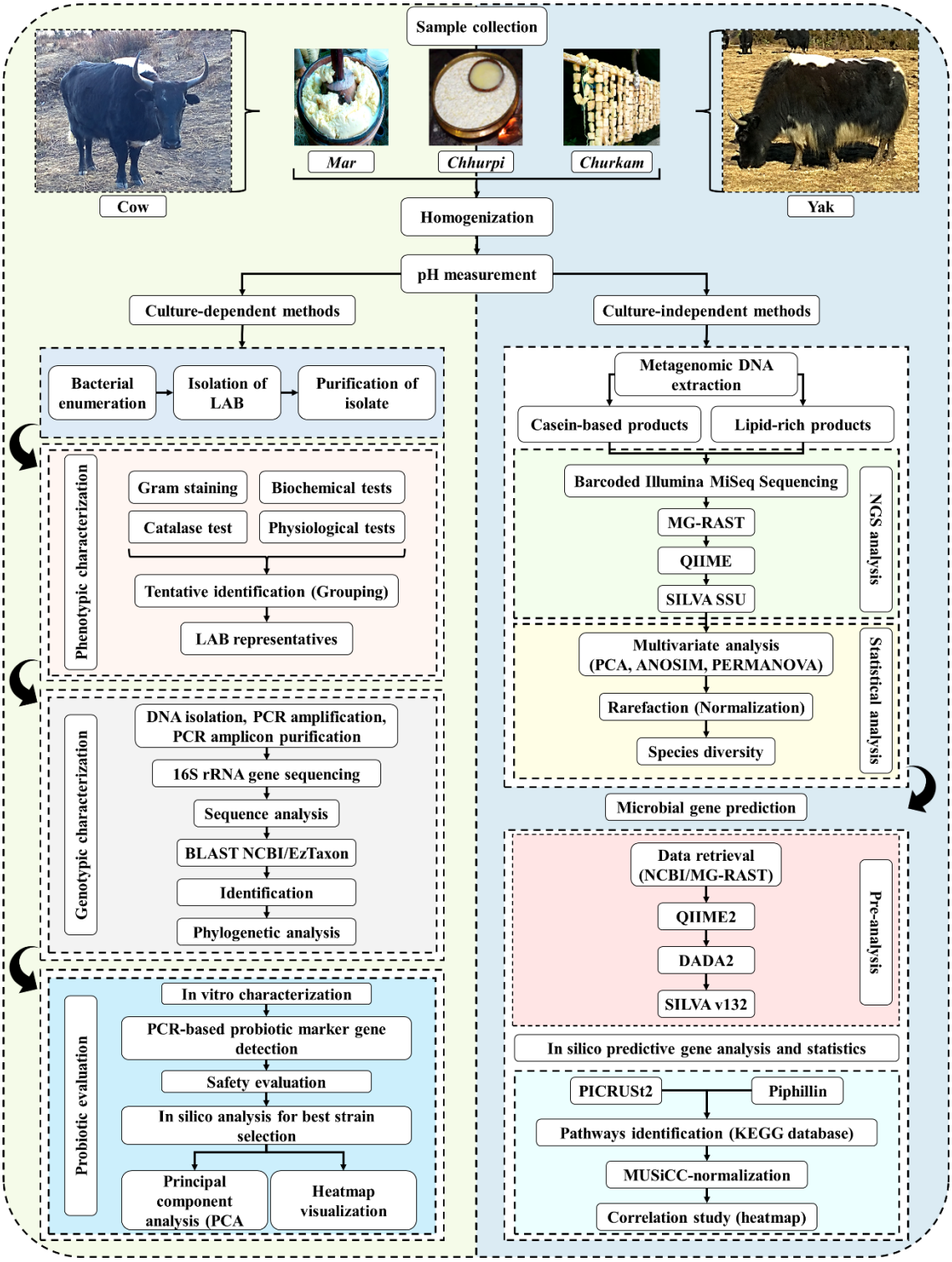
Another part of the Thesis was to study the functional properties using culture-independent data as speculated by bioinformatics tools. Here, we employed two commonly used pipelines- PICRUSt2 and Piphillin. QIIME2 was used for analyzing the raw reads (retrieved from NCBI/MG-RAST) and DADA2 was applied for generation of amplicon sequence variants (ASVs), which were then clustered using SILVA v132. Functional prediction was carried out simultaneously using PICRUSt2 and Piphillin, resulting in 6520 and 5201 KO (KEGG orthologs) features from PICRUSt2 and Piphillin predictive respectively. The KO profiles were then normalized using MUSiCC and mapped to 178 and 157 KEGG pathways from PICRUSt2 and Piphillin respectively. A high abundance of metabolism was observed from both prediction which indicates an active metabolic activity in the NFM products as proved of their high bacterial diversity. Since both the pipelines uses two different principles in indirect gene function prediction, significant differences between the two prediction pipelines were observed. However, a consolidated outcome from both the pipelines was considered for further analysis. Predominance of metabolism of amino acid, carbohydrate, lipid etc have also been reported to be associated with aroma and flavour development- an important feature in dairy industry. Since these natural products are an outcome of consortia of bacteria as observed from their huge bacterial diversity, some bacteria and their metabolic activity may have acted as precursors for other important fermenters or

inhibited unwanted bacteria; this may be correlating to the presence of functional features like metabolism of cofactors and vitamins- ubiquinone and other terpenoid-quinone biosynthesis and lipoic acid metabolism. Furthermore, correlation study was carried out to understand the possible interaction between the bacteria and functionalities. A high correlation of LAB has been well reported in cheeses as they are usually the predominant microorganisms in fermented milk products. Additionally, the presence of acetic acid bacteria in the NFM products is also of great importance as they have been well reported to be associated with many functional features like the production of secondary metabolites, volatile compounds, and essential amino acids in other fermented foods. The application of bioinformatics tools in prediction of indirect functional genes/features has been well applied in fermented milk products as supplementary analysis. Though these are just speculations based on in silico analysis, however, in the absence of shotgun metagenomics study, possible metabolic activity can be assumed using PICRUSt2 and Piphillin which was achieved in this present study. Therefore, in future studies following this present work, techniques such as shotgun metagenomics and metabolomics can be carried out for more in-depth holistic functionality and metabolite profiling.

Highlights of findings

- Culture-dependent analysis revealed the presence of *Enterococcus durans*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Lactococcus lactis* subsp. *cremoris*, *Levilactobacillus brevis*, *Loigolactobacillus coryniformis* subsp. *torquens*, *Lentilactobacillus parabuchneri*, and *Lactococcus lactis* subsp. *hordniae*.
- Culture-independent analysis based on high-throughput sequencing revealed the predominance of Firmicutes followed by Proteobacteria. Predominant species includes: lactic acid bacteria (LAB)- *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Latilactobacillus sakei* (Basonym: *Lactobacillus sakei*), *Lactococcus lactis*, *Lactococcus raffinolactis*, and *Leuconostoc mesenteroides*; acetic acid bacteria (AAB)- *Acetobacter lovaniensis*, *Acetobacter pasteurianus*, *Acetobacter syzygii*, *Acetobacter tropicalis*, *Gluconobacter cerinus*, and *Gluconobacter oxydans*; and few Non-LAB/AAB- *Bacillus cereus*, *Pseudomonas fluorescens*, *Staphylococcus cohnii*, and *Staphylococcus kloosii*.
- One promising LAB strains with potential probiotic properties was isolated, identified and characterized- *Levilactobacillus brevis* AcCh91.
- Predictive metabolic pathways analysis using PICRUSt2 and Piphillin reveals the presence of many functional features which are important in fermentation as well as in milk products such as flavour and aroma development, among others.

Schematic workflow of the complete PhD work accomplished in this Thesis



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CURRICULUM VITAE

Name	**:**	H. Nakibapher Jones Shangpliang
Email	**:**	nakibapher19@gmail.com hnjones.shangpliang@gmail.com
Address	**:**	Lumaha, Khliehshnong, Sohra (Cherrapunjee), East Khasi Hills, Meghalaya- 793108
Contact	**:**	+91 – 8906239770/7076884656

PhD Thesis **:** Phenotypic and Genotypic Identification of Microorganisms from Some Naturally Fermented Milk Products of Arunachal Pradesh

Qualification:

Degree	College and affiliating University	Month and year of joining	Month and year of passing	Division with total % of marks
M.Sc. (2 years)	Sikkim University	August 2012	August 2014	I (70.3%)

Research Experience:

Expertise in phenotypic and genotypic characterization of bacteria, molecular biology techniques which includes DNA isolation (including metagenomic DNA extraction), PCR amplification, PCR amplicons purification; DNA sequence analysis (Sanger sequencing), Phylogenetic tree construction, amplicon sequence analysis (Next-generation sequencing), bioinformatics tools like MG-RAST, QIIME, QIIIME2, PICRUSt, PICRUSt2, Piphillin etc.

Awards/Fellowships:

- Securing 1st Division (4th Rank) in BSc (*Microbiology*, Hons.) with 72.63%, Shillong College, Shillong (North Eastern Hill University, Shillong, Meghalaya).
- Presented the Silver Medal in MSc (*Microbiology*), Batch 2012-2014, Department of Microbiology, Sikkim University, Gangtok, Sikkim.
- Qualified the National Eligibility Test (NET) conducted by ARSB on 5 August 2016 in the Discipline of *Agricultural Microbiology*.
- Qualified GATE (Graduate Aptitude Test in Engineering) 2018 in *Life Sciences*, organized by Indian Institute of Technology Guwahati on behalf of the National Coordination Board (NCB) - GATE for the Department of Higher Education, Ministry of Human Resource Development (MHRD), Government of India.
- Junior & Senior Research Fellow under the project entitled: "Ethno-microbiology of some naturally fermented milk products of Sikkim and Arunachal Pradesh using culture-independent methods" sanctioned to Prof. Dr. Jyoti Prakash Tamang, from 2014 – 2018.
- Junior & Senior Research Fellow under the project entitled: "DBT-AIST International Center for Translational and Environmental Research (DAICENTER)" under Prof. Dr. Jyoti Prakash Tamang, from 2018 – 2022.

Oral/poster presented at National/International conferences: 08

Workshop/Training:

- Workshop on Mendeley and Plagiarism Software organized by Department of Zoology, Sikkim University on 24th – 25th October 2016.
- Internship on bioinformatics analysis (next generation sequencing NGS data analysis) at the Department of Biochemical Engineering and Biotechnology, IIT-Delhi, under the supervision of Prof. Durai Sundar, from March 4th to July 12th, 2019.
- Successfully completed three months advanced training in Biotechnology under DAILAB STAR Invitation Program from October 1st to December 28th, 2019, at the Biomedical Research Institute, AIST, Tsukuba Science City, Japan, under the supervision of Dr. Sunil C. Kaul and Dr. Renu Wadhwa.

Publications:

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2. **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. (Impact Factor: 5.640).
3. **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): 1-10. doi: 10.1038/s41598-018-19524-6. (Impact Factor: 4.379).
4. Bhutia, M.O., Thapa, N., **Shangpliang, H.N.J.** and Tamang, J.P., 2021. Metataxonomic profiling of bacterial communities and their predictive functional profiles in traditionally preserved meat products of Sikkim state in India. *Food Research International* 140: 110002. doi: 10.1016/j.foodres.2020.110002. (Impact Factor: 6.475).
5. Bhutia, M.O., Thapa, N., **Shangpliang, H.N.J.** and Tamang, J.P., 2021. High-throughput sequence analysis of bacterial communities and their predictive functionalities in traditionally preserved fish products of Sikkim, India. *Food Research International* 143: 109885. doi: 10.1016/j.foodres.2020.109885. (Impact Factor: 6.475).
6. **Shangpliang, H.N.J.** and Tamang, J.P., 2021. Phenotypic and genotypic characterisation of lactic acid bacteria isolated from exotic naturally fermented milk (cow and yak) products of Arunachal Pradesh, India. *International Dairy Journal* 118: 105038. doi: 10.1016/j.idairyj.2021.105038. (Impact Factor: 3.032).

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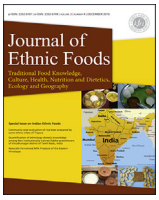

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Original article

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

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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).

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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.

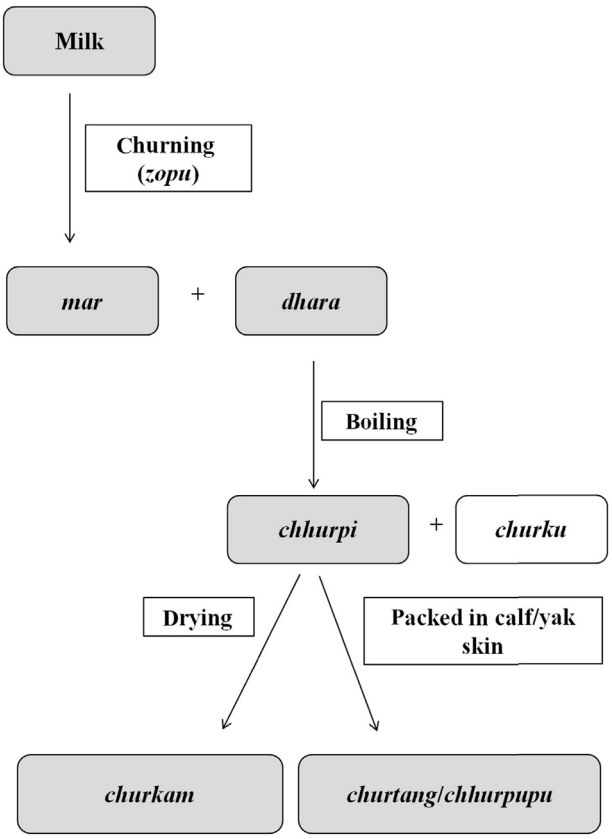

Fig. 1. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Arunachal Pradesh.

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

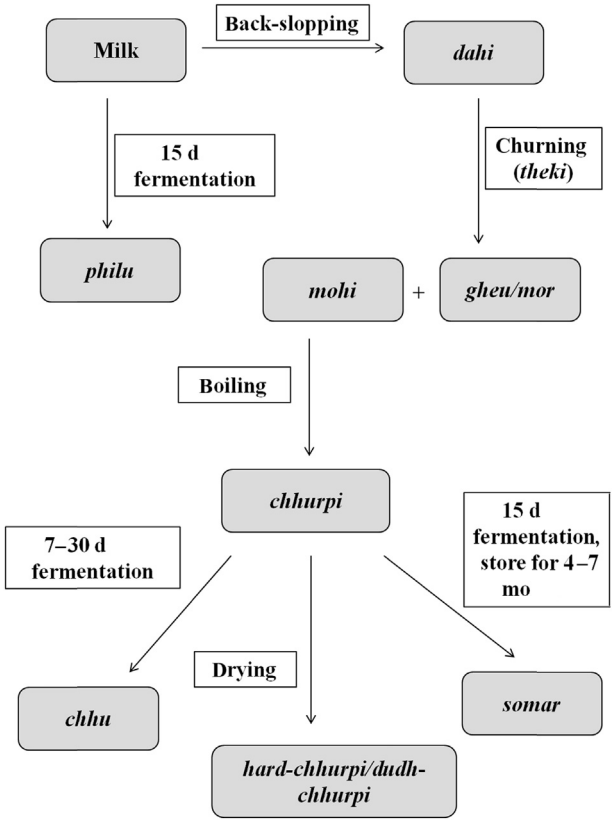

Fig. 3. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Sikkim and Darjeeling Hills in India, and Nepal.

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*.

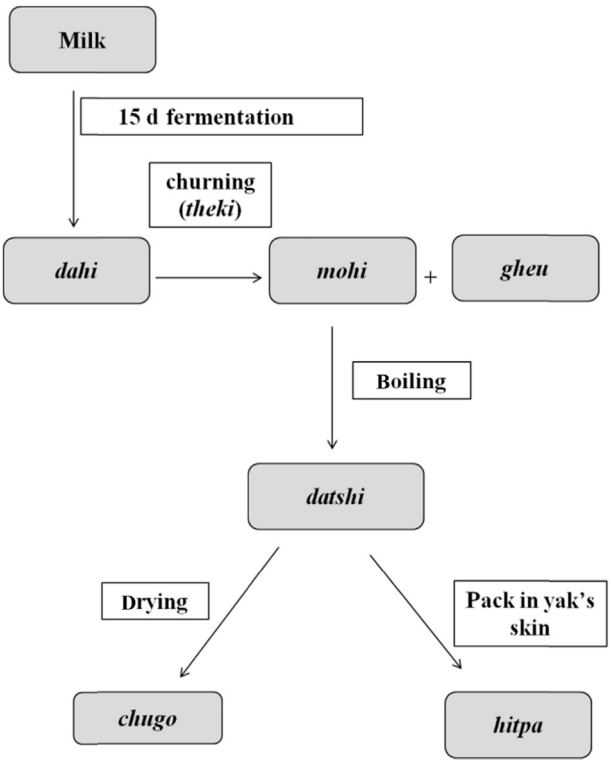

Fig. 5. Flowchart of the traditional method of preparation of ethnic naturally fermented milk products in Bhutan.

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

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ORIGINAL RESEARCH
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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

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****Correspondence:***
Jyoti P. Tamang
jyoti_tamang@hotmail.com

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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

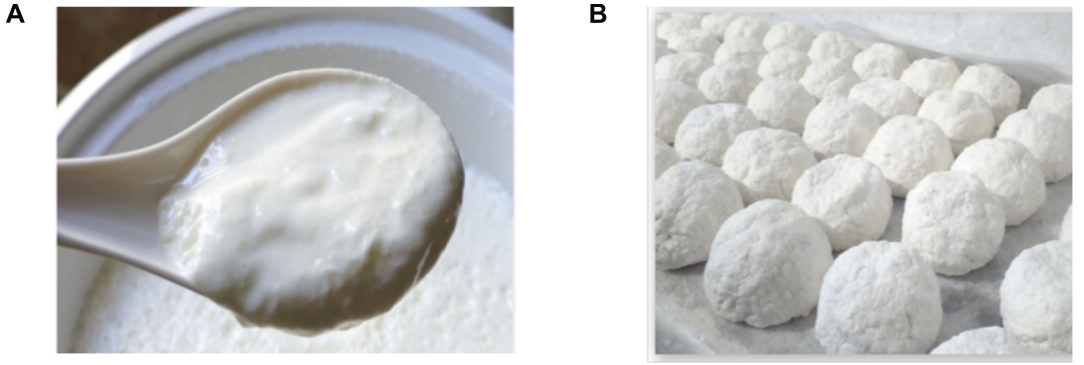

FIGURE 1 | (A) *Dahi* and **(B)** *datshi*.

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

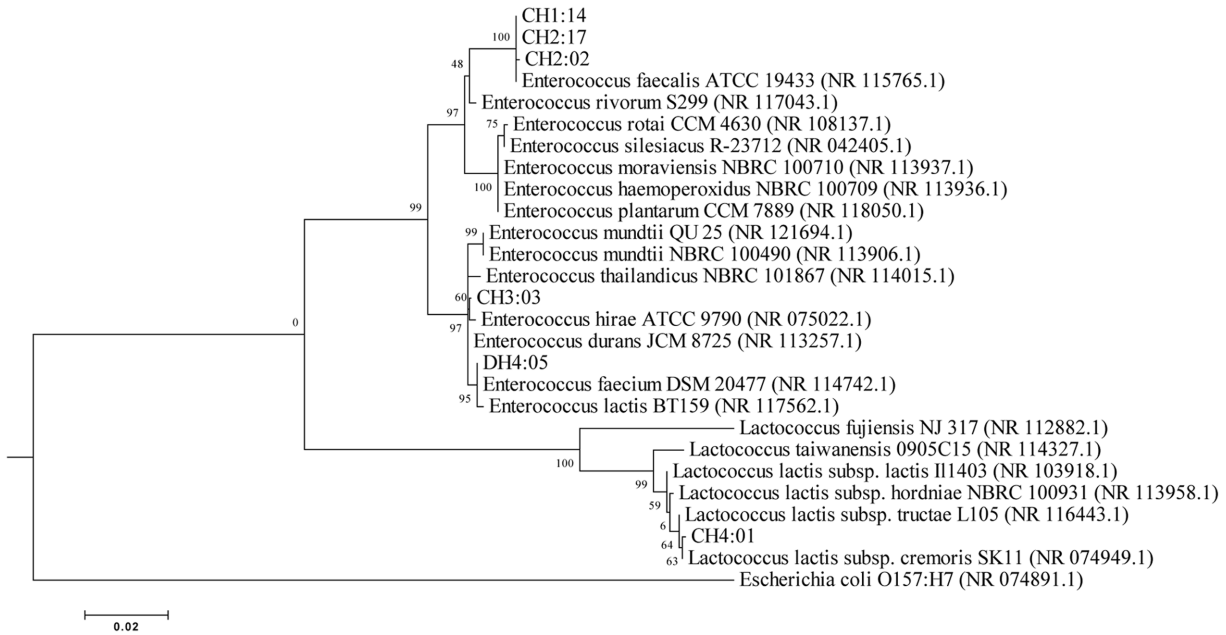

FIGURE 2 | Phylogenetic tree based upon the Neighbor-Joining of 16S rDNA sequences (*E. coli* 84 to 1437) derived by PCR with the primer 27F and 1492R.

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.

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SCIENTIFIC REPORTS

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Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing

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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)

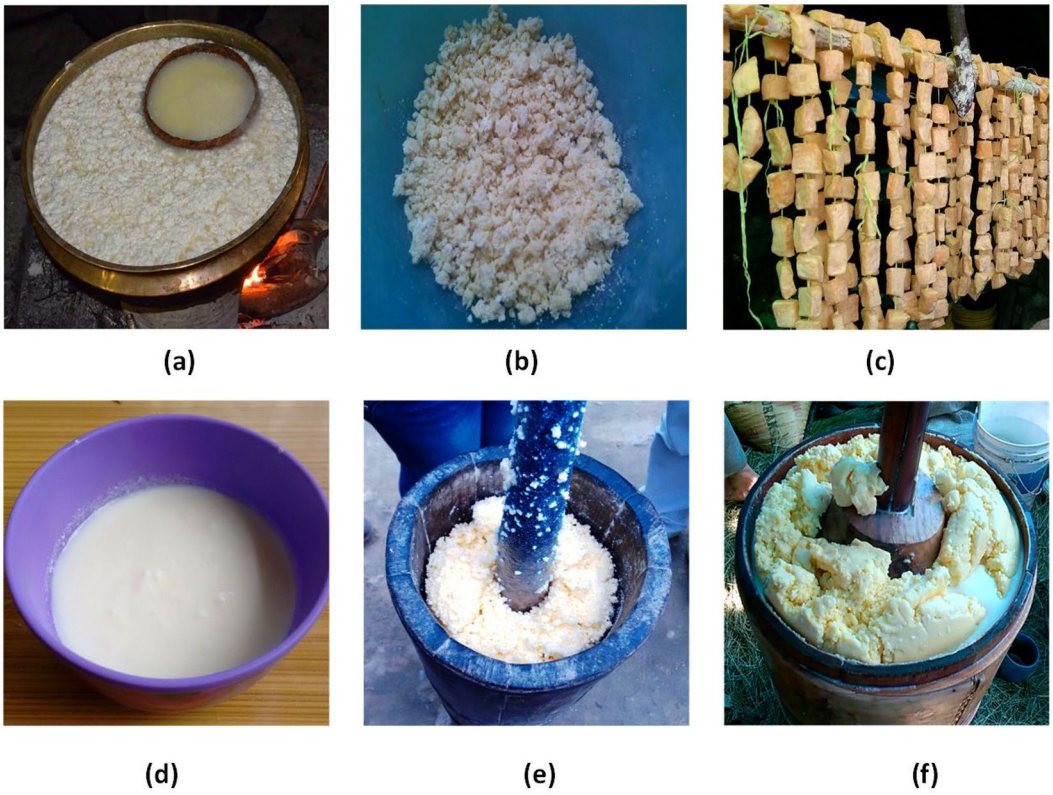

Figure 1. (**a**) *Chhurpi* of Arunachal Pradesh (AP); (**b**) *Chhurpi* of Sikkim; (**c**) *Churkam* of AP; (**d**) *Dahi* of Sikkim; (**e**) *Gheu* of Sikkim; (**f**) *Mar* of AP.

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).

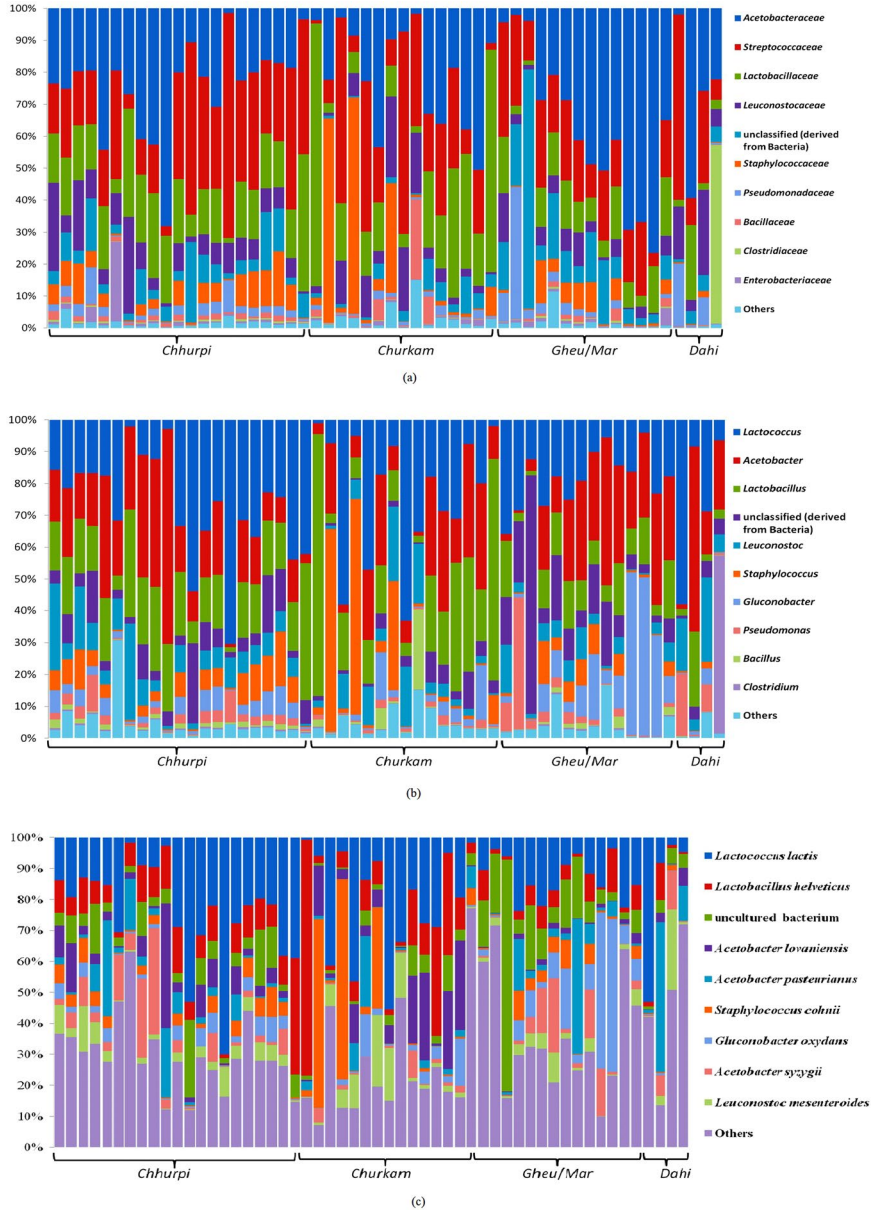

Figure 2. The overall bacterial composition of NFMs: *chhurpi*, *churkam*, *gheu*/*mar* and *dahi* at different taxonomic levels (**a**) Family, (**b**) Genus and (**c**) Species.

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).

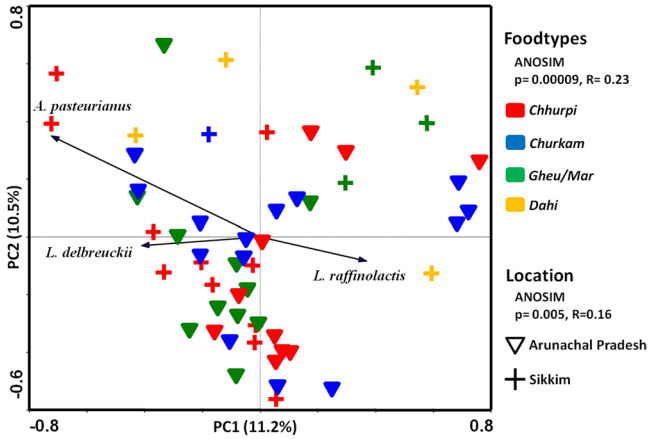

Figure 3. PCA plot shows the difference in bacterial community structure among the NFM products of Arunachal Pradesh and Sikkim. Arrow indicates the species direction. Significant difference is shown by ANOSIM analyzed with 10,000 permutations using Bray-Curtis distances.

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*.

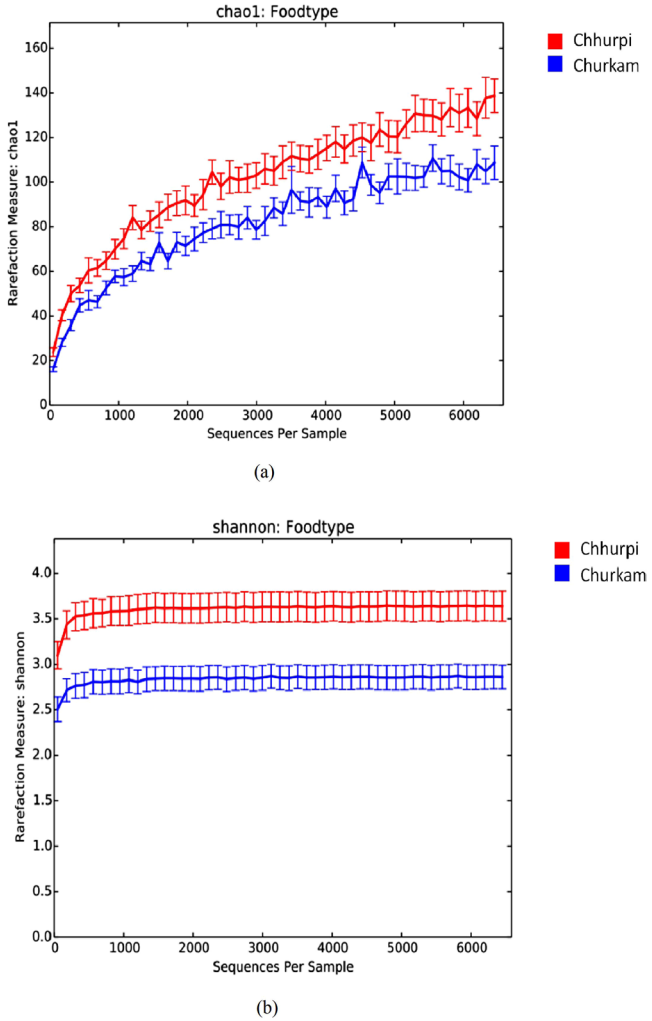

Figure 4. Difference in the bacterial alpha diversity indices of *chhurpi* and *churkam* (**a**) Chao1 species richness and (**b**) Shannon Diversity Index.

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

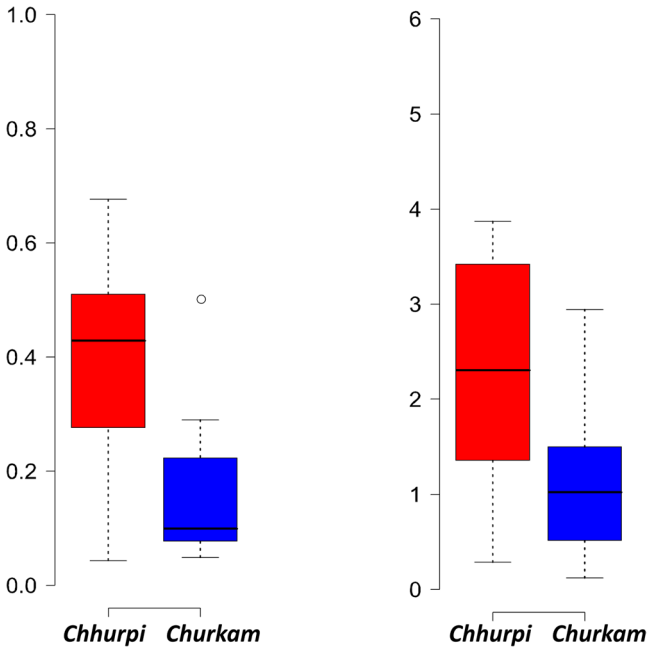

Figure 5. Boxplot showing the difference in the relative abundance of (**a**) *Clostridiaceae* and (**b**) *Pseudomonadaceae* between *chhurpi* and *churkam*.

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.

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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.

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High-throughput sequence analysis of bacterial communities and their predictive functionalities in traditionally preserved fish products of Sikkim, India

Meera Ongmu Bhutia [a], Namrata Thapa [b,*], H. Nakibapher Jones Shangpliang [a], Jyoti Prakash Tamang [a,**]

[a] DAICENTER (DBT-AIST International Centre for Translational and Environmental Research) and Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok 737102, Sikkim, India
[b] Biotech Hub, Department of Zoology, Nar Bahadur Bhandari Degree College, Sikkim University, Tadong 737102, Sikkim, India

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ABSTRACT

Traditionally preserved fish products viz. *suka ko maccha*, a smoked fish product, *sidra* and *sukuti,* sun-dried fish products are commonly consumed in Sikkim state in India. Bacterial communities in these fish products were analysed by high-throughput sequence (HTS) method supported by bioinformatics tool. Metataxonomic of the overall bacterial communities in samples revealed the abundance of phylum Firmicutes followed by Proteobacteria. *Psychrobacter* was abundant genus in all traditionally preserved fish products of Sikkim, followed by *Bacillus, Staphylococcus, Serratia, Clostridium, Enterobacter, Pseudomonas, Rummeliibacillus, Enterococcus, Photobacterium, Myroides, Peptostreptococcus, Plesiomonas* and *Achromobacter*. Product-wise distribution showed that *Bacillus* was abundant in *suka ko maacha* and *sidra* samples, whereas *Psychrobacter* was abundant in *sukuti* samples. Unique genus to each product was observed on the basis of analysis of shared operational-taxonomic-unit (OTU) contents, Alpha diversity indices showed significantly differences among the samples, and also showed maximum coverage as per Good's coverage (0.99). Beta diversity showed clustering of bacterial compositions between *suka ko maacha* and *sidra,* whereas *sukuti* showed scattering pattern among the other samples, indicating a diverse population in *suka ko maacha* and *sidr*a samples. Non-parametric analysis of abundant genera and predictive functionalities showed the complex bacterial inter-dependencies with predictive functionalities mostly in metabolism (79.88%).

1. Introduction

Nature harbours microorganisms in various biome-systems which synchronize the ecological diversity of various bio-resources including food ecosystems (Gibbons & Gilbert, 2015). People living nearby coastal regions, lakes and rivers traditionally preserve perishable fish by fermentation (Zang, Xu, Xia, & Regenstein, 2020), sun drying (Thapa, 2016), smoking (Olaleye & Abegunde, 2015) and salting (Tamang, Holzapfel, & Watanabe, 2016) for consumption as seasonings, condiments, curries and side-dishes. In Asia, traditionally preserved fish products are country-specific with various vernacular names for products such as *jeot kal* in Korea, *shottsuru* and *shiokara* in Japan, *yucha* in China *sukuti, sidra, ngari, hentak, tungtak* and *shidal* in India, *patis* in Philippines, *nam pla* and *pla ra* in Thailand (Devi, Deka, & Jeyaram, 2015; Koo et al., 2016; Thapa, 2016; Tamang et al., 2016; Tamang et al., 2020; Zang et al., 2020). These products are commonly prepared as fish-sauces in South Asia as condiments and seasonings except in India, Bangladesh, Nepal and Bhutan. Traditional methods of fermentation of fish from sea and rivers into various fish products are also common in Africa (Anihouvi, Kindossi, & Hounhouigan, 2012) and in few European countries (Skåra, Axelsson, Stefánsson, Ekstrand, & Hagen, 2015). Traditionally preserved fish products have some functional and autochthonous microorganisms (Lee, Jung, & Jeon, 2014; Zhang et al., 2016; Speranza et al., 2017; Zang et al., 2020), which may contribute to the formation of distinctive flavour, texture, and taste of the products (Wang, Xia, Gao, Xu, & Jiang, 2017; Bao et al., 2018). Traditionally

* Corresponding author at: Department of Zoology, Nar Bahadur Bhandari Degree College, Sikkim University, Tadong 737102, Sikkim, India.
** Corresponding author at: Department of Microbiology, Sikkim University, Tadong 737102, Sikkim, India.
E-mail addresses: namumani@hotmail.com (N. Thapa), jptamang@cus.ac.in (J.P. Tamang).

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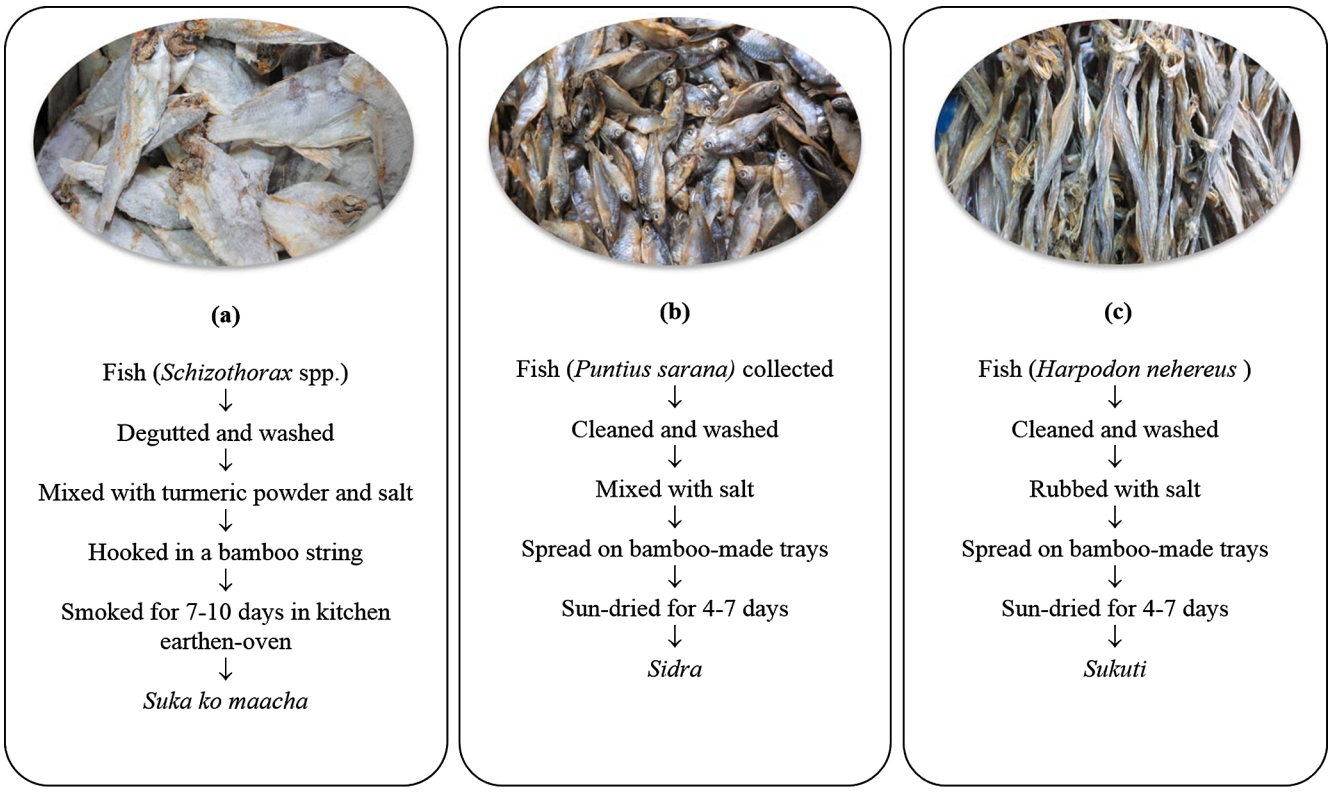

Fig. 1. Traditional methods of preparation of smoked and sun-dried fish products of Sikkim (a) *Suka ko maccha*; (b) *Sidra* and (c) *Sukuti*.

preserved fish products are also popular food items in Indian diets which include *ngari* (Thapa, Pal, & Tamang, 2004; Abdhul et al., 2014; Devi et al., 2015; Majumdar et al., 2015), *hentak* (Thapa et al., 2004; Aarti et al., 2017), *tungtap* (Thapa et al., 2004; Rapsang, Kumar, & Joshi, 2011), *shidal* (Majumdar, Roy, Bejjanki, & Bhaskar, 2016), *lona ilish* of Tripura (Majumdar & Basu, 2010; Das, Kumar, & Nayak, 2020); *sukuti* and *sidra* (Thapa, Pal, & Tamang, 2006), *bordia, karati* and *lashim* (Thapa, Pal, & Tamang, 2007), and *namsing* (Chowdhury, Goswami, Hazarika, Pathak, & Barooah, 2019). One of the important steps in traditional preparation of fish products is salting, a process that leads to preservation of fish with reduction of water content and pH (Zang et al., 2018). Water activity also influences the microbial composition effecting the shelf-life of the product (Kumar et al., 2017; Nagwekar, Tidke, & Thorat, 2017). The process of salting during the preparation of fish products may facilitate the predominance of halophilic and halotolerant bacteria (Samad, Jimat, & Shukor, 2017).

However, information on bacterial communities in traditionally preserved fish products is limited. Several bacterial genera have been reported in traditional fermented, sun-dried/smoked fish products of Asia, which include *Enterococcus, Lactobacillus, Weissella, Pediococcus*, *Tetragenococcus*, *Bacillus, Micrococcus, Staphylococcus*, *Haloanaerobium, Halomonas, Salinivibrio* and *Salimicrobium* (Thapa et al., 2006; Lee et al., 2014; Wang et al., 2017; Song et al., 2018; Das et al., 2020). Application of high-throughput sequence (HTS) analysis (Cox et al., 2017) with bioinformatics tool including pair-end read merger (PEAR) software (Zhang, Kobert, Flouri, & Stamatakis, 2014) and Quantitative Insights Into Microbial Ecology (QIIME2) (Boylen et al., 2019) are more reliable and accurate to study the bacterial diversity in fermented foods (Ercoloni, 2013; Tamang et al., 2020). Similarly, application of computational approach using the updated version of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) algorithm software (Douglas et al., 2020) provides predictive gene functionality of bacterial communities in different ecosystems (Langille et al., 2013; De Filippis, Parente, & Ercolini, 2017). Recently, bacterial communities in some fermented and sun-dried fish products of Asian countries have been profiled by high-throughput analysis (Song et al., 2018; Du, Zhang, Gu, Song, & Gao, 2019; Jiang et al., 2019).

Suka ko maacha is a smoked fish (*Schizothorax* spp.) product (Fig. 1a), *sidra* is a sun-dried fish (*Puntius sarana*) product (Fig. 1b), and *sukuti* is also a sun-dried fish (*Harpodon nehereus*) product (Fig. 1c). During traditional processing, fish are caught from rivers and cleaned, rubbed with salt, hooked in a bamboo-made sticks and are hung above the kitchen oven for smoking/sun-drying for 4–10 days (Fig. 1a-c). Moisture contents of *suka ko maacha*, *sidra* and *sukuti* are 7.6–11.3% (Bhutia, 2020), hence products may be stored for 6–8 months at room temperature in moist-free place. These traditionally preserved fish products are eaten as curry, fried, pickles and soup in local diets. Earlier report on microorganisms of *suka ko maacha, sidra* and *sukuti* showed species of *Lactococcus, Leuconostoc, Enterococcus, Pediococcus,* and *Weissella* revealed by limited phenotypic and biochemical tests (Thapa et al., 2006). Since these fish products are traditionally prepared without proper hygienic practices under natural conditions, food safety of products remains an important concern (Tamang et al., 2020). Moreover, profiling of bacterial communities in traditionally preserved fish products of Sikkim by high-throughput sequence analysis have not reported yet. Hence, we aimed to profile the bacterial communities in three different traditionally preserved fish products of Sikkim viz. *suka ko maacha, sidra* and *sukuti* by high-throughput sequence analysis. We also aimed to analyse predictive functionality based on marker gene sequences of bacteria in these products for predictive pathways and other functional properties by using PICRUSt2 (Douglas et al., 2020).

2. Materials and methods

2.1. Sample collection

Nine samples of traditionally preserved fish products: *suka ko maacha*, a smoked fish product (3 samples), *sidra*, a sun-dried fish product (3) and *sukuti*, a sun-dried fish product (3) were collected from different vendors in local markets of Gangtok, Singtam and Namchi of Sikkim in

India. The products were aseptically collected in sterile poly-bags kept in an icebox carrier, transported to the laboratory and stored at −20 ℃ for further analysis.

2.2. Homogenization of sample

Ten grams of each sample was homogenized in a 90 ml of sterile 0.1 M phosphate buffer saline (pH 6.4) using Stomacher 400 Circulator (Seward, UK) at 200 rpm for 2 min. After homogenization, the big debris was allowed to settle down for 5 min and the homogenate was used for DNA extraction.

2.3. Metagenomic DNA extraction

DNA was extracted from nine samples of fish products (3 each from *suka ko maacha*, *sidra* and *sukuti*) using Nucleospin® Food kit (MacherеyNagel GmbH & Co.KG, Düren, Germany) as per the manufacturer's instructions. Extracted DNA was then quantified using spectrometer (Eppendorf, USA). DNA quality was also checked on 0.8% agarose gel electrophoresis and visualized under Gel Doc EZ imager (BioRad, USA).

2.4. Sequencing using MiSeq Illumina platform

The 50 ng of DNA was used to amplify the V3-V4 region of the 16S rRNA gene using the primers pairs 341F 5′-GCCTACGGGNGGCWGCAG-3′ and 785R 5′-ACTACHVGGGTATCTAATCC-3′. The PCR amplification was achieved using PCR master mix containing DNA template, primers and KAPA HiFi HotStart ready mix (KAPA Biosystems, USA) with a final concentration of 100 nM (Klindworth et al., 2013). The PCR reaction was initially denatured at 95 °C for 5 min followed by 25 cycles at 95 °C for 30 sec, 55 °C for 45 sec and 72 °C for 30 sec, respectively, and finally extended for 7 min at 72 °C. Amplicons were purified using AMPure XP beads (Beckman Coulter, USA) to remove unused primers. Sequencing libraries were prepared using additional 8 cycles of PCR with Illumina barcoded primers with a read length of 2 × 300 bp and finally sequencing was run in an Illumina-MiSeq platform (Illumina, USA).

2.5. Bioinformatics analysis

Raw sequences generated from Illumina-MiSeq platform were extracted and imported into QIIME2 version 2019.10 (Bolyen et al., 2019) with paired-end reads via manifest-format (Paired End Fastq Manifest Phred33V2, https://docs.qiime2.org/2019.10/tutorials/importing/). First, the quality of the raw reads was checked using Fastqc (Andrews, 2010) and accordingly the adapter sequences were then trimmed by Trim Galore algorithm (Krueger, 2017). We have chosen to use an alternative joining method of the raw paired-end sequences using PEAR (Paired-End reAd mergeR) program (Zhang et al., 2014), which was then followed by importing the sequences into QIIME2 environment as single-end sequences (SingleEndFastqManifestPhred33 format). Quality filtering of the joined reads was then passed using q2-quality-filter (Bokulich et al., 2013) which was followed by denoising steps using deblur algorithm (q2-deblur denoise-16S) (Amir et al., 2017) against a positive filter (Greengenes 13_8). The resulting sub-operational-taxonomic-unit (sOTUs) were then aligned with multiple alignment using fast Fourier transform (mafft) (Katoh, Misawa, Kuma, & Miyata, 2002) (via q2-alignment). Approximately-maximum-likelihood phylogenetic tree from alignments of nucleotide sequences was created using fasttree2 (Price, Dehal, & Arkin, 2010) (via q2-phylogeny) for diversity analysis. Taxonomic assignment was achieved using the Greengenes reference databases (13_8_99% OTUs) (McDonald et al., 2012a) via q2-feature-classifier classify-consensus-vsearch (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). The taxonomic BIOM (biological observation matrix) (McDonald et al., 2012a) file was then exported and collapsed at different taxonomic level for further analysis.

2.6. Predictive gene functionality

The representative sequences along with their frequency tables were clustered in accordance to the Greengenes reference database (13_8_99% OTUs) via q2-vsearch-cluster-features-closed-reference (Rognes et al., 2016). Prediction of functional composition of marker gene (16S rRNA gene), based on the abundances of gene families in the samples, was deduced as per the standard integrated genomes database using PICRUSt2 algorithm (Douglas et al., 2020). Here, the clustered sequences were first aligned by multiple-sequence alignment of 16S sequences with HMMER (Howard Hughes Medical Institute, 2018), where the most likely placements of the 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 & Stamatakis, 2019) were applied. Castor R package (Louca & Doebeli, 2018) was applied in PICRUSt2 pipeline for prediction of gene families with the default run (maximum parsimony). Metagenome prediction was run using (metagenome_pipeline.py) (Ye & Doak, 2009) and the output features were mapped into KEGG (Kyoto Encyclopaedia of Genes and Genomes) database for systematic analysis of gene functions (Kanehisa & Goto, 2000). Pathway levels of defined gene functionality with high-level function (Level 1) viz., metabolism, environmental information processing, genetic information processing, human diseases, cellular processes, organismal system and drug development were interpreted, which was further divided into lower Level 2 (Scala, Serra, Marwah, Saarimäki, & Greco, 2019) and were mapped with gene copy numbers (reads) using 16S rRNA sequences profile inferred by PICRUSt2 against KEGG database in samples (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017).

2.7. Statistical analysis

Alpha diversity metrics including observed OTUs (defined as qualitative measures of a community with abundance), Shannon's diversity index, Faith's Phylogenetic Diversity, Evenness) were studied using q2-core-metrices-phylogenetic (Kim, 2017). Beta diversity (Bray-Curtis distance) was estimated using q2-diversity (Astudillo-Melgar, Ochoa-Leyva, Utrilla, & Huerta-Beristain, 2019). Alpha and beta diversities were conducted using hypothesis testing [ANOSIM (Analysis of Similarities and PERMANOVA (permutational multivariate analysis of variance)] (Anderson, 2001) and were checked in QIIME2-2018.10 environment (McMurdie & Holmes, 2013). Student's *t*-test was also applied for alpha diversity metrics. Graphical representation of shared and unique genera (with > 1% relative abundance) among the three different samples, obtained from each sample in triplicate, were visualized using iGraph R-package. Beta diversity was analysed using Bray-Curtis dissimilarities and by using PASTv4, PCA (principal component analysis) plot was constructed. Statistical analysis for predictive functional features was applied using STAMP (statistical analysis of metagenomic profiles) software by ANOVA (analysis of variance) hypothesis testing method (Parks, Tyson, Hugenholtz, & Beiko, 2014). Significance of genera and predictive functional features among the samples was also analysed using White's non-parametric *t*-test, (Parks et al., 2014). Non-parametric correlation (Spearman's correlation) of the abundant genera with the predictive functionalities was tested using SPSS v20 and the graphical data visualization was represented as a heatmap.

3. Results

3.1. Bacterial communities

We obtained a total raw sequence reads of 3,179,621 from sequencing out of which, 2,993,524 quality-filtered reads were analysed, and then denoised using deblur algorithm producing a total of 71,5415 sOTUs. Taxonomic classification of the generated sOTUs was achieved using Greengenes database and was represented in three

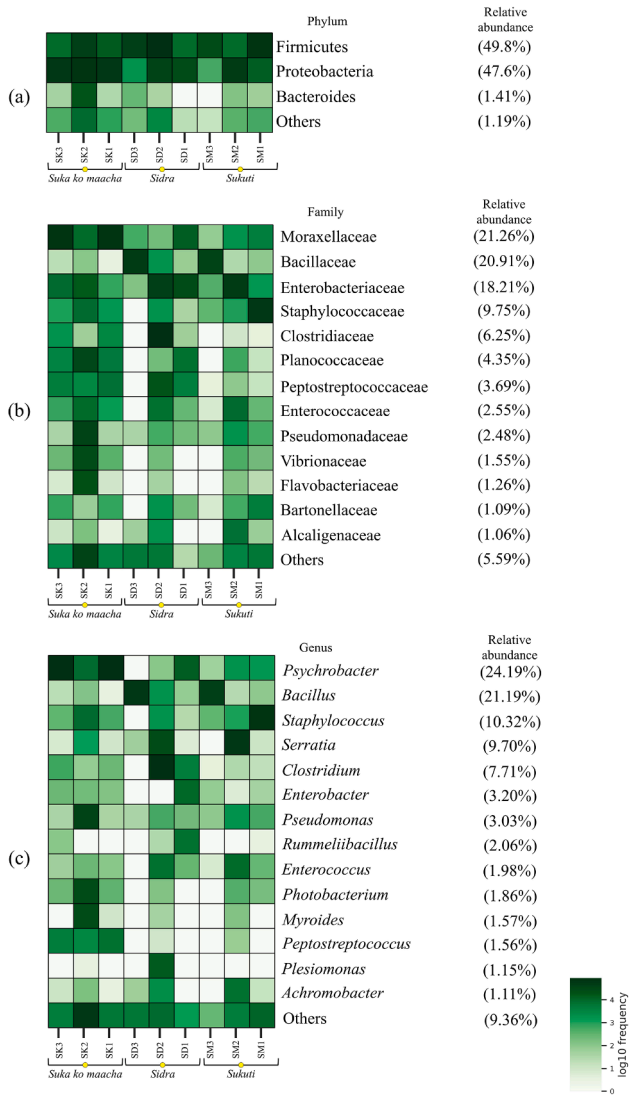

Fig. 2. Heat-map representation of bacterial diversity in traditionally preserved fish products of Sikkim distributed at four taxonomic levels - (a) Phylum (b) Family and (c) Genus. The sub-OTUs-frequency table was plotted using q2-feature-table heatmap and normalized by log10 frequency with each of the taxon relative abundances shown.

taxonomic levels- phylum, family and genus. Reads were then normalized as relative percentages for data visualization and taxonomic abundances with $>1\%$ were shown in the graphs and those $<1\%$ were grouped as 'others'. It is noteworthy that we captured a maximum diversity from sequencing of the samples. Metataxonomic study of bacterial communities in all nine samples showed Firmicutes as the most abundant phylum (Fig. 2a), Moraxellaceae as the abundant family (Fig. 2b), *Psychrobacter* as the abundant genus (Fig. 2c). In samples of *suka ko maccha*, Firmicutes was the most abundant phylum followed by phylum Proteobacteria (Fig. 3a). Similarly, Firmicutes was also found abundant phylum in *sidra* samples (Fig. 3b). Bacillaceae was abundant family in both *suka ko maacha* and *sidra* samples, with *Bacillus* as the most abundant genus (Fig. 3a-3b). Contrastingly, in *sukuti* samples, we observed the phylum Proteobacteria as the most abundant phylum followed by Firmicutes and Bacteroidetes (Fig. 3c). *Sukuti* was predominated by the family Moraxellaceae which was represented by *Psychrobacter* as the most abundant genus. Graphical representation of the shared and unique genera was visualized using iGraph-package where we observed several genera unique to each product. *Acinetobacter*, *Achromobacter* and *Kushneria* were found to be unique genera in *suka ko maacha* samples. *Rummeliibacillus*, *Clostridium, Enterobacter* and *Plesiomonas* were unique genera found in *sidra*. *Cetobacterium*, *Proteus*, *Oceanimonas*, *Peptostreptococcus*, *Vagococcus*, *Photobacterium*, *Myroides* and *Vibrio* were unique genera in samples of *sukuti*. *Enterococcus*, *Serratia* and *Bacillus* were shared genera between *suka ko maacha* and *sidra*. *Staphylococcus* and *Pseudomonas* were shared between *suka ko maacha* and *sukuti*. The core genus shared amongst all nine samples was *Psychrobacter* (Fig. 4). Huge diversity of bacteria with<1% abundance was detected in all samples (Supplementary Table 1).

3.2. Diversity

Alpha and beta diversities were calculated in QIIME2-2019.10 environment to understand the bacterial community structure in traditionally preserved fish products. Alpha diversity metrics showed significant ($p > 0.05$) between *sidra* and *sukuti* as per ace, chao1, Fisher's alpha index (Table 1). However, we observed significance ($p > 0.05$) in terms of Shannon indices between *suka ko maacha* and *sukuti*. Good's coverage of 0.99 was observed (Table 1). Beta diversity showed clustering of bacterial compositions between *suka ko maacha* and *sidra,* whereas *sukuti* showed scattering pattern among the other samples, indicating a diverse population from *suka ko maacha* and *sidr*a samples as represented by PCA plot (Fig. 5).

3.3. Predictive gene functionality

The functional potentials of the annotated genes inferred by PICRUSt2 were mapped against KEGG database for systematic analysis of gene functions. The predictive functionality of samples of *suka ko maacha, sidra* and *sukuti* were primarily classified into six categories - metabolism (79.87%), genetic information processing (11.89%), cellular processes (4.35%), environmental information processing (3.21%), human diseases (0.34%), and organismal systems (0.31%). Analysis of sub-pathways level of metabolism revealed 36 KEGG pathways in bacterial genes of samples (Supplementary Table 2). The predictive functionality across the products were relatively similar. Significant differences amongst the predicted pathways were analyzed by STAMP ($p < 0.05$). Metagenome contribution of genera with $> 1\%$ abundance was also computed to acquire the sOTUs-functionality. Geraniol degradation was contributed only by *Psychrobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Photobacterium*, *Myroides*, *Plesiomonas* and *Achromobacter*. Phosphotransferase system (PTS) was contributed by *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Photobacterium*, *Peptostreptococcus*, *Plesiomonas* and *Achromobacter*. Among the human diseases-related pathways, *Staphylococcus aureus* infection was found to be associated with the genera *Bacillus*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *Enterococcus*, *Peptostreptococcus* and *Achromobacter*. However, only one genus *Photobacterium* is associated with *Vibrio cholerae* infection pathway. STAMP analysis resulted in the following significant pathways: amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, glycolysis/gluconeogenesis, carbon fixation pathways, oxidative phosphorylation, biotin metabolism, nicotinate and nicotinamide metabolism, one carbon pool by folate, seleno-compound metabolism, geraniol degradation), protein export, ribosome biogenesis in eukaryotes and phosphotransferase system (Fig. 6).

3.4. Non-parametric analysis of abundant genera and predictive functionality

White's non-parametric *t*-test analysis of abundant genera showed significance differences between *sukuti* samples with that of *sidra* and *suka ko maacha*, where abundances of *Psychrobacter* and *Peptostreptococcus* were significantly higher in *sukuti* than in *sidra* and *suka ko*

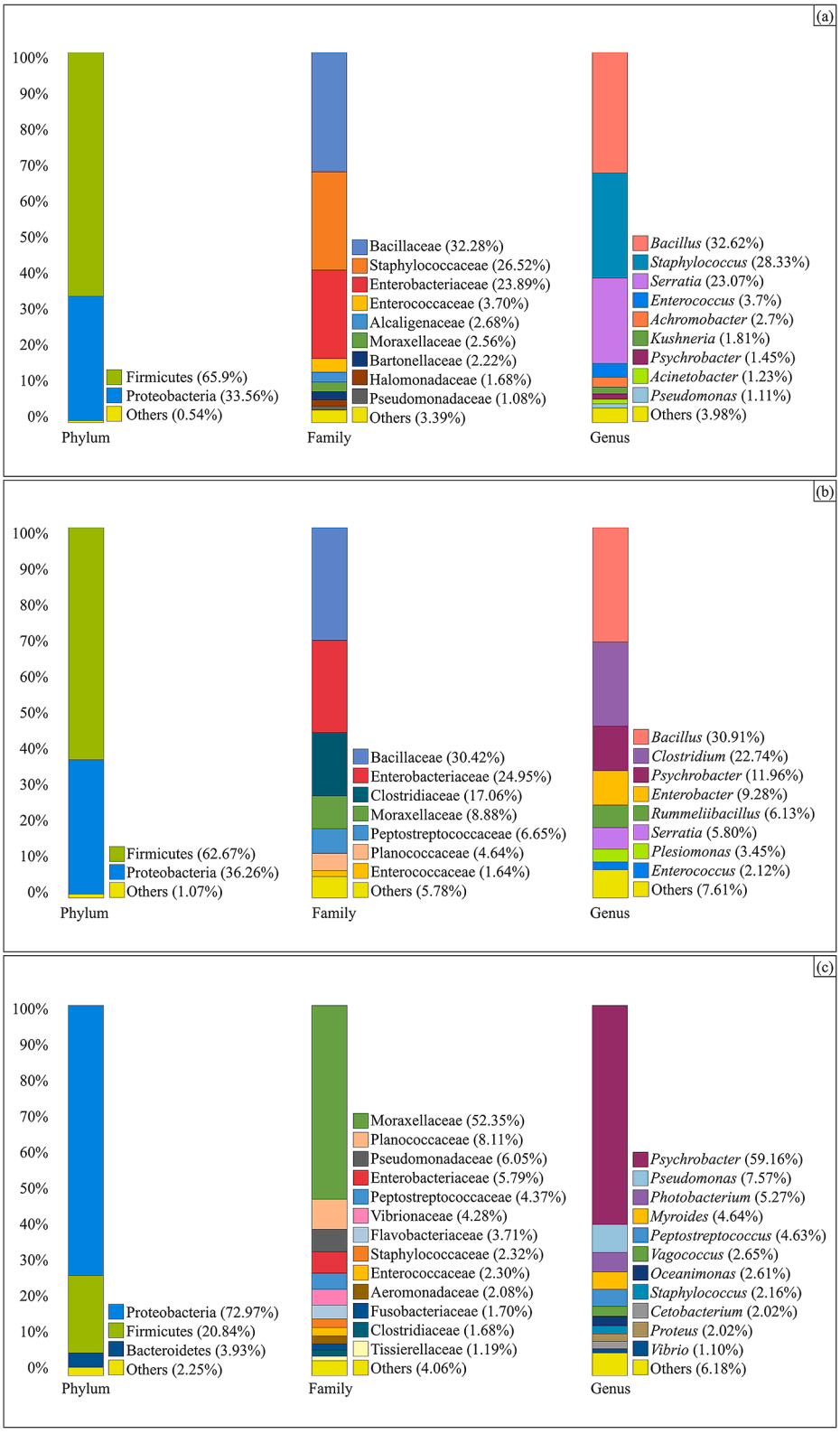

Fig. 3. Bar-chart representation of the bacterial composition of each of the traditionally preserved fish products (a) *Suka ko maacha* (b) *Sidra* and (c) *Sukuti*. Bacterial distribution is represented in three taxonomic levels - Phylum, Family and Genus.

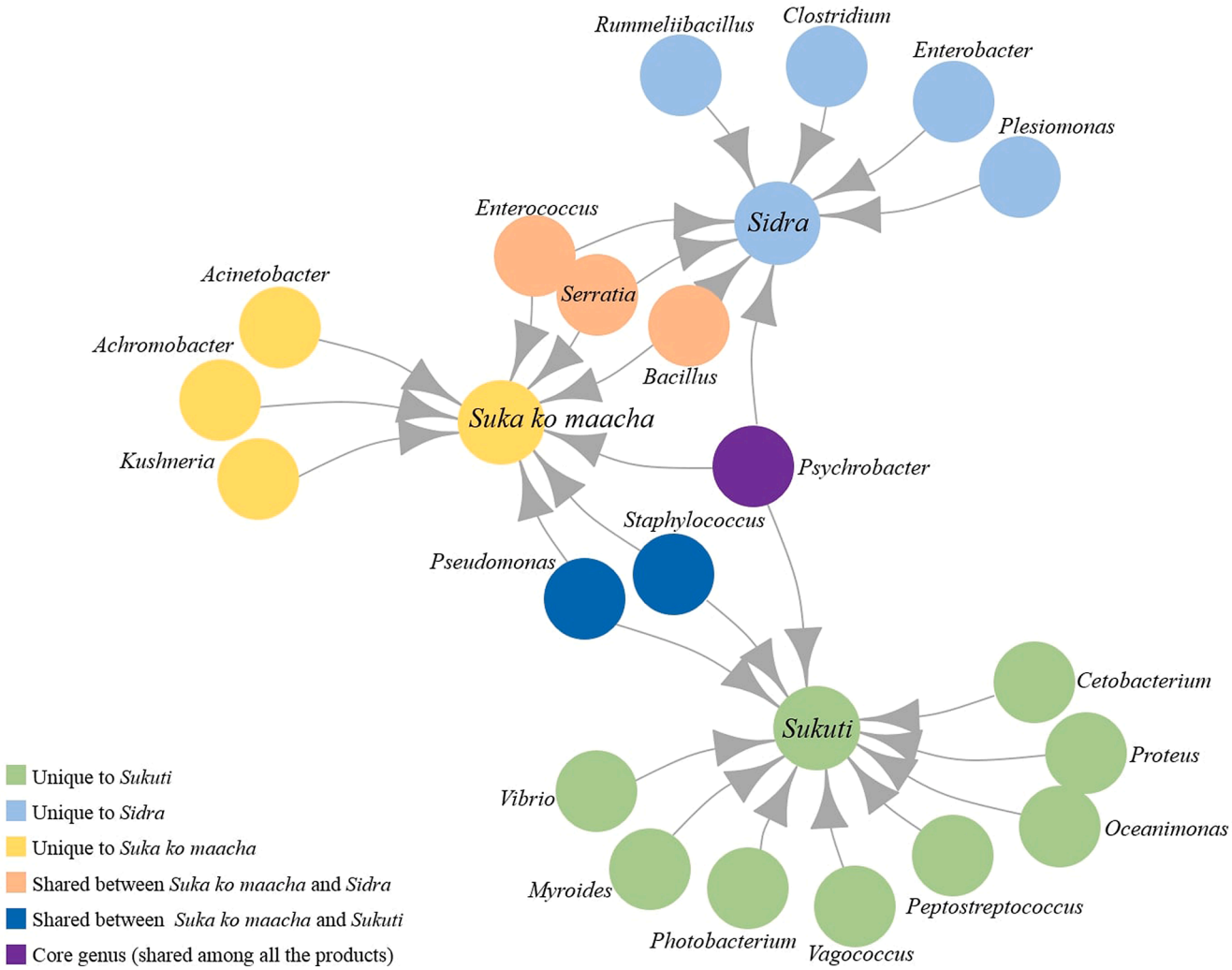

Fig. 4. Graphical representation of the shared genera among the three traditionally preserved fish products. Each circle represents the genera that was obtained from the 16S-targeted amplicon sequencing study where the difference in the shared and unique genera amongst the tree products are shown.

Table 1
Alpha diversity profiles of the traditionally preserved fish products of Sikkim.

Alpha diversity metrices	*Suka ko maacha* (SM)	*Sidra* (SD)	*Sukuti* (SK)	p-value		
				SM vs SD	SD vs SK	SM vs SK
Ace	261.95 ± 146.88	214.79 ± 147.56	393.13 ± 89.83	0.444	0.034	0.138
Chao1	257.94 ± 147.06	212.44 ± 143.61	390.07 ± 88.16	0.45	0.032	0.136
Fisher Alpha	33.41 ± 20.33	26.3 ± 17.74	50.36 ± 9.64	0.337	0.037	0.164
Shannon	2.71 ± 0.79	3.74 ± 1.48	5.55 ± 0.42	0.124	0.151	0.02
Simpson	0.64 ± 0.15	0.78 ± 0.24	0.95 ± 0.01	0.131	0.329	0.068
Good's coverage	0.99	0.99	0.99			
Observed OTUs	249.33 ± 140.75	208 ± 140.38	383.33 ± 85.54			

maacha (Fig. 7). No significant differences of predictive functional features were observed between *suka ko maacha* and *sidra*, however, significant differences was observed in *sukuti* samples with other products. Several predictive functional features including fructose and mannose metabolism, D-alanine metabolism, glycerolipid metabolism, selenocompound metabolism, nitrotoleune degradation, galactose metabolism, starch and sucrose metabolism, sphingolipid metabolism, amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, and *Staphylococcus aureus* infection were observed significanlty higher in *sidra*; whereas geraniol degradation, lipopolysaccharide biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthesis, glutathione metabolism and biotin metabolism were significantly higher in *sukuti* (Fig. 7). On the other hand, significant differences were also observed between *suka ko maacha* and *sukuti*, where galactose metabolism, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, taurine and hypotaurine metabolism, ascorbate and aldarate metabolism, *Staphylococcus aureus* infection, and butanoate metabolism were significantly higher in *suka ko maacha*; whereas biotin metabolism, fatty acid degradation, riboflavin metabolism, nicotinate and nicotinamide metabolism, and lipopolysaccharide biosynthesis were significantly higher in *sukuti* (Fig. 7).

Non-parametric correlation study of the predominant genera with the predicted microbial functional features was calculated using Spearman's correlation. Significant positive correlation was observed between biosyntesis of ansamycins with the genera *Clostridium*, *Enterococcus* and *Achromobacter*. *Psychrobacter* and *Peptostreptococcus* showed significant positive correlation with several predictive functional features that included synthesis and degradation of ketone bodies, biotin metabolism, nicotinate and nicotinamide metabolism and lipopolysaccharide biosynthesis (Fig. 8). Contrastingly, these two genera also

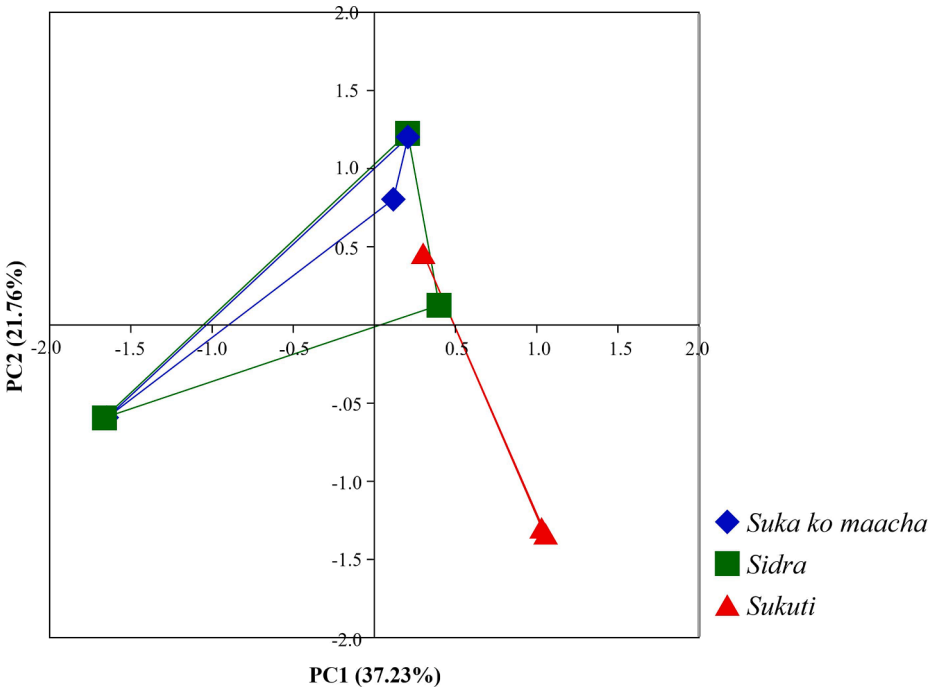

Fig. 5. Beta diversity comparison as per Bray-Curtis dissimilarities matrix represented by Principal Component Analysis (PCA).

showed to exhibit significant negative correlation with D-alanine metabolism, fructose and mannose metabolism, starch and sucrose metabolism, and galactose metabolism. Tyrosine metabolism, toluene degradation, cyanoamine acid metabolism was also observed to have a significant correlation with *Psychrobacter* (Fig. 8). Additionally, *Bacillus* showed significant positive correlation with alanine, aspartate and glutamate metabolism, D-Arginine and D-ornithine metabolism and inositol phosphate metabolism and exhibited a significant negative correlation with geraniol degradation, riboflavin metabolism, tyrosine metabolism, toluene degradation and phenylalanine metabolism. Glycerolipid metabolism showed a significant negative correlation with *Pseudomonas* and *Photobacterium*. Significant negative correlation was also observed between lysine degradation and *Enterococcus*. Additionally, significant negative correlation was also observed between *Achromobacter* and D-glutamine and D-glutamate metabolism; and between *Plesiomonas* and tryptophan metabolism. Lastly, *Achromobacter* showed a significant positive correlation with styrene degradation (Fig. 8).

4. Discussion

4.1. Bacterial community and diversity

We applied high-throughput sequence method supported by the bioinformatics software to profile the bacterial communities in *suka ko maacha, sidra* and *sukuti*, which revealed a huge diversity of both Gram-positive and Gram-negative bacteria including lactic acid bacteria (LAB) and non-LAB bacterial community. Abundance of Firmicutes in fermented fish products of Asia was reported earlier (Zang et al., 2018; Ohshima et al., 2019), which was also observed in traditionally preserved fish products of Sikkim. Shared genera analysis showed Gram-negative bacterium *Psychrobacter* as the core abundant genus in all three different fish products of Sikkim with significantly higher in abundance in *sukuti*. This difference in abundance may be due to different types of raw fish used to prepare each product (Zang et al., 2020). *Psychrobacter* was reported to be associated with diverse habitats such as fermented milk products, cold-storage meat products, fish products, fermented seafoods, and clinical sources (Bakermans et al., 2006; Bjerke et al., 2019). The process of salting during the traditional method of preparation of *suka ko maacha*, *sidra* and *sukuti* might have favoured several halophilic or halotolerant bacteria (Samad et al., 2017). *Psychrobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Photobacterium*, *Peptostreptococcus*, and *Achromobacter* have also been reported from various types of fermented fish and salted fish products (Daroonpunt, Itoh, Kudo, Ohkuma, & Tanasupawat, 2016; Kobayashi et al., 2016; Zhang et al., 2016; Zheng et al., 2017; Osimani et al., 2019; Ohshima et al., 2019; Keisam, Tuikhar, Ahmed, & Jeyaram, 2019; Karyantina, Anggrahini, Utami, & Rahayu, 2020). In *suka ko maacha* and *sukuti*, we detected genera associated with halophilic habitats that included *Pseudomonas* and *Staphylococcus*. Similarly, in *sidra*, halotolerant genera such as *Serratia* and *Plesiomonas* were detected. *Kushneria*, another halophilic bacterium reported from traditional cured meat products (Zou & Wang, 2010), was also detected from *suka ko maacha*. Most of the bacteria reported from salted fish products are known to be moderately halophilic or halotolerant in nature (Visciano, Schirone, Tofalo, & Suzzi, 2012; Phewpan et al., 2020; Xu, Zang, Regenstein, & Xia, 2020). *Acinetobacter, Myroides, Serratia* and *Plesiomonas* are considered as opportunistic pathogens or as food-spoiling bacteria in fish products (Thomas, Johney, & Ragunathan, 2018; Kahraman et al., 2017; Zotta, Parente, Ianniello, De Filippis, & Ricciardi, 2019; Zhong et al., 2019). However, *Myroides* was detected only in *sukuti* samples, *Serratia* and *Plesiomonas* were detected in *sidra* samples; and *Acinetobacter* was detected only in *suka ko maacha* samples. The occurrence of these different pathogenic groups in fish products might have contaminated during the traditional preparation processes including handling, storage and transportation. (Doeun, Davaatseren, & Chung, 2017). We observed a higher abundance of *Clostridium* in *sidra* samples, an anaerobic bacterium, which is responsible for causing food-borne gastrointestinal diseases (Freedman, Shrestha, & McClane, 2016). *Photobacterium* is a food spoilage bacterium reported in fresh meat and fish (Fuertes-Perez, Hauschild, Hilgarth, & Vogel, 2019). Fish and its products are regarded as a potential source of pathogenic and spoilage bacteria such as species of *Vibrio, Staphylococcus Photobacterium, Escherichia, Aeromonas, Salmonella, Plesiomonas, Listeria* and *Clostridium* which may cause infections and intoxication in humans (Novotny, Dvorska, Lorencova, Beran, & Pavlik, 2004). Interestingly, HTS analysis revealed the

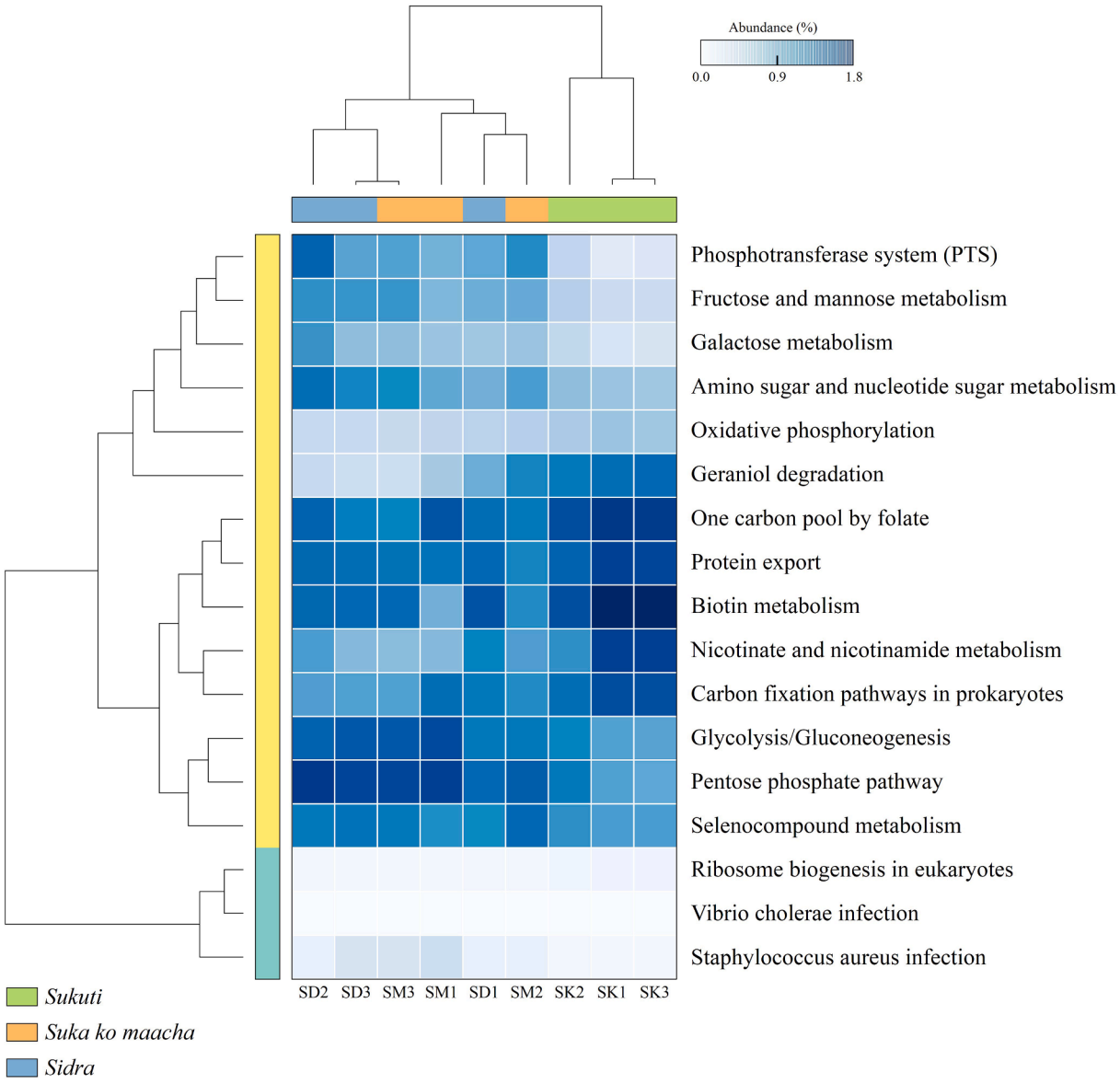

Fig. 6. Heatmap (using STAMP software) showing the statistically significant ($p < 0.05$) predictive functionality observed in the traditionally preserved fish products through PICRUSt2 against KEGG database.

presence of *Rummeliibacillus* only in *sidra* samples. Though *Rummeliibacillus* has been reported from diverse geographical locations (Vaishampayan et al., 2009), it has also been isolated from fish gut and have been successfully used as a probiotic for fishes (Tan, Chen, & Hu, 2019). *Cetobacterium* has been reported in Greenlandic dried fish (Hauptmann et al., 2020), and species belonging to this genus have been associated with fish gut microbiota (Li et al., 2015). Other genera which are known to be included as fish gut microbiota includes *Pseudomonas*, *Vibrio*, *Photobacterium*, *Clostridium*, *Acinetobacter*, *Bacillus*, and *Achromobacter* (Egerton, Culloty, Whooley, Stanton, & Ross, 2018). Though *Vibrio* is commonly found in marine fish, however, its presence has also been reported from freshwater-based fish products of China such as *yucha* (Zhang et al., 2016) and *chouguiyu* (Yang et al., 2020). *Oceanimonas*, known to be marine bacterium, is a halophilic in nature (Liu et al., 2019), was observed only in *sukuti* samples.

4.2. Predictive functionality

Predicting microbial functions from 16S rRNA genes have been widely studied using PICRUSt (Ortiz-Estrada, Gollas-Galván, Martínez-Córdova, & Martínez-Porchas, 2019), and it has been improved to PICRUSt2 (Douglas et al., 2020). Application of PICRUSt2 algorithm and correlation analysis showed complex bacterial inter-dependencies with predictive metabolic pathways mostly the metabolism (79.88%) category in traditionally preserved fish microbiome data, which included carbohydrate metabolism, amino acid metabolism and lipid metabolism as predominant super-pathways. Microbial metabolism is an important process that adds to flavour development in fermented fish products (Mouritsen, Duelund, Calleja, & Frøst, 2017; Xu et al., 2018). Among many important predictive microbial functions, *Bacillus* showed significant positive correlation with amino acid metabolisms in *suka ko maacha* and *sidra*. *Bacillus* may be responsible for flavour development in these products (Wang et al., 2017; Zang, Xu Xia, & Regenstein, 2020). *Psychrobacter,* which was significantly predominant in *sukuti*, showed positive correlation with other amino acid metabolism. Perhaps, *Psychrobacter* detected in *sukuti* was one of the main contributors to metabolic activities, that may lead to flavour development in these products (Zhao & Eun, 2020). During the decaying period in the fermentation process, microorganisms contribute to flavour compounds by undergoing amino acid metabolism (Ardö, 2006). The lipases secreted by

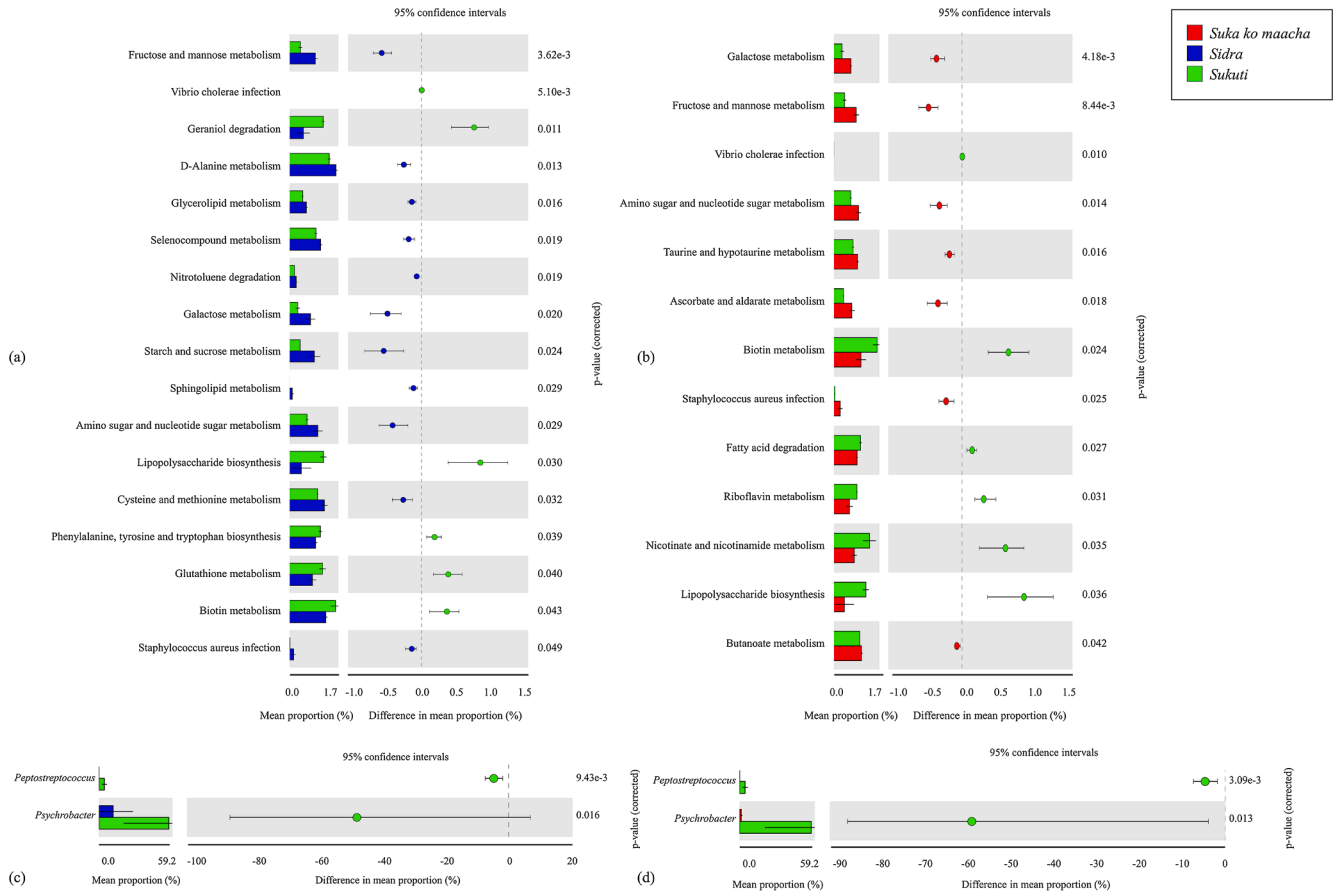

Fig. 7. Non-parametric analysis of the predominant genera and predictive microbial functions among three different fish products: (a) significant predictive microbial functions between *sukuti* and *sidra*, (b) significant predictive microbial functions between *sukuti* and *suka ko maacha*, (c) significant predominant genera between *sukuti* and *sidra* and (d) significant predominant genera between *sukuti* and *suka ko maacha*.

microbes contribute to the development of flavour (Gilles, 2009; Xu et al., 2018) in the products due to the degradation of lipids to free fatty acids (Xu et al., 2020). Galactose metabolism also contributes to the production of aromatic compounds that are associated with flavour compounds (Lee et al., 2015). Geraniol is another important flavour compound that is associated with flavour developments (Chen et al., 2010). Fish microbiota harbours a high level of carbohydrate and amino acid metabolisms (Zhang et al., 2016), which are associated with taste and aroma of the fermented products (Lee et al., 2014). Apart from major metabolic functions that are related to flavour development, PICRUSt2 analysis detected human-disease-related pathways- *Vibrio cholerae* infection and *Staphylococcus aureus* infection. As per sOTUs contribution, we speculated that the presence of some pathogenic genera may contribute to these predictive features. Many pathogenic bacteria are associated with *ngari* and *hentek*, fermented fish products of North-East India (Keisam et al., 2019), a huge concern regarding the safety of these products. However, these speculations were entirely based on bioinformatics tools that was used in this study.

5. Conclusion

Perishable fish are traditionally preserved in India for consumption by smoking, salting and sun drying, which may facilitate the growth of diverse microbiota. High-throughput sequence analysis has given more insight into the microbial diversity of the traditionally preserved fish products of India where such studies have not been applied before. *Psychrobacter* was detected in all products, where it was significantly higher in *sukuti* samples. Microbial predictive functionality studies also showed the abundance of many metabolic pathways relating to flavour development. Additionally, the detection of many pathogenic bacteria does pose a huge concern regarding the safety of these products; a need to establish safety measure in the production, storage and transportation of these fish products.

6. Data availability

The sequences obtained from HTS analysis were submitted to National Center for Biotechnology Information (NCBI) which are available under Bio project ID PRJNA600094 with Sequence Read Archive (SRA) accession numbers: SRR10857170, SRR10857171, SRR10857172, SRR10857173, SRR10857174, SRR10857175, SRR10857176, SRR10857177 and SRR10857178.

Authors contribution

Conceptualization (NT and JPT); Data curation (MOB and HNJS); Formal analysis (MOB and HNJS); Funding acquisition (JPT); Investigation (MOB, NT and HNJS); Methodology (MOB and HNJS); Project administration (JPT); Resources (NT and JPT); Software (MOB and HNJS); Supervision (NT and JPT); Validation (MOB, HNJS, NT and JPT); Visualization (MOB and HNJS); Roles/Writing - original draft (MOB and HNJS); Writing - review & editing (NT and JPT)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

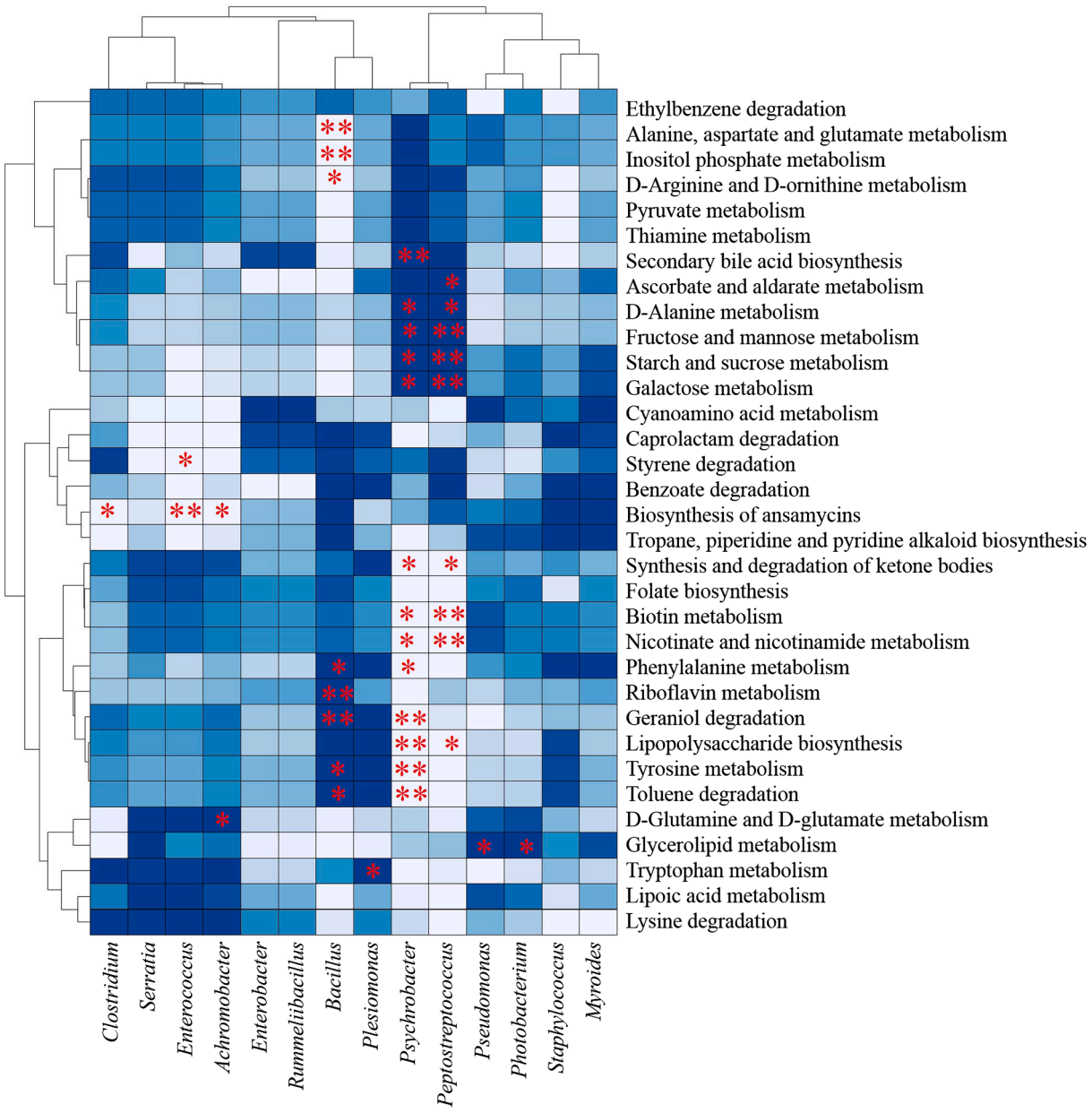

Fig. 8. Non-parametric Spearman's correlation analysis between the predominant genera observed in the three different fish products with the predicted microbial functions. Spearman's Rho coefficient values −1 to 1 depicts the correlation between the two variables, and significant differences is shown, where *indicates *p*-value < 0.05 and **indicates *p*-value < 0.01.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109885.

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Metataxonomic profiling of bacterial communities and their predictive functional profiles in traditionally preserved meat products of Sikkim state in India

Meera Ongmu Bhutia [a], Namrata Thapa [b,*], H. Nakibapher Jones Shangpliang [a], Jyoti Prakash Tamang [a,*]

[a] DAICENTER (DBT-AIST International Centre for Translational and Environmental Research) and Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok 737102, Sikkim, India
[b] Biotech Hub, Department of Zoology, Nar Bahadur Bhandari Degree College, Tadong 737102, Sikkim, India

A B S T R A C T

Traditionally preserved meat products are common food items in Sikkim state of India. We studied the high-throughput sequencing of four traditionally preserved meat products viz. beef *kargyong*, pork *kargyong*, yak *satchu* and *khyopeh* to profile the bacterial communities and also inferred their predictive functional profiles. Overall abundant OTUs in samples showed that *Firmicutes* was the abundant phylum followed by *Proteobacteria* and *Bacteroidetes*. Abundant species detected in each product were *Psychrobacter pulmonis* in beef *kargyong*, *Lactobacillus sakei* in pork *kargyong*, *Bdellovibrio bacteriovorus* and *Ignatzschinera* sp. in *yak satchu* and *Lactobacillus sakei* and *Enterococcus* sp. in *khyopeh*. Several genera unique to each product, based on analysis of shared OTUs contents, were observed among the samples except in *khyopeh*. Goods coverage recorded to 1.0 was observed, which reflected the maximum bacterial diversity in the samples. Alpha diversity metrics showed a maximum bacterial diversity in *khyopeh* and lowest in pork *kargyong* Community dissimilarities in the products were observed by PCoA plot. A total of 133 KEGG predictive functional pathways was observed in beef *kargyong*, 131 in pork *kargyong*, 125 in yak *satchu* and 101 in *khyopeh*. Metagenome contribution of the OTUs was computed using PICTRUSt2 and visualized by BURRITO software to predict the metabolic pathways. Several predictive functional profiles were contributed by abundant OTUs represented by *Enterococcus*, *Acinetobacter*, *Agrobacterium*, *Bdellovibrio*, *Chryseobacterium*, *Lactococcus*, *Leuconostoc*, *Psychrobacter*, and *Staphylococcus*.

1. Introduction

Perishable flesh of domesticated animal is traditionally preserved by smoking (Plavsic, Okanovic, Gubic, & Njezic, 2015), sun-drying (Aksoy, Karasu, Akcicek, & Kayacan, 2019), salting (Uğuz, Soyer, & Dalmiş, 2011) and fermentation (Tamang, Holzapfel, & Watanabe, 2016) to prolong the shelf-life as well as to enhance the delicacy as foods. Consumption of different types of meat products is dietary culture of many people in the world such as *jerky* of America (Nummer et al., 2004), *pastrima* and *sucuk* of Turkey (Kaban, 2013), *botillo* of Spain (Fontán, Lorenzo, Martínez, Franco, & Carballo, 2006), *alheira* of Portugal (Ferreira et al., 2006), *nham* of Thailand (Santiyanont, 2019) and *biltong* of South Africa (Petit, Caro, Petit, Santchurn, & Collignan, 2014). During the natural preservation of perishable meat by smoking/sun-drying and fermentation, diverse types of microbiota including autochthonous microorganisms appear, which may affect the sensory properties of the products (Woods, Kozak, Flynn, & O'Gara, 2019). Dominant species of lactic acid bacteria present in fermented, smoked and cured meat products of the world are *Lactobacillus sakei, Lb. curvatus, Lb. plantarum, Pediococcus pentosaceus, Enterococcus faecium, Leuc. carnosum, Leuc. gelidum, Leuc. pseudomesenteroides, Weissella* (Laranjo, Elias, & Fraqueza, 2017; Li et al., 2019; Nguyen et al., 2013; Oki, Rai, Sato, Watanabe, & Tamang, 2011); the other main group of non-lactic bacteria are *Kocuria*, micrococci, and coagulase-negative staphylococci (Marty, Buchs, Eugster-Meier, Lacroix, & Meile, 2012; Quijada et al., 2018; Wang, Zhang, Ren, & Zhan, 2018) and Enterobacteriaceae (Mainar, Stavropoulou, & Leroy, 2017). Bacteria present in smoked, salted, sun-dried and fermented meat products have functional roles as probiotics (Laranjo, Potes, & Elias, 2019), as well as non-functional roles as spoilage (Dave & Ghaly, 2011) and food-borne (Heredia & García, 2018).

Majority of Indian populace are vegetarians, however, consumption of meats is common dietary culture in Indian Himalayan regions of Jammu & Kashmir, Ladakh, Himachal Pradesh, Uttarakhand, Darjeeling hills, Sikkim and Arunachal Pradesh (Tamang, 2010). Livestock plays a subsidiary role in the mixed farming system in Sikkim, the Himalayan state of India, which includes cattle, sheep, goats, pigs, yaks, poultry,

* Corresponding authors at: DBT-Biotech Hub, Department of Zoology, Nar Bahadur Bhandari Degree College, Sikkim University, Tadong 737102, Sikkim, India (N. Thapa). Department of Microbiology, Sikkim University, Tadong 737102, Sikkim, India (J. Tamang).
E-mail addresses: namumani@hotmail.com (N. Thapa), jptamang@cus.ac.in (J. Prakash Tamang).

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etc., used for meat, milk and milk products (Tamang, 2010). Yak (*Bos grannies* L.), is domesticated in high altitudes of Sikkim between 2100 m to 4500 m for milk, milk products, meat and wool. Ethnic peoples living in Sikkim, traditionally prepare and consume varieties of ethnic fermented foods and beverages including traditionally preserved, smoked and fermented meat products. In Sikkim, about 88.3% are non-vegetarians (Tamang, et al., 2007). Three different types of meats are traditionally preserved in Sikkim which include a traditional sausage-like product, locally called *kargyong* (Fig. 1a & b), smoked and sun-dried yak meat product called *satchu* (Fig. 1c), and fermented yak meat product known as *khyopeh* (Fig. 1d). *Kargyong* is traditionally prepared by mixing lean meats of pork/beef/yak with required amount of salt, garlic and ginger and are stuffed into the intestine of animals locally called *gyuma* as natural casings. Both ends of casing are then tied up with a rope, boiled for around 30 min in an open cooker and hooked in a bamboo stick and smoked above the traditional earthen oven for 10–15 days (Rai, Palni, & Tamang, 2009). Unlike other sausages, no nitrates and nitrides are added during preparation of *kargyong*. *Satchu* is prepared by slicing red meat (yak/beef/buffalo) into long strands and mixed with turmeric, salt and oil, which is then dried or smoked for 7–10 days (Rai et al., 2009). *Khyopeh* is a naturally fermented meat product of Sikkim, which is prepared from yak. During the preparation of *khyopeh*, chopped meats and innards of yak are mixed with required amount of salt, and the mixtures are stuffed into the rumen, which is previously removed from slaughtered yak. Filled up rumen is tied up with a twine and hung into a bamboo stripes for natural fermentation for

Fig. 1. Traditional methods of preparation of different traditionally preserved meat products of Sikkim. (a) beef *kargyong*; (b) pork *kargyong*; (c) *satchu* and (d) *khyopeh*.

4–6 months above earthen oven (Bhutia, Thapa, & Tamang, 2020). *Kargyong* and *satchu* are commonly eaten as fried side-dish or made into thick curry. *Khyopeh* is a strong-flavoured product with soft or hard texture and brown in colour, which is consumed by the ethnic people dwelling in high altitudes of Sikkim as soup or curry in meal. Beef-*kargyong* and pork-*kargyong* are sold in local markets, whereas, *satchu* and *khyopeh* are prepared at household for home consumption. The average pH value and moisture content of beef *kargyong* is 5.8 and 14.1%, pork *kargyong* is 5.5 and 11.2%, *satchu* is 5.4 and 8.5%, and *khyopeh* is 5.9 and 2.5%, respectively (Bhutia, 2020).

Based on phenotypic characteristics and biochemical tests, some bacterial genera present in two traditionally preserved meat products viz. *kargyong* and *satchu* were previously reported, which included *Lactobacillus, Leuconostoc, Enterococcus, Bacillus, Staphylococcus* and *Micrococcus* (Rai, Tamang, & Palni, 2010). Till date there is no report on bacterial community structure analyzed by high-throughput sequencing (HTS) method in traditionally preserved meat products of India except in *sa-um*, a fermented pork meat of Mizoram in India (De Mandal et al., 2018). However, there are few reports on application of HTS method to profile the microbial community in various fermented/smoked meat products of other countries (Połka, Rebecchi, Pisacane, Morelli, & Puglisi, 2015; Wang et al., 2018). Sequence-based taxonomy or meta-taxonomic tool, mostly high-throughput amplicon sequencing method (Cox et al., 2017) supported by bioinformatics tools, is more accurate to profile the microbial community in fermented foods (Ercolini, 2013; Tamang et al., 2020). Similarly, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) algorithm (Douglas et al., 2020) infers the predictive gene functionality of bacterial communities in different ecosystems (Langille et al., 2013; De Filippis, Parente, & Ercolini, 2017). Hence, the present paper is aimed to profile the bacterial communities using HTS method in four different types of traditionally preserved meat products of Sikkim viz. sausage-like products, beef *kargyong* and pork *kargyong*, smoked meat product, yak *satchu,* and fermented yak meat product, *khyopeh.* It is also aimed to predict the various metabolisms of OTUs obtained from sequence-data. To our updated knowledge, this is the first report on metataxonomic profiling of bacterial community and their predictive functionality in traditionally preserved meat products of India.

2. Materials and methods

2.1. Sample collection

Twelve samples of traditionally preserved meat products viz. beef *kargyong* (3 samples) and pork *kargyong* (3 samples) were collected from different local markets (Gangtok, Namchi and Geyzing) in Sikkim in India, and yak *satchu* (3 samples) and *khyopeh* (3 samples) were collected from different households in Lachen and Lachung regions of North Sikkim. The products were aseptically collected in pre-sterile poly-bags, kept in ice-box carrier and were transported to the laboratory, stored at −20 °C for further analysis.

2.2. Homogenization of sample

The 10 g of each sample was homogenized in a 90 ml of sterile 0.1 M phosphate buffer saline (pH 6.4) using Stomacher 400 Circulator (Seward, UK) at 200 rpm for 2 min. After homogenization, the big debris were allowed to settle down for 5 min and the homogenate was used for DNA extraction.

2.3. DNA extraction

DNA was extracted from each sample using Nucleospin Food kit (MachereyNagel, Germany) according to manufacturer's protocol. Concentration of genomic DNA of each sample was quantified using Eppendorf BioSpectrometer (USA) and the bands were visualized in agarose gel (0.8%) using Bio-Rad Gel Doc EZ Imager (USA).

2.4. High-throughput sequencing analysis

The 50 ng of DNA was used to amplify 16S rRNA hyper variable region V3-V4 using 341F-GCCTACGGGNGGCWGCAG and 785R-ACTACHVGGGTATCTAATCC primers (Thijs et al., 2017). The reaction mixture containing DNA template, amplicon PCR forward and reverse primers and KAPA HiFi HotStart ready mix (KAPA Biosystems, USA) was made to a final concentration of 100 nM (Klindworth et al., 2013) The PCR reaction was initially involved the denaturation of 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 45 sec and 72 °C for 30 sec, respectively, and finally extended for 7 min at 72 °C. The amplicons were then purified using AMPure XP beads (Beckman Coulter, USA) to remove unused primers. Additionally, 8 cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries with a read length of 2 × 300 bp and sequencing was carried using Illumina-MiSeq platform (Illumina, USA).

2.5. Bioinformatics analysis

2.5.1. Bacterial community

Raw demultiplexed sequences were checked for their quality using FastQC (Andrews, 2010) and the adapter sequences were trimmed off using Trim Galore (Krueger, 2017). The quality-checked adapter-free paired-end reads were assembled using Paired-End reAd merger (PEAR) (https://cme.h-its.org/exelixis/web/software/pear) software (Zhang, Kobert, Flouri, & Stamatakis, 2013). Taxonomic analysis was carried on in Quantitative Insights Into Microbial Ecology (QIIME2)-2019.4 (https://qiime2.org) software (Bolyen et al., 2019) imported as single-end reads via manifest-format (Single End Fastq Manifest Phred 33). After importing into QIIME2, initial filtering process, based on the quality score, was applied using q2-quality-filter q-score script (Bokulich et al., 2013). The 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). Taxonomy was assigned to the resulting sub-operational-taxonomic-unit (sOTUs) using the q2-feature-classifier classify-consensus-vsearch (Rognes, et al., 2016) against a Naive Bayes classifier pre-trained on Greengenes 13_8 99% OTUs full-length sequences (McDonald et al., 2012a; Bokulich et al., 2018) (https://docs.qiime2.org/2020.8/data-resources/). The resulting BIOM (biological observation matrix) file (McDonald et al., 2012b) was then further collapsed at different taxonomic levels.

2.5.2. Predictive functionality

Prediction of functional profiles of metagenome using marker gene (16S rRNA gene), based on the abundances of gene families in the samples, was inferred using PICRUSt2 (Douglas et al., 2020). Here, we used the Greengenes-clustered representative sequences and placed into a reference tree, that contained 20,000 full 16S rRNA genes from bacterial genomes in the Integrated Microbial Genomics (IMG) database (Markowitz et al., 2012). Firstly, phylogenetic placement of the representative sequences was aligned by multiple-sequence alignment of 16S sequences with HMMER (http://www.hmmer.org). Determination of the optional position of these representative sequences in the reference tree was applied using EPA-ng (Barbera et al., 2019) and Genesis Applications for Phylogenetic Placement Analyses (GAPPA) omics (Czech & Stamatakis, 2019). Castor R package (Louca & Doebeli, 2018) was applied for prediction of the gene families using a default parameter (maximum parsimony) to predict the gene copy number for sOTUs (Nearing, Douglas, Comeau, & Langille, 2018) and were mapped into KEGG (Kyoto Encyclopaedia of Genes and Genomes) database for systematic analysis of gene functions (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017; Karlsen, Schulz, & Almaas, 2018). Functional contributions of OTUs were visualized using BURRITO software

(McNally, Eng, Noecker, Gagne-Maynard, & Borenstein, 2018). Pathway levels of defined gene functionality with high-level function (Level 1) viz., metabolism, environmental information processing, genetic information processing, human diseases, cellular processes, organismal system and drug development were interpreted, which were further divided into lower Level 2 of sub-pathway categories (Scala, Serra, Marwah, Saarimäki, & Greco, 2019).

2.6. Statistical analysis

Alpha diversity indices were calculated through QIIME2 for Chao1, Goods coverage, Fisher alpha, Simpson, observed OTUs (Kim, 2017) (via q2- diversity alpha), alpha-rarefaction (via q2-diversity alpha-rarefaction), Faith's Phylogenetic Diversity and Shannon Diversity (Faith, 1992) using rarefied (3875, being the lowest reads) sequences per sample (McCoy, & Matsen, 2013). Raw reads were normalised to relative abundances and data visualization were created using MS-Excel 365. Using iGraph R-package, we then constructed a simple network analysis, representing the shared and unique genera (with > 1% relative abundance) among the four different processed meat products. Comparison of the inter-diversity relationship among the samples was achieved by measuring beta diversity using q2-diversity beta plugin in QIIME2 environment (Faith, Minchin, & Belbin, 1987), and Principal Coordinates Analysis (PCoA) ordination plot was plotted using PASTv4.0.

3. Results

3.1. Bacterial communities

A total of 170,114 raw reads were obtained from sequencing, out of which 81,538 quality-filtered reads with 350 bp uniform length were then denoised using deblur algorithm producing a total of 19,675 denoised reads. Denoised reads of all the individual samples were 5061, 6778, 3875 and 3961 for beef *kargyong*, pork *kargyong,* yak *satchu* and *khyopeh*, respectively. Overall bacterial diversity in samples of four traditionally preserved meat products, detected at > 1% abundance, showed *Firmicutes* as the most abundant phylum followed by *Proteobacteria, Bacteroidetes*, and other phyla in including both classified and unclassified with < 1% abundance (Fig. 2a). At the family level, Moraxellaceae was the abundant family (Fig. 2b), at the genus level, Psychrobacter was the abundant genus (Fig. 2c) and at species-level, unknown species of *Enterococcus* was abundant followed by *Lactobacillus sakei* in the samples (Fig. 2d). About 38 species, detected at < 1% abundance, were observed in samples (Supplementary Table 1). In samples of beef *kargyong*, Proteobacteria, Moraxellaceae and *Psychrobacter pulmonis* were abundant phylum, family and species, respectively (Fig. 3a). *Firmicutes,* Lactobacillaceae and *Lactobacillus sakei* were the abundant phylum, family and species in pork *kargyong,* respectively (Fig. 3b). In *satchu* samples, Proteobacteria, Weeksellaceae and unknown species of *Ignatzschineria* were abundant phylum, family and species, respectively (Fig. 3c). Interestingly, *Firmicutes* was the only phylum detected in samples of *khyopeh,* with the abundance of family Enterococcaceae and the unknown species of *Enterococcus* (Fig. 3d).

We observed several genera unique to each product, based on analysis of shared OTUs contents, among the samples. *Streptococcus*, *Arthrobacter*, *Kurthia*, *Agrobacterium*, *Vitreoscilla*, *Erwinia*, *Sphingobacterium* and *Paraclostridium* were unique to beef *kargyong* (Fig. 4). Similarly, *Rothia, Micrococcus, Stenotrophomonas, Corynebacterium* and *Citrobacter* were unique genera in pork *kargyong*. *Novosphingobium, Bdellovibrio, Brevundimonas, Clostridium, Ketogulonicigenium, Ignatzschineria, Enterobacter* and *Luteolibacter* were unique genera in yak *satchu*. However, no unique genus was observed in *khyopeh*. Interestingly, *Staphylococcus* was the common genus which was observed in all meat products (Fig. 4).

Denoised reads were rarefied at sampling depth of 3875, which was the lowest denoised reads of yak *satchu*, however, denoised reads of

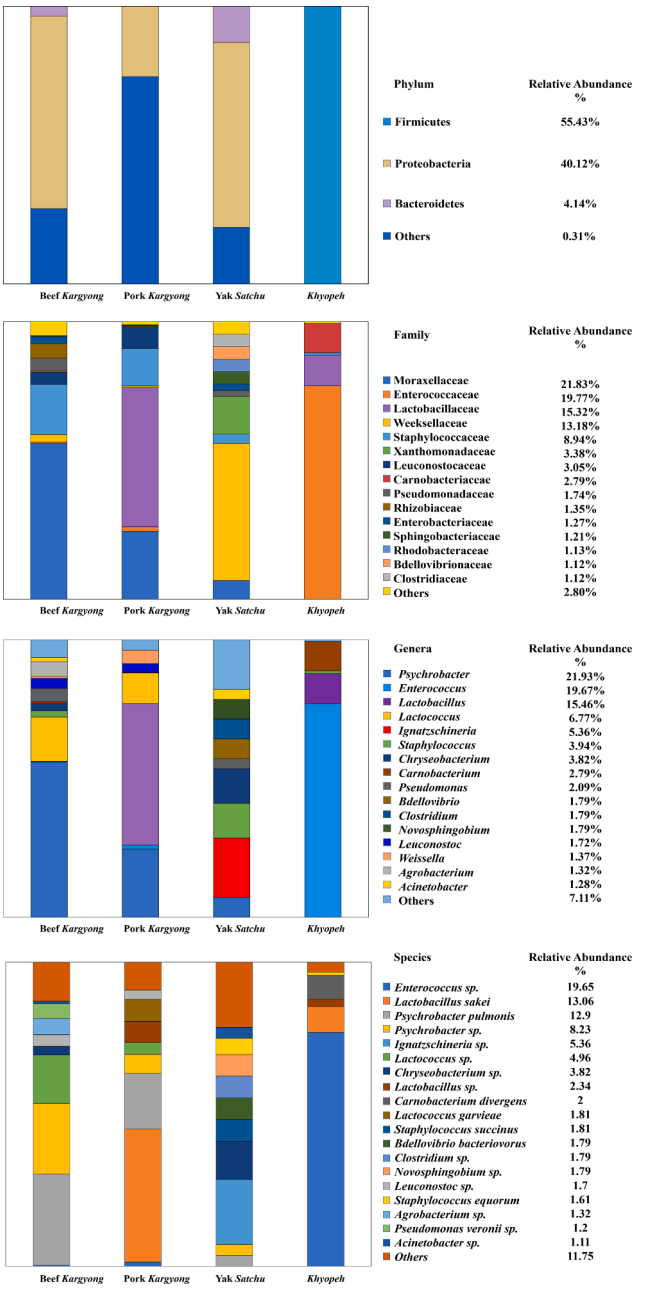

Fig. 2. Bar-plot representation of the bacterial composition of the traditionally preserved meat products of Sikkim at different taxa-levels: phylum, family, genus and species.

khyopeh were 3961; beef *kargyong*, 5061; and pork *kargyong*, 6778) for alpha diversity calculation (Fig. 5). Goods coverage (Table 1) recorded 0.99 to 1.0 showing the maximum bacterial diversity in the samples. Alpha diversity metrics showed a maximum bacterial diversity in *khyopeh* and lowest in pork *kargyong* (Table 1). The inter-diversity relationship among the samples was plotted using PCoA-based Bray-Curtis dissimilarities matrix in QIIME2 (Fig. 6). We observed a clear dispersion of the bacterial communities among the four traditional meat products.

3.2. Predictive functionality

Predictive metabolic pathways, inferred by PICRUSt2 algorithm for 16S-based bacterial members of traditionally preserved meat products of Sikkim showed 133 metabolic predictive functional pathways in beef

Table 1
Alpha diversity metrics of bacterial community in traditionally preserved meat products of Sikkim (rarefied at 3875 sequencing depth).

Samples	Observed_OTUs	Chao1	Fisher_alpha	Goods_coverage	Shannon	Simpson
Beef *kargyong*	136.0	140.3871	27.4325	0.9956	4.8213	0.9239
Pork *kargyong*	110.0	119.3704	21.0741	0.9941	4.1897	0.8857
Yak *satchu*	236.0	236.0000	55.3664	1.0000	6.4278	0.9749
Khyopeh	246.0	246.0000	58.4432	1.0000	6.8326	0.9764

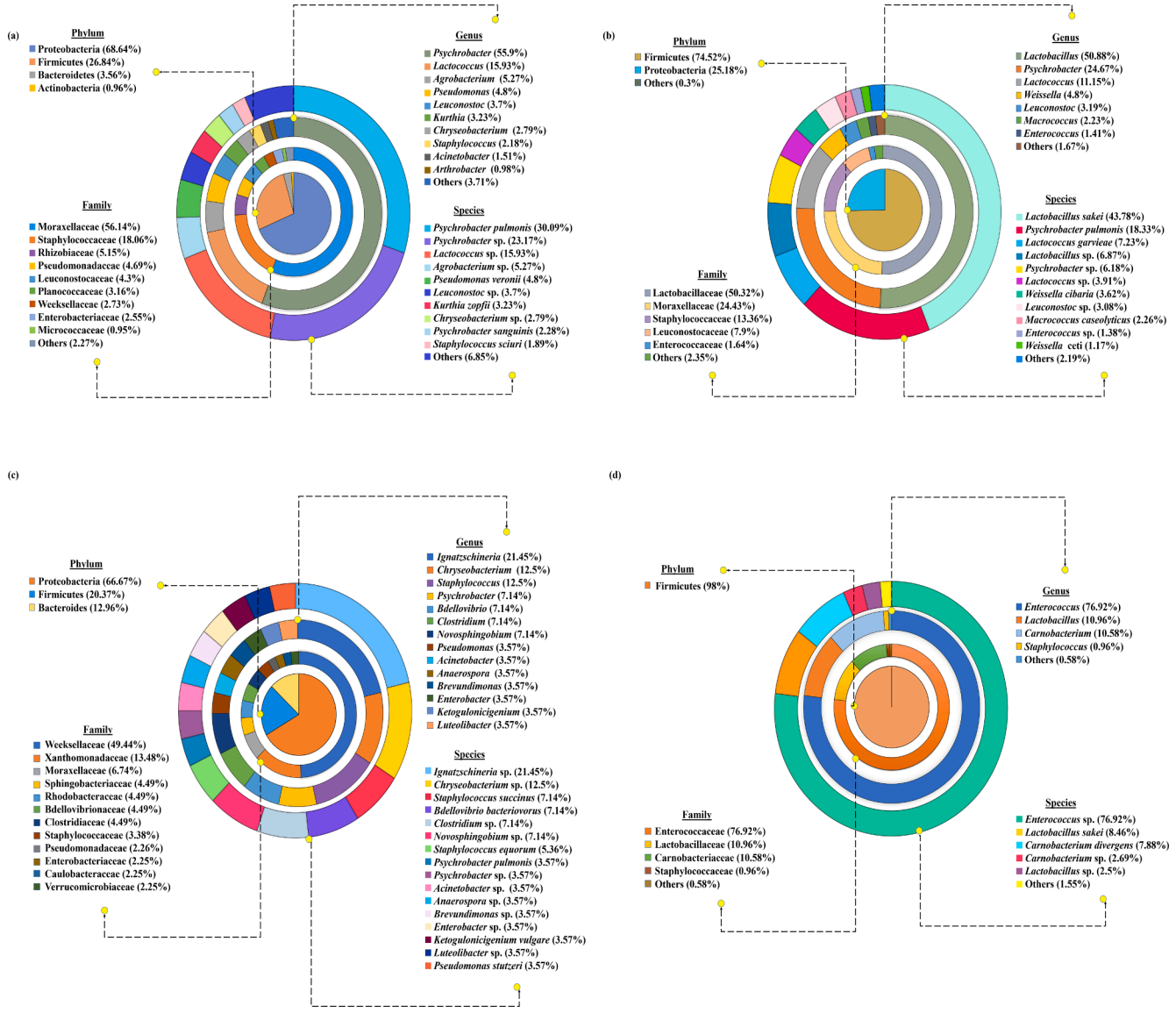

Fig. 3. Bacterial diversity of traditionally preserved meat products represented as doughnut-chart - (a) beef *kargyong* (b) pork *kargyong* (c) yak *satchu* and (d) *khyopeh*.

kargyong, 131 in pork *kargyong*, 125 in yak *satchu* and 101 in *khyopeh* with a relative abundance of > 1% (Fig. 7) as well as < 1% (Supplementary Table 2). The major predictive functionalities in traditionally preserved meat products of Sikkim was metabolism, which was 80.7% in beef *kargyong*, 77.21% in pork *kargyong*, 78.36% in yak *satchu* and 76.78% in *khyopeh*, respectively (Supplementary Table 2). Among the sub-pathways, predictive carbohydrate metabolism was abundant in *khyopeh* (18.47%), followed by pork *kargyong* (15.74%), beef *kargyong* (14.12%) and yak *satchu* (14.04%), respectively. Metagenome contribution of the OTUs was also inferred by PICRUSt2 and visualized by BURRITO software, showing the genera-predictive functionality relationship (Fig. 8). *Lactococcus* (detected in beef *kargyong* and pork *kargyong*) contributed to about 117 metabolic pathways. *Chryseobacterium* (detected in beef *kargyong* and yak *satchu*) contributed to about 117 metabolic pathways. *Bdellovibrio* (detected in yak *satchu*) contributed to about 109 metabolic pathways. *Leuconostoc* (detected in beef *kargyong* and pork *kargyong*) contributed about 102 metabolic pathways. *Agrobacterium* (detected in beef *kargyong*) contributed to about 114 metabolic pathways and *Acinetobacter* (detected in beef *kargyong*, pork *kargyong* and yak *satchu*) contributed to about 118 metabolic pathways. Among the major predictive metabolic pathways; biosynthesis of ansamycin, D-Glutamine and D-glutamate metabolism, fatty acid metabolism and D-alanine metabolisms were observed in all the samples) (Fig. 8).

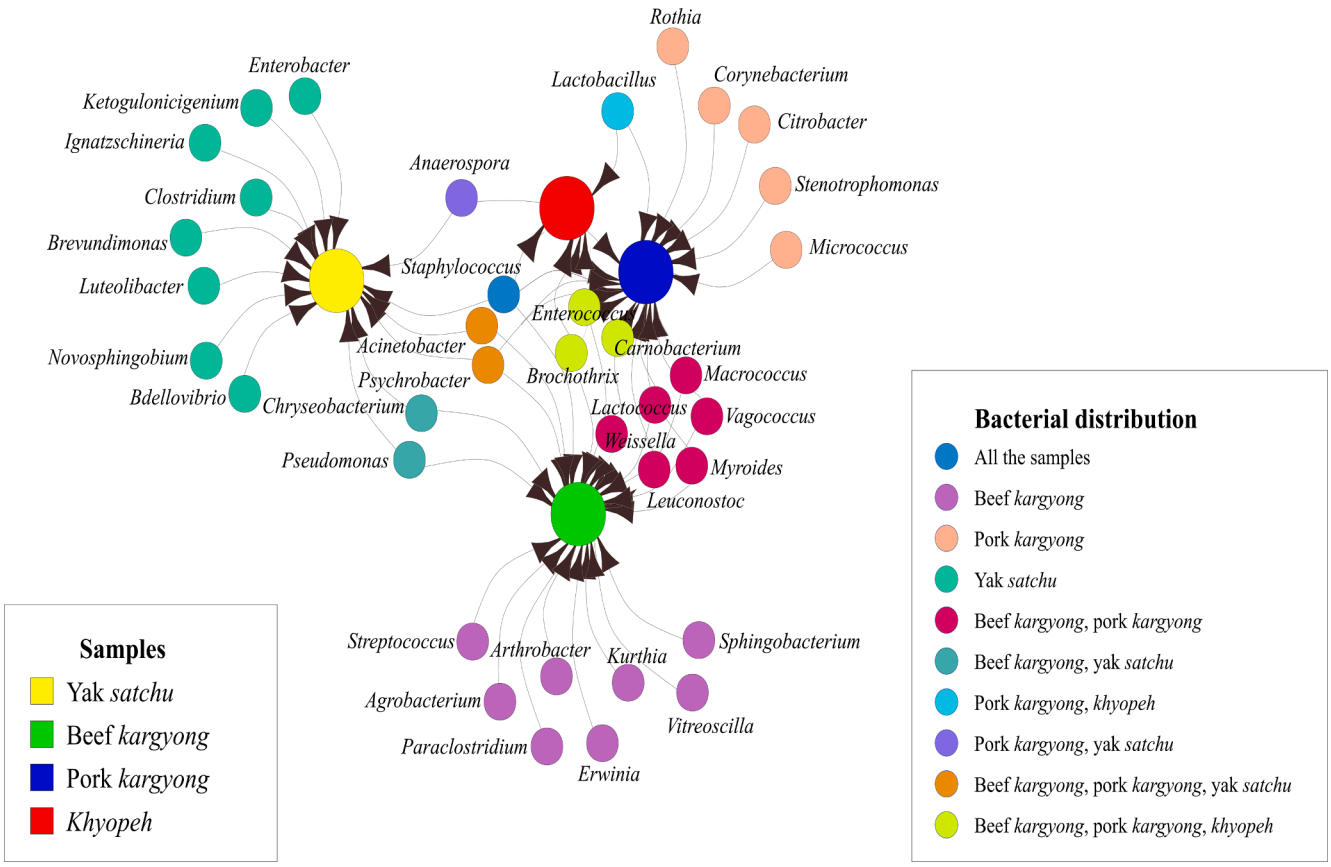

Fig. 4. Distribution of shared and unique genera in traditionally preserved meat products of Sikkim as represented by a simple network analysis using iGraph R-package. Each sample is represented in blue, green, red and yellow circles, while all genera (shared/unique) are represented in smaller circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

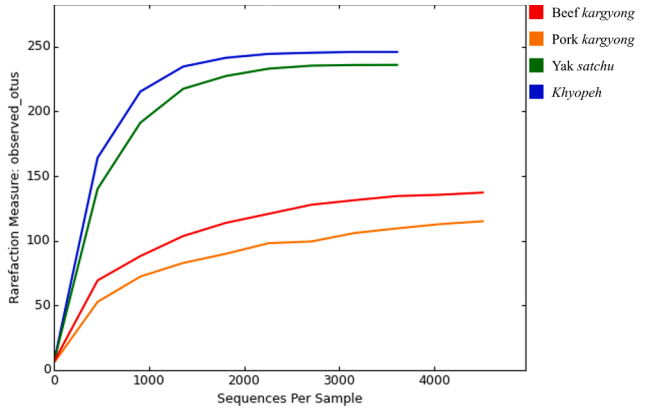

Fig. 5. Rarefaction curves showing observed operational taxonomic units (OTUs) of bacterial diversity of beef *kargyong, pork kargyong,* yak *satchu* and *khyopeh.*

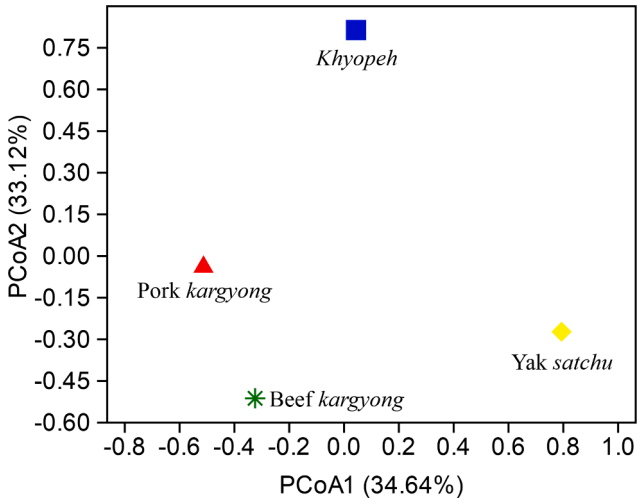

Fig. 6. Beta diversity comparison of the microbial diversity of traditionally preserved meat products as per Bray-Curtis dissimilarities matrix represented by Principal Coordinates Analysis (PCoA).

4. Discussion

4.1. Bacterial diversity

Phylum *Firmicutes* was abundant in pork *kargyong* and *khyopeh,* whereas phylum *Proteobacteria* was abundant in beef *kargyong* and yak *satchu,* respectively. Similar observation of phyla distributing in fermented sausages was reported by Huang et al. (2020). *Psychrobacter pulmonis*, Gram-negative bacterium, was abundant in beef *kargyong,* which is psychrotolerant and halotolerant bacterium (Wu, Zhan, Shao, & Liu, 2013) and is also associated with meat products when stored at cold temperature (Zhang et al., 2012). *Lactobacillus sakei* was the most abundant bacterium in pork *kargyong.* Though *Lb. sakei* is generally isolated from vegetable sources (Lee et al., 2018), however, it was also reported from meat products such as sausage prepared from llama meat of Northwest Argentina (Fontana et al., 2016), fermented sausage (Zagorec, & Champomier-Vergès, 2017) and *mum,* fermented sausage of

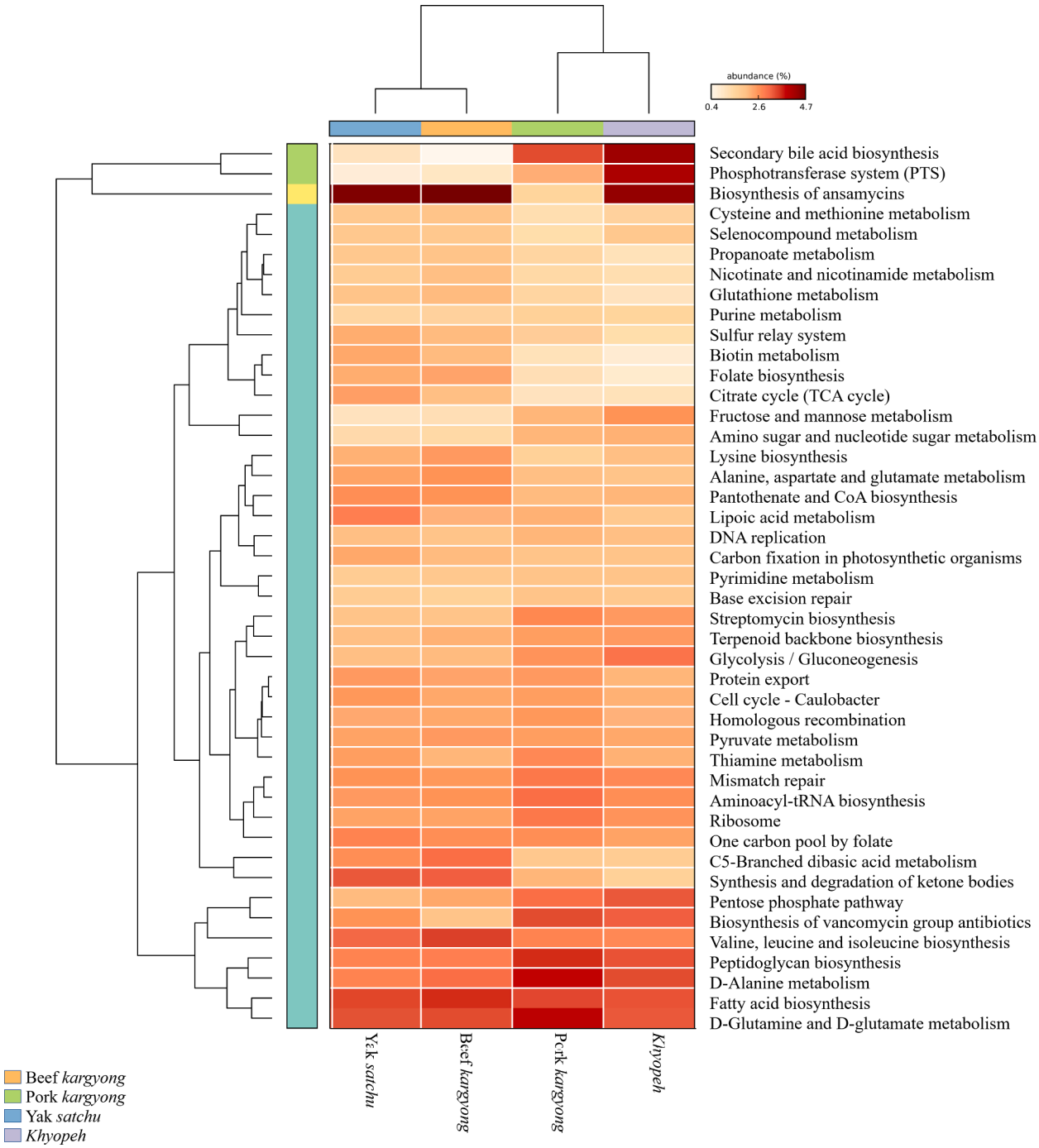

Fig. 7. Heatmap representation showing the predictive metabolic pathways (KEGG pathways) observed in the traditionally preserved meat products as inferred by PICRUSt2.

Thailand (Wanangkarn et al., 2014). *Lb. sakei* has several probiotic attributes such as acidification, tolerance to bile salt, reduction of biogenic amine levels and adherence to intestinal cells (Laranjo et al., 2019). Gram-negative bacterium *Ignatzschineria* was abundant in yak *satchu*, which is commonly associated with larvae of flesh flies (Barker et al., 2014). Probably this bacterium might have contaminated during storage under unhygienic conditions. Another Gram-negative bacterium *Bdellovibrio bacteriovorus* was also detected in yak *satchu* samples, which is a predatory bacterium and and may act as a bio-control agent for Gram-negative pathogenic bacteria (Negus et al., 2017). Detection of anti-pathogenic bacterium *Bdellovibrio bacteriovorus* in traditionally smoked meat *satchu* by OTUs sequences is remarkable findings in this study. Since *Bdellovibrio bacteriovorus* has also antibiotic and probiotic properties (Shatzkes et al., 2017; Bratanis, Andersson, Lood, & Bukowska-Faniband, 2020), it may be isolated from traditional meat products of India by culture method using selective medium (Ottaviani et al., 2020) for further research in future. Interesting, the predominance of phylum *Firmicutes* in *khyopeh* may be due to supplement of gut microbiome from yak rumen, since during preparation of *khyopeh*, chopped innards of yak are stuffed into the rumen of dead yak, and fermented spontaneously for 4–6 months. *Firmicutes* is the predominant bacterial phylum in the yak rumen (Liu et al., 2019). The abundant bacterium in *khyopeh* was *Enterococcus* spp., followed by *Lactobacillus sakei* and *Carnobacterium divergens*. Species of *Enterococcus* have been reported from rumen of yak

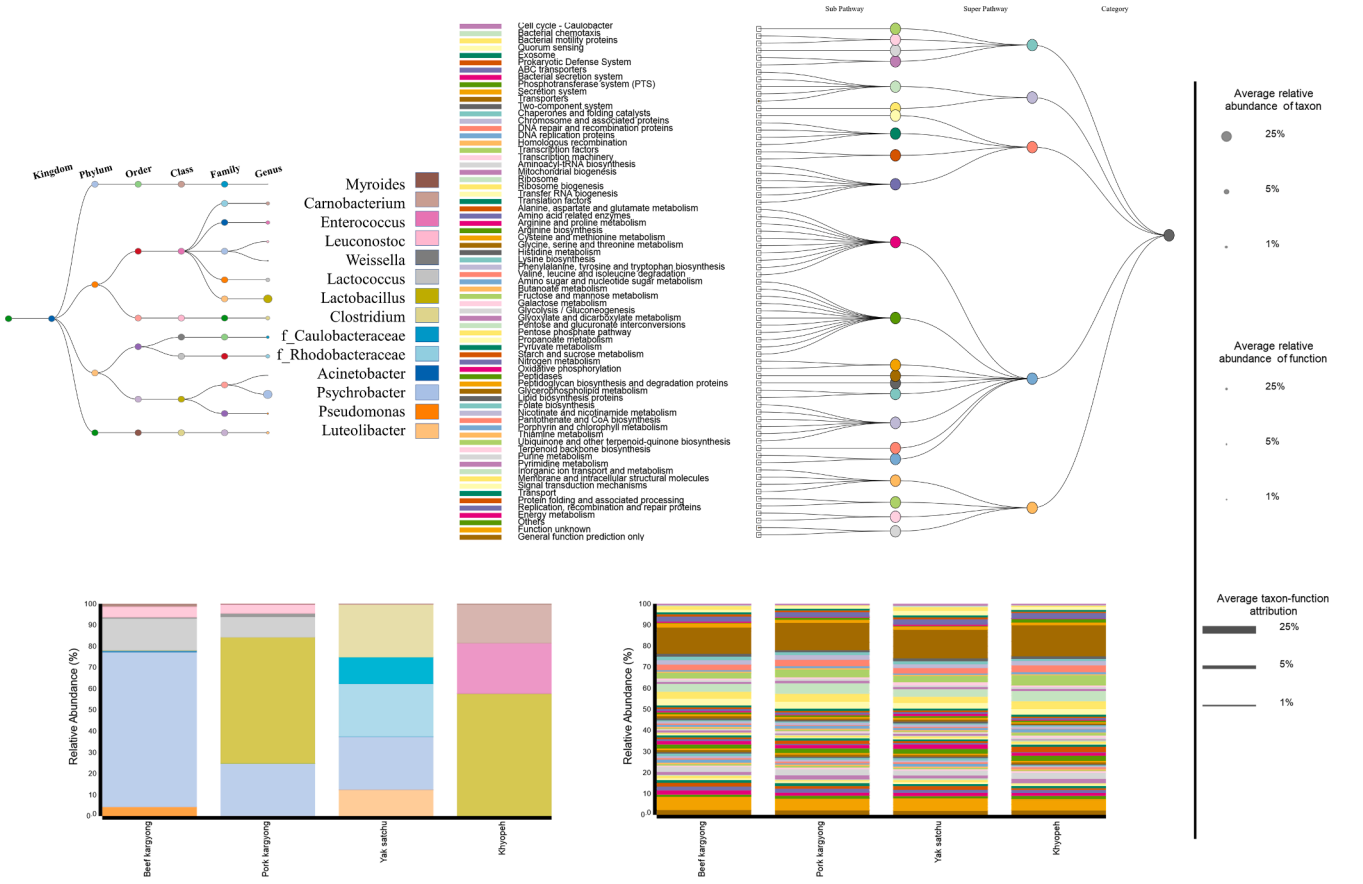

Fig. 8. Bacterial diversity and their associated predictive functional pathway as inferred by PICRUSt2 and visualized by BURRITO.

(Li et al., 2018), which stimulate the growth of rumen microbiome (Mamuad et al., 2019), and also produce antimicrobial agents in rumen (Wang et al., 2018). *Carnobacterium divergens* can tolerate to freezing and high-pressure conditions and grow anaerobically (Leisner, Laursen, Prévost, Drider, & Dalgaard, 2007) and is reported from frozen vacuum packaged meat product (Zhang, Gänzle, & Yang, 2019). *Carnobacterium divergens* is considered as a biopreservative due to its ability to inhibit pathogenic bacteria in foods (Mokrani, Essid, Hassouna, Jihene, & Abdeljalil, 2018).

Staphylococcus was the core genus present in all samples of traditionally preserved meat products of Sikkim. *Staphylococcus sciuri, S. succinus and S. equorum* were detected with > 1% abundance in this study. *Staphylococcus aureus* was not detected in any samples. Coagulase negative group of *Staphylococcus* genus was isolated from fermented and dried meat products such as *kitoza* of Madagascar (Ratsimba et al., 2017), dry fermented sausage of Spain (Quijada et al., 2018) and Chinese dry/smoked-cured sausage (Wang et al., 2018). Among the unique genera detected in beef *kargyong*, genus *Vitreoscilla*, a Gram-negative bacterium, has been reported as the source of bacterial haemoglobin (VHb) (Veseli, dos Santos, Juárez, Stark, & Pombert, 2018) with antibiotics production (Mejía et al., 2018). *Rothia*, *Micrococcus*, *Stenotrophomonas*, *Cornybacterium* and *Citrobacter* were found only in pork *kargyong*. Probably these genera are residential bacteria in end products (Møretrø & Langsrud, 2017).

Based on metataxonomic result, some bacterial genera detected in traditionally preserved meat products belonged to beneficial groups of bacteria in meat fermentation (Laranjo et al., 2019; Negus et al., 2017) such as *Lactobacillus*, *Enterococcus*, *Lactococcus, Leuconostoc*, *Bdellovibrio*, *Novosphingobium*, and *Leuconostoc*. However, some spoilage bacteria such as *Pseudomonas* (Stellato et al., 2017), *Brochotrix* (Illikoud et al., 2019) and *Clostridium* (Adam, Flint, & Brightwell, 2010) were also detected in some samples of meat products. Major food borne bacterial pathogens such as *Salmonella, Listeria, Campylobacter* etc. were not detected by HTS method in any sample. Moreover, no record of food poisoning by consuming these traditionally preserved meat products have been reported in Sikkim.

Bacterial communities detected in samples of beef *kargyong*, pork *kargyong*, yak *satchu* and *khoypeh* by HTS analysis were viable, since the same products have been culturally analysed and found the viable microbial load as 10^5 to 10^7 cfu/g (Bhutia, 2020). Moreover, any fermented food products are dietary sources of live microbiota (Rezac, Kok, Heermann, & Hutkins, 2018). Information on bacterial community in lesser-known traditionally preserved meat products of the Himalayas by culture-independent method may help to isolate some functional culturable bacteria for preservation of as microbial resources and also for further studies on their technological and functional properties, including development of starter cultures.

Alpha diversity result, based on abundance of observed OTUs in yak *satchu* and *khyopeh*, showed more diversity in comparison to beef *kargyong* pork *kargyong*. Goods coverage showed the maximum coverage of sequencing depth in samples, which indicated the maximum diversity captured (Sims, Sudbery, Ilott, Heger, & Ponting, 2014). We visualized a scattering PCA-plot of the OTU abundances among the different types of the traditionally preserved meat products, which indicates the community dissimilarities in the products (Hugerth & Andersson, 2017).

4.2. *Predictive functionality*

Application of omics to predict functionality of species' metabolism from sequence-data may help to understand the adaptive responses of microbiota in foods (Hadadi, Pandey, Chiappino-Pepe, Morales, Gallart-Ayala, Mehl, Ivanisevic, Sentchilo, & van der Meer, 2020). Hence, we

inferred the possible predictive functionality in OTUs of bacteria present in beef *kargyong*, pork *kargyong,* yak *satchu* and *khoypeh* by PICRUSt2 software (Douglas et al., 2020). Six functional gene groups at Level 1 were categorised to infer the different predictive pathways including metabolism, genetic information processing, environmental information processing, cellular processes, human diseases and organismal systems in meat samples, which are similar to the previous reports on predictive functionality of beef steaks with marker genes encoding for amino acid and lipid metabolism (Yang, Zhu, Zhang, Liang, & Luo, 2018). At Level 2 category, our findings indicated the higher abundance of carbohydrate metabolism followed by metabolisms of amino acids, cofactors and vitamins, terpenoids, polyketides, lipids, and xenobiotics biodegradation. The observation of higher carbohydrate metabolism rate in these samples indicates a vigorous microbial metabolism. During the processing of these meat products, microorganisms may break carbohydrates resulting into formation of volatile organic compounds that can affect the sensory properties of the meat products (Ferrocino et al., 2018). Metagenome contribution of the OTUs was shown using BURRITO software (McNally et al., 2018) for visual relationship between bacteria and the predictive functionality. Several predictive metabolisms were contributed by abundant OTUs represented by *Enterococcus*, *Acinetobacter*, *Agrobacterium*, *Bdellovibrio*, *Chryseobacterium*, *Lactococcus*, *Leuconostoc*, *Psychrobacter*, and *Staphylococcus*. Abundance of amino acid metabolism in the samples was observed, which may be due to prolong fermentation or drying/smoking during traditional processing of the meat samples resulting into development of the distinct aroma in the products (Perea-Sanz, Montero, Belloch, & Flores, 2019). Amino acid metabolism is one the main factors contributing to development of the organoleptic property of meat products (Flores, 2018). Carbohydrate metabolism in meat is correlated with *Firmicutes*, whereas high metabolism of amino acid and lipid is due to abundance of *Proteobacteria* in meat samples (Stellato et al., 2016). We assumed that genes related to carbohydrate metabolism were overrepresented in traditional meat products of Sikkim, indicating the microbial community associated with it might be more essential to carbohydrate degradation (Leroy, Vermassen, Ras, & Talon, 2017). We also predicted genes responsible for metabolism of vitamins such biotin, vitamin B6, folate and thiamine. Production of vitamin B-complex has been reported in meat products (Gille & Schmid, 2015).

The predictive genes encoding for biogenic amines such as histidine and tyramine were detected at relatively low abundance in the samples. Low concentration of biogenic amines in food is very important to indicate the safety of foods for consumption (Ruiz-Capillas & Herrero, 2019). PICRUSt2 analysis of OTUs of bacteria in meat samples predicted the pathways associated with human diseases at relatively low abundance (<1%) such as genes encoding for *Staphylococcus aureus* infection, beta-lactam resistance and epithelial cell signalling in *Helicobacter pylori* infection, whereas the metataxonomic did not identify these organisms at the diversity level. Hence, it could be other factors, probably proteins which may be triggering the signalling of pathways causing the diseases (Sebastian-Leon et al., 2014). Since all predictive metabolic pathways are based on the bacterial community present in samples, these predictions showcase the ability of the samples to serve as good source of nutrients or pose as unsafe for consumption (Eetemadi et al., 2020).

5. Conclusion

Fresh meat and traditionally preserved meat products are popular in the Himalayan regions of India. We studied *meta*-taxonomy of beef *kargyong*, pork *kargyong*, yak *satchu* and *khyopeh* by high-throughput sequencing tool, which revealed the bacterial community including both beneficial and pathogenic in these products. Gene functionality of OTUs of bacteria inferred by PICRUSt2 software against KEGG database predicted various metabolic pathways which are essential for the survival of the bacterial community in these meat products. Since this is the first report on metataxonomic analysis and predictive gene functionality of traditional meat products of the Himalayan regions of India, the findings in this study may be helpful to isolate the beneficial bacteria using culture method for further improvement of these traditional meat products. We believe, this information may be shared to producers for proper maintenance of hygienic conditions during traditional processing of meat products, and also to consumers to know about the food safety and health benefits of these meat products.

Data availability

The sequences obtained from high-throughput sequencing were submitted to National Center for Biotechnology Information (NCBI), which are available under Bio project ID PRJNA555473 with Sequence Read Archive (SRA) Number: SRR9714957, SRR9714958, SRR9714959 and SRR9714960.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.110002.

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Phenotypic and genotypic characterisation of lactic acid bacteria isolated from exotic naturally fermented milk (cow and yak) products of Arunachal Pradesh, India

H. Nakibapher Jones Shangpliang, Jyoti Prakash Tamang*

DAICENTER (DBT-AIST International Centre for Translational and Environmental Research) and Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Tadong, Gangtok 737102, Sikkim, India

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ABSTRACT

Exotic naturally fermented milk of cow and yak products of Arunachal Pradesh in India such as mar, chhurpi and churkam were analysed for identification of lactic acid bacteria (LAB). The pH of samples was 5.32 ± 0.01 to 6.62 ± 0.01 with viable LAB count of 6.27 ± 0.01 to 6.49 ± 0.02 log cfu g^{-1}. A total of 307 LAB isolates were isolated from 30 samples, and out of which 76 isolates were randomly grouped on the basis phenotypic characteristics, and were identified using 16S rRNA gene sequence analysis into 9 species of LAB from cow-milk products, and 5 LAB species from yak-milk products, respectively. *Enterococcus durans* was the predominant species along with *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *hordniae*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Levilactobacillus brevis*, *Loigolactobacillus coryniformis* subsp. *torquens* and *Lentilactobacillus parabuchneri*. Chhurpi (cow-milk) showed the higher species richness and diversity among the products.

1. Introduction

Naturally fermented milk (NFM) products are traditionally prepared from the milk of different domesticated animals such as cow, yak, buffalo, donkey, mare, camel, goat, and ewe (Faccia, D'Alessandro, Summer, & Hailu, 2020), which are culturally considered as ethnic foods by different ethnic communities of the world (Tamang et al., 2020). Although most of the artisan milk products do share the similar type of production methods and product characteristics (Zhong et al., 2016), the nomenclature of the products differ due to different local vernacular languages/dialects used for each NFM product. Several common or community-specific NFM products are consumed by various ethnic groups of people around the world, which include chhu, dahi, lassi, chhurpi, misti dahi, mohi, philu, shoyu, somar, srikhand, mar, gheu are prepared and consumed in India (Dewan & Tamang, 2006, 2007; Shangpliang, Rai, Keisam, Jeyaram, & Tamang, 2018; Tamang et al., 2000); airag, koumiss, kurut, tarag of Kyrgyzstan, Turkey, Mongolia and China (Ataseve & Ataseve, 2018; Uchida, Hirata, Motoshima, Urashima, & Arai, 2007); amabere amaruranu, amasi, ergo, fènè, gariss, kefir, kule naoto, leben, lben, mabisi, mafi, masai, mursik, mutandabota, nunu, omashikwa, pendidam, nyarmie, sethemi, suusac, and zabady of African countries (Akabanda et al., 2013; Jans et al., 2017; Parker et al., 2018).

Firmicutes is the most abundant phylum present in majority of NFM products of the world mostly belonging to lactic acid bacteria (LAB) *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Weissella* (Shangpliang et al., 2018; Terzić-Vidojević et al., 2020; Zhong et al., 2016). Phylum *Proteobacteria* (mostly belonging to genera *Enterobacter*, *Citrobacter*, *Klebsiella*, *Buttiauxella*, *Aeromonas*, *Acetobacter* and *Acinetobacter*) is also reported in some NFM products (Moonga et al., 2020; Zhong et al., 2016).

The Indian state of Arunachal Pradesh is geographically located in the Eastern Himalayan region bordering with Tibet in China, Bhutan and Myanmar. Monpa, also known as Brokpa, are the indigenous people of India residing in Tawang and West Kameng districts of Arunachal Pradesh. Cattle rearing, mostly cows in lower altitude and yaks in high mountains, is the major pastoralism in these regions. Traditional preparation of NFM products from cow and yak milk are artisan processes practised by Monpa of Arunachal Pradesh (Rai, Shangpliang, & Tamang, 2016). Basically, there are two types of exotic NFM products prepared from raw milk (cow/yak), based on their nature, the first type is lipid-based butter-like

* Corresponding author. Tel.: +91 9832061073.
E-mail address: jptamang@cus.ac.in (J.P. Tamang).

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product, locally known as mar (Supplementary material Fig. S1a), and the second type is the casein-based cottage-cheese like product, which is again of two varieties: soft cheese-like product chhurpi (Supplementary material Fig. S1b) and the hard variety called churkam (Supplementary material Fig. S1c). Mar is used as butter for frying vegetable and meats, chhurpi is consumed as curry and pickle and churkam is used as masticator due its characteristic gumminess and chewiness.

Culture-dependent methods have been used in studying the culturable bacteria in many NFM products (Akabanda et al., 2013; Dewan & Tamang, 2006, 2007; Nyambane, Thari, Wangoh, & Njage, 2014; Yu et al., 2015). Usually, the combination of phenotypic, biochemical tests and genotypic identification using 16S rRNA gene sequencing is used for LAB identification in most of the fermented foods (Tilahun et al., 2018). Previously we analysed samples of mar, chhurpi and churkam of Arunachal Pradesh using a culture-independent method with a high-throughput sequencing (HTS) tool and observed that LAB belong to phylum *Firmicutes* were significantly present in mar, chhurpi and churkam samples (Shangpliang et al., 2018). However, isolation of culturable LAB present in samples of mar, chhurpi and churkam by culture-dependent method is also essential to know the dominance of culturable LAB for preservation of these bacteria as genetic resources. Moreover, the traditional production of these exotic artisan NFM product is already is in peril due to rapid modernisation of the region. Hence, the present study is aimed to isolate and identify the culturable LAB isolated from exotic naturally fermented cow-milk and yak-milk products of Arunachal Pradesh in India, viz. mar, chhurpi and churkam, by phenotypic characteristics and 16S rRNA gene sequencing method.

2. Materials and methods

2.1. Samples

A total of 30 samples of NFM products (6 cow-milk mar, 4 yak-milk mar; 6 cow-milk chhurpi, 4 yak-milk chhurpi, 6 cow-milk churkam and 4 yak-milk churkam) were collected from West Kameng and Tawang districts of Arunachal Pradesh in India (Table 1). All samples were collected in pre-sterilised containers and transported to the laboratory in an ice-box cooler and stored at 4 °C for immediate microbiological analysis.

2.2. Analysis of pH

One gram of sample was dissolved in 10 mL sterilised physiological saline (0.85% NaCl) and the pH of all samples were determined using a pH meter (GeNei™, Bangalore, India) and calibrated with standard buffers. The pH value was represented as mean ± SD values of triplicates sets.

2.3. Enumeration and isolation of LAB

Hard samples (churkam) were first cut into small pieces with sterile scalpel before homogenisation and soft samples of mar and chhurpi samples were directly homogenised. All samples were homogenised in a stomacher (400, Seward, London, UK) using stomacher bags in a ratio of 10:100 (w/v) dissolved in physiological solution (0.85% NaCl) and serial dilution (10^{-1} to 10^{-8}) was made. One millilitre of homogenised mixture was transferred into MRS (Man-Rogosa-Sharpe) agar plate (M641, HiMedia, India), pH 6.2 (Yang et al., 2018) with 1% $CaCO_3$ by pour plate method and incubated in an anaerobic jar for 48 h at 30 °C. The number of colonies was counted as colony forming units (cfu g^{-1}) presented as log values with mean ± SD values of triplicate sets. Colonies were randomly selected and purified twice using the streak plate method. Purified colonies were checked then stored in 20% glycerol at −80 °C.

2.4. Phenotypic and biochemical characterisation

A total of 307 isolates of LAB isolates were isolated from 30 samples of mar, chhurpi and churkam. Preliminary characterisation including colony morphology, cell morphology, Gram stain, catalase test and ability of the colonies to produce light halo zone in the MRS media supplemented with 1% $CaCO_3$ (Dewan & Tamang, 2007) were performed for presumptive selection of LAB. The ability of LAB isolates to produce gas from glucose was used to differentiate homo-fermenters from hetero-fermenters (Carr, Chill, & Maida, 2002). Homo-fermenters were then differentiated, based on the cellular morphology and the ability to grow at 10 °C, 15 °C, 45 °C and 6.5% NaCl. Hetero-fermenters were further differentiated by the arginine hydrolysis test. Sugar fermentation test was performed following the method described by Holzapfel and Wood (2012). Based on phenotypic tests, biochemical and physiological profiles, all 307 isolates were tentatively identified up to genus level or groups, out of which 76 representative strains were randomly selected for further identification.

2.5. Genotypic characterisation

2.5.1. DNA extraction

DNA of LAB was extracted using an enzymatic-heating lysis method as described by Jeyaram, Romi, Singh, Devi, and Devi (2010) with slight modifications. A pure colony was inoculated in MRS broth and incubated at 30 °C for 16–18 h. The 2 mL of the culture broth was then transferred to 2 mL micro-centrifuge tube and centrifuged (Microcentrifuge, MicroCL 21R, ThermoFisher Scientific, Carlsbad, CA, USA) at 8000×*g* for 5 min. The supernatant was discarded, and the remaining pellet was then washed with sterile 0.5 M NaCl two times, followed by immediate washing with sterile deionised water (MilliQ H_2O). The pellet was suspended in 1 × TE buffer (pH 8), and 10 μL of lysozyme (2 mg mL^{-1}) was added to the solution. The cell suspension was then incubated at 37 °C for 30 min for enzyme activation, followed by immediate heating at 98 °C for 15 min. The suspension was centrifuged at 10,000×*g* for 10 min at 4 °C and the supernatant was transferred to a sterile micro-centrifuge tube. DNA was quantified using Eppendorf BioSpectrometer (Hamburg, Germany). Quantified DNA was stored at −20 °C until required and DNA purity of 1.8–2.2 was used for PCR reaction.

2.5.2. PCR amplification

Identification of LAB isolates was carried out using Sanger sequencing of 16S rRNA gene (Heather & Chain, 2016). The PCR reaction was carried out in a 50 μL reaction volume using GoTaq® Green Master Mix (M7122, Promega, Wisconsin, USA) containing the required dNTPs (dATPs, dTTPs, dGTPs, dCTPs), $MgCl_2$; primers 27F 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R 5′- GTTACCTTGTTACGACTT-3′ (Lane, 1991) and about 30–50 ng of the DNA template. The PCR amplification was carried out using a SimpliAmp™ Thermal Cycler (Cat No. A24811, ThermoFisher Scientific, Carlsbad, CA, USA) with the following conditions: initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C (denaturation) for 1 min, 55 °C (annealing process) for 1 min and 72 °C (elongation process) for 1.5 min. Lastly, PCR amplification was set to a final elongation process of 72 °C for 10 min and a stoppage process at 4 °C.

2.5.3. Purification of the PCR amplicons

The PCR amplicons were purified using PEG (polyethylene glycol)-NaCl (sodium chloride), 20% (w/v) PEG, 2.5 M NaCl (Schmitz & Riesner, 2006) with slight modifications. Briefly, 0.6 volumes of PEG-NaCl solution was mixed with the PCR amplicons and incubated at 37 °C for 30 min. The mixture was centrifuged at 10,000×*g*, 4 °C for 30 min, the supernatant was carefully discarded, the pellet was then washed twice with freshly prepared and cold 70% ethanol and was allowed to air-dry overnight. Finally, 20 μL nuclease free water was used to suspend the purified DNA. Agarose (1.2%) gel electrophoresis was visualised using a Gel Doc™ EZ Imager (Bio-Rad, Hercules, CA, USA).

2.5.4. 16S rRNA gene sequencing

The purified PCR amplicons was subjected to sequencing using the primer pairs 27F 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R 5′-GTTACCTTGTTACGACTT-3′ (Lane, 1991). Two sequencing PCR reactions were carried out for each primer. A final volume of 50 μL reaction volume containing 0.2 μM primer, 0.2 mM dNTPs (dATPs, dTTPs, dGTPs, dCTPs), 2.0 mM MgCl2, 0.5 mg mL^{-1} and 0.04 U μL^{-1} Taq DNA polymerase. The PCR conditions used for sequencing included an initial denaturation of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min (denaturation), 40 °C for 2 min (annealing), 72 °C for 1 min (elongation) and a final elongation of 72 °C for 10 min. Sequencing was performed using an automated DNA analyser (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA).

2.6. Bioinformatics analysis

Raw sequences were checked for their quality using Sequence Scanner v2.0, a software from Applied Biosystems, https://www.thermofisher.com/in/en/home/life-science/sequencing/sanger-sequencing/sanger-dna-sequencing/sanger-sequencing-data-analysis.html. Good quality sequencing reads were then assembled using ChromasPro v1.34, http://technelysium.com.au/wp/chromas/. Chimera-check was performed using a programme called Mallard (Ashelford, Chuzhanova, Fry, Jones, & Weightman, 2006). Identity was acquired by aligning the sequences with BLAST (basic local alignment search tool) https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch (Altschul, Gish, Miller, Myers, & Lipman, 1990) and EzTaxon, https://www.ezbiocloud.net/(Kim et al., 2012) databases. Phylogenetic relationship of the identified species was carried out after aligning the sequences with clustalW (Thompson, Higgins, &

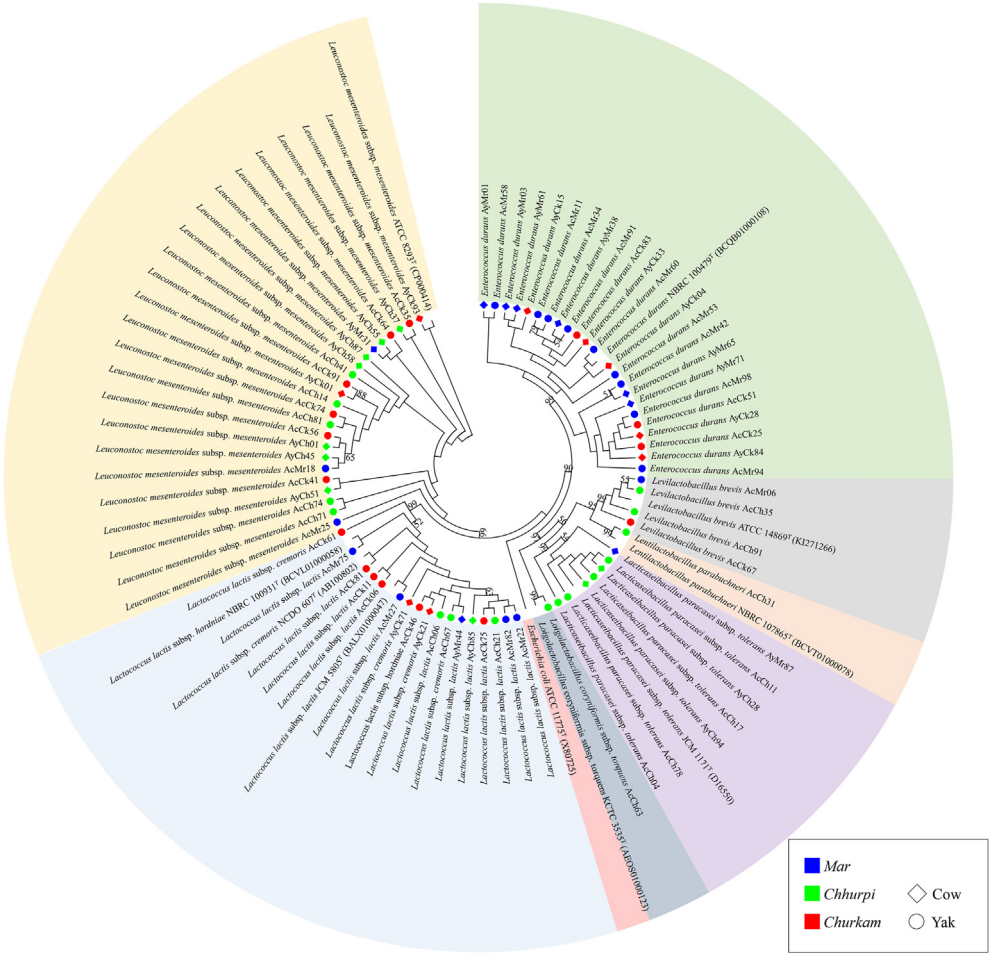

Fig. 1. Phylogenetic diversity analysis of lactic acid bacteria species isolated from naturally fermented milk products of Arunachal Pradesh, India. The evolutionary history was inferred using the neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. The analysis involved 86 nucleotide sequences (including type strains). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. The isolates were also depicted in colours where they were originally isolated from: blue-mar, green-chhurpi and red-churkam; diamond for cow and circle for yak products. *Escherichia coli* ATCC 11775(T) was used as an outgroup for phylogenetic tree construction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Gibson, 1994). Phylogenetic tree using neighbour-joining (Saitou & Nei, 1987) was constructed by Molecular Evolutionary Genetics Analysis version 7 (MEGA7.0.26) (Kumar, Stecher, & Tamura, 2016).

2.7. Statistics

Frequency of the isolates was calculated using MS Excel v365. Diversity indices [Simpson diversity index (H_{Si}), Shannon diversity index (H_{Sh})] were calculated using PAST v4 (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer, Harper, & Ryan, 2001).

Shannon's diversity index was given as follows:

$$H_{Sh} = -\sum_{i=1}^{S} p_i \ln(p_i)$$

and Simpson's diversity index was given as follows:

$$D = \sum_{i=1}^{S} p_i^2$$

$$H_{Si} = 1 - D$$

where, S is the number of species p_i is the number of the given species divided by the total number of isolates observed, and D is Simpson's index (Daly, Baetens, & De Baets, 2018).

3. Results

The pH of naturally fermented cow-milk products (mar, chhurpi and churkam) was 5.32 ± 0.01 to 6.55 ± 0.01; and that of yak products (mar, chhurpi and churkam) was 5.40 ± 0.01 to 6.62 ± 0.01, respectively (Table 1). The LAB populations in NFM of cow products and yak products were 6.27 ± 0.01 log cfu g^{-1} to 6.40 ± 0.01 log cfu g^{-1}, and 6.27 ± 0.01 log cfu g^{-1} to 6.49 ± 0.02 log cfu g^{-1}, respectively (Table 1). A total of 76 representative strains of LAB were randomly selected on the basis of their biochemical and physiological profiles (Supplementary material Tables S1–S4).

Based on the 16S rRNA gene sequence result, a phylogenetic tree was constructed using the neighbour-joining method (Fig. 1) and identification of LAB strains was carried out using good quality sequences ranging from 1048 bp to 1471 bp, which were aligned using EzTaxon and NCBI database (Supplementary material Tables S5–S7). Overall, 9 species of LAB were identified from samples of naturally fermented cow-milk products, and 5 species of LAB from samples of naturally fermented yak-milk products, respectively (Fig. 2a). All the 16S rRNA gene sequences were deposited in GenBank-NCBI under the accession numbers: MK203740-MK203744; MK182827-MK182841; MT305879-MT305898; MT305901-MT305936.

Enterococcus durans was the predominant species in samples of cow and yak-milk mar and yak-milk churkam, whereas it was not detected in any sample of chhurpi (Fig. 2b). Other LAB present in naturally fermented cow-milk samples were *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *hordniae*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Levilactobacillus brevis*, *Loigolactobacillus coryniformis* subsp. *torquens* and *Lentilactobacillus parabuchneri*, except *Lacticaseibacillus paracasei* subsp. *tolerans* which was not detected in mar samples (Fig. 2b). Whereas, distribution of LAB species in naturally fermented yak-milk products was variable with absence of few genera (Fig. 2b). A simple network analysis using iGraph-R-package showing the shared and unique identified LAB species among the NFM products is shown in Fig. 2c. Based on Shannon's and Simpson's diversity indices, chhurpi (cow) showed a relatively high diversity index $H_{Sh} = 1.56$ and $H_{Si} = 0.85$, with mar (yak) showed the least diversity index of $H_{Sh} = 0.84$ and HSi = 0.58

Table 1
Sample details, pH and lactic acid bacteria load of naturally fermented milk products of Arunachal Pradesh, India.

NFM	Source of milk	Nature of samples	Place of collection	pH (Mean ± SD)	Log cfu g^{-1} (Mean ± SD)
Mar	Cow	Butter-like	Cheghar, Tawang	6.52 ± 0.01	6.29 ± 0.03
Mar	Cow	Butter-like	Samchin, Tawang	6.52 ± 0.01	6.39 ± 0.04
Mar	Cow	Butter-like	Kudung, Tawang	6.53 ± 0.01	6.40 ± 0.01
Mar	Cow	Butter-like	Tawang, Tawang	6.53 ± 0.01	6.35 ± 0.08
Mar	Cow	Butter-like	Bomdila, West Kameng	6.53 ± 0.02	6.38 ± 0.01
Mar	Cow	Butter-like	Dirang, West Kameng	6.55 ± 0.01	6.39 ± 0.04
Mar	Yak	Butter-like	Samchin, Tawang	6.56 ± 0.02	6.47 ± 0.02
Mar	Yak	Butter-like	Cheghar, Tawang	6.61 ± 0.01	6.49 ± 0.02
Mar	Yak	Butter-like	Dirang, West Kameng	6.62 ± 0.01	6.48 ± 0.03
Mar	Yak	Butter-like	Bomdila, West Kameng	6.62 ± 0.01	6.43 ± 0.03
Chhurpi	Cow	Soft, cheese-like	Cheghar, Tawang	5.32 ± 0.01	6.28 ± 0.02
Chhurpi	Cow	Soft, cheese-like	Samchin, Tawang	5.32 ± 0.01	6.29 ± 0.01
Chhurpi	Cow	Soft, cheese-like	Kudung, Tawang	5.32 ± 0.02	6.32 ± 0.02
Chhurpi	Cow	Soft, cheese-like	Tawang, Tawang	5.33 ± 0.01	6.27 ± 0.01
Chhurpi	Cow	Soft, cheese-like	Bomdila, West Kameng	5.33 ± 0.02	6.33 ± 0.02
Chhurpi	Cow	Soft, cheese-like	Dirang, West Kameng	5.35 ± 0.01	6.29 ± 0.01
Chhurpi	Yak	Soft, cheese-like	Samchin, Tawang	5.35 ± 0.01	6.30 ± 0.03
Chhurpi	Yak	Soft, cheese-like	Cheghar, Tawang	5.41 ± 0.01	6.27 ± 0.01
Chhurpi	Yak	Soft, cheese-like	Dirang, West Kameng	5.42 ± 0.01	6.34 ± 0.05
Chhurpi	Yak	Soft, cheese-like	Bomdila, West Kameng	5.42 ± 0.02	6.36 ± 0.03
Churkam	Cow	Hard-mass, masticator	Cheghar, Tawang	5.71 ± 0.01	6.29 ± 0.02
Churkam	Cow	Hard-mass, masticator	Samchin, Tawang	5.71 ± 0.01	6.35 ± 0.04
Churkam	Cow	Hard-mass, masticator	Kudung, Tawang	5.71 ± 0.01	6.34 ± 0.03
Churkam	Cow	Hard-mass, masticator	Tawang, Tawang	5.71 ± 0.01	6.3 ± 0.04
Churkam	Cow	Hard-mass, masticator	Dirang, West Kameng	5.72 ± 0.01	6.38 ± 0.03
Churkam	Cow	Hard-mass, masticator	Bomdila, West Kameng	5.72 ± 0.01	6.34 ± 0.11
Churkam	Yak	Hard-mass, masticator	Samchin, Tawang	5.82 ± 0.01	6.31 ± 0.06
Churkam	Yak	Hard-mass, masticator	Cheghar, Tawang	5.82 ± 0.01	6.28 ± 0.04
Churkam	Yak	Hard-mass, masticator	Dirang, West Kameng	5.87 ± 0.02	6.34 ± 0.03
Churkam	Yak	Hard-mass, masticator	Bomdila, West Kameng	5.85 ± 0.02	6.36 ± 0.04

Table 2
Frequency and species diversity indices of lactic acid bacteria strains isolated from the exotic naturally fermented milk products (cow and yak) of Arunachal Pradesh.

Species/diversity indices	Mar		Chhurpi		Churkam	
	Cow (%)	Yak (%)	Cow (%)	Yak (%)	Cow (%)	Yak (%)
Enterococcus durans	56.25	66.67	0.00	0.00	18.75	55.56
Leuconostoc mesenteroides subsp. *mesenteroides*	12.50	11.11	31.25	70.00	37.50	22.22
Lactococcus lactis subsp. *lactis*	25.00	11.11	12.50	10.00	25.00	0.00
Lactobacillus paracasei subsp. *tolerans*	0.00	11.11	25.00	20.00	0.00	0.00
Lactococcus lactis subsp. *cremoris*	0.00	0.00	6.25	0.00	6.25	22.22
Lactobacillus brevis	6.25	0.00	12.50	0.00	6.25	0.00
Lactobacillus coryniformis subsp. *torquens*	0.00	0.00	6.25	0.00	0.00	0.00
Lactobacillus parabuchneri	0.00	0.00	6.25	0.00	0.00	0.00
Lactococcus lactis subsp. *hordniae*	0.00	0.00	0.00	0.00	6.25	0.00
Species diversity indices						
Simpson diversity index (D)	0.64	0.58	0.85	0.51	0.80	0.67
Shannon diversity index (H)	1.01	0.84	1.56	0.70	1.39	0.88

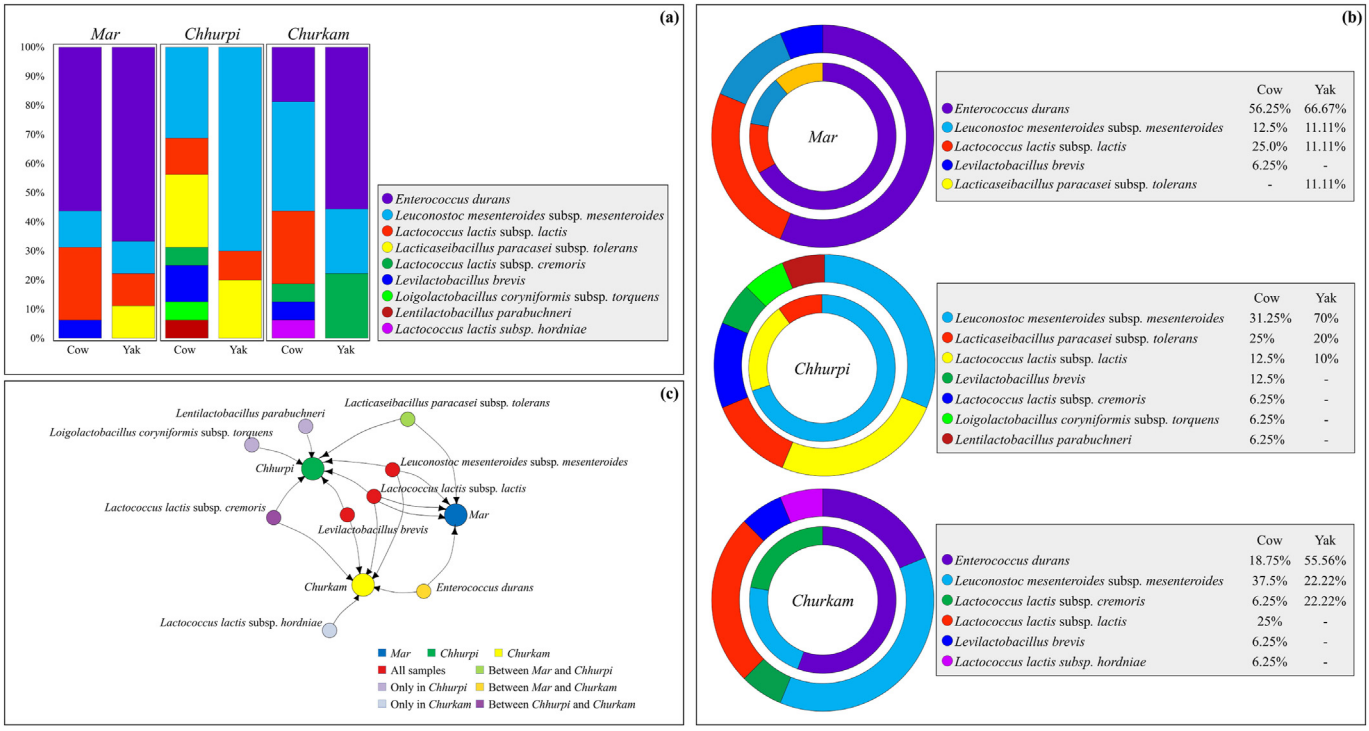

Fig. 2. Data visualisation of the lactic acid bacteria (LAB) strains isolated from naturally fermented milk (NFM) products of Arunachal Pradesh: (a) overall bar-graph representation of identified LAB strains among mar, chhurpi and churkam samples from both cow-milk and yak-milk products; (b) individual representation (doughnut-chart) of LAB isolated from each NFM products and (c) simple network analysis showing the shared and unique identified LAB species among the NFM products.

(Table 2). Altogether, cow-based products showed a higher diversity with $H_{Sh} = 1.73$ and $H_{Si} = 0.82$ in comparison with yak products $H_{Sh} = 1.28$ and $H_{Si} = 0.72$.

4. Discussion

There may a possible threat to production of exotic artisan NFM products by the ethnic communities due to rapid development and modernisation in Indian state of Arunachal Pradesh, resulting in near extinction of these rare exotic NFM products in the Himalayas. Hence, we isolated the culturable LAB from samples of mar, chhurpi and churkam using a culture-dependent method.

Casein-based products chhurpi and churkam are slightly acidic in nature in comparison with the lipid-rich product, mar, due to predominance of LAB, since NFM products are mostly dominated by LAB (Dewan & Tamang, 2007; Tamang et al., 2000; Zhong et al., 2016). LAB are the major members of various fermented milk products including yoghurt, cheese, and other fermented milk products (Macori & Cotter, 2018; Wirawati, Sudarwanto, Lukman, Wientarsih, & Srihanto, 2019).

LAB population of NFM samples was 6.27 ± 0.01 to 6.49 ± 0.02 log cfu g^{-1}, which is similar to previous culture-dependent study of chhurpi of Arunachal Pradesh (Tomar et al., 2009). LAB isolated from NFM products of Arunachal Pradesh were phenotypically identified into *Enterococcus*, *Lactococcus*, *Leuconostoc* and lactobacilli, which were confirmed by the molecular identification tool using 16S rRNA gene sequencing method, a universal housekeeping gene for bacterial identification for bacterial identification (Janda & Abbott, 2007). Recently, *Lactobacillus* members were re-classified under the new nomenclature (Zheng et al., 2020). Hence, *Lactobacillus paracasei* subsp. *tolerans* was reclassified as *Lacticaseibacillus paracasei* subsp. *tolerans*; *Lactobacillus brevis* as *Levilactobacillus brevis*; *Lactobacillus coryniformis* subsp. *torquens* as *Loigolactobacillus coryniformis* subsp. *torquens*;

and *Lactobacillus parabuchneri* as *Lentilactobacillus parabuchneri* (Zheng et al., 2020).

Overall identification by 16S rRNA gene sequencing revealed the identity of 7 genera viz., *Enterococcus*, *Lacticaseibacillus*, *Lactococcus*, *Lentilactobacillus*, *Leuconostoc*, *Levilactobacillus* and *Loigolactobacillus*, with 9 species in samples of naturally fermented cow-milk products and 5 species in samples of naturally fermented yak-milk products, respectively. *E. durans* was the predominant species in samples of mar and churkam, however it was not detected in samples of chhurpi. High-throughput sequence analysis reported the presence of *E. durans* at <1% in chhurpi (Shangpliang et al., 2018). Hence, we speculated that due to the random selection of isolated strains and owing to its low number, *E. durans* may not have been cultivated while plating from samples of chhurpi. Enterococci are primarily associated with the human gastrointestinal tract (Graham, Stack, & Rea, 2020), which are also reported from fermented milk products (Terzić-Vidojević et al., 2020). *E. durans* has been isolated and identified from various fermented milk products (Chen et al., 2010; Shangpliang, Sharma, Rai, & Tamang, 2017), and may be responsible for ripening of cheese and development of flavour compounds for sensory properties of the product (Nami et al., 2019). However, species belonging to the genus *Enterococcus* have yet to be recommended for the Qualified Presumption of Safety (QPS) list as well as the Generally Regarded as Safe (GRAS) list (Graham et al., 2020). Though few strains of *Enterococcus* are used as probiotics (Li et al., 2018), more updates are needed to test the safety and efficacy of this genus (Hanchi, Mottawea, Sebei, & Hammami, 2018).

L. mesenteroides subsp. *mesenteroides* was present in all NFM products with 70% prevalence in samples of yak-milk *chhurpi*. *Leuc. mesenteroides* is commonly isolated from fermented milk products (Arakawa et al., 2016). *Leuc. mesenteroides* has many potential probiotic traits such as antimicrobial properties (Liu, Kim, Kwak, & Kang, 2017), antioxidant activities and cholesterol-lowering effects (Macori & Cotter, 2018) and contributing to aroma development (Özcan et al., 2019). *Levilactobacillus brevis* (Basonym: *Lactobacillus brevis*; Zheng et al., 2020) has been reported from chhu, a traditional cheese-like product of Sikkim in India through culture-dependent study (Dewan & Tamang, 2006). *Lev. brevis* is one of the LAB species that have been granted QPS status (Leuschner et al., 2010). *Lentilactobacillus parabuchneri* (Basonym: *Lactobacillus parabuchneri*; Zheng et al., 2020) is one of the main non-starter LAB, commonly reported from fermented dairy products (Terzić-Vidojević et al., 2020). *Lacticaseibacillus paracasei* (Basonym: *Lactobacillus paracasei*; Zheng et al., 2020) has been isolated from Greek artisanal dairy products (Meng et al., 2018). Some strains are known to have probiotic traits including bacteriocins production (de Almeida Júnior et al., 2015); cholesterol-lowering properties (Albano et al., 2018); inhibiting adherence of *Escherichia coli* and *Salmonella typhimurium*, *Yersinia enterocolitica* (Damodharan, Palaniyandi, Suh, & Yang, 2019). *L. lactis* is associated with many fermented milk products (Allam, Darwish, Ayad, Shokery, & Darwish, 2017; de Freitas Martins et al., 2020; Shangpliang et al., 2017), and has probiotic traits (Yerlikaya, 2019).

Species richness represented the absolute number of the species present in the sample (Daly et al., 2018). We observed the higher species diversity in casein-based products (chhurpi and churkam) in comparison with lipid-based products (mar), indicating casein may be a good medium for bacterial proliferation in comparison with lipids (Zhang et al., 2011).

Enterococcus, Lacticaseibacillus, Lactococcus, Lentilactobacillus, Leuconostoc, Levilactobacillus and *Loigolactobacillus,* detected in samples of mar, chhurpi and churkam by culture-dependent method were also detected in the same products earlier by culture-independent method using HTS analysis (Shangpliang et al., 2018). Application of culture-independent method is to profile the microbial community present in a sample depending on expression of DNA represented by Operational Taxonomic Units (OTUs), where maximum species can be captured, especially those which are difficult to identify through culture-dependent methods (Zapka et al., 2017). Information on bacterial community in NFM of Arunachal Pradesh by culture-independent method has guided us to isolate the predominant culturable LAB for preservation of these bacteria as microbial resources and also for further studies on their technological and functional properties, including development of starter cultures.

5. Conclusion

Exotic naturally fermented cow-milk and yak-milk products, viz. mar, chhurpi and churkam, are important dietary items of the ethnic people of Arunachal Pradesh in the Indian Himalayas. Since the traditional production of these exotic artisan milk product is already in peril, isolation and identification of several species of LAB may help to preserve the microbial resources present in exotic foods of the Himalayas. Moreover, exploration of isolated strains for their potential probiotic applications as well as for development of starter culture may be carried out in future. Additionally, a more in-depth study of other culturable/non-culturable microorganisms including yeasts may also be of great importance to explore from these exotic NFM products.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2021.105038.

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