Study of Bacterial Diversity in Traditional Starter Cultures of the Eastern Himalayas

A Thesis Submitted

To

Sikkim University

For the Degree of Doctor of Philosophy

By

Pooja Pradhan

Supervisor: Professor Dr. Jyoti Prakash Tamang

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

JULY 2020

DECLARATION

I declare that the present Ph.D thesis entitled **"Study of Bacterial Diversity in Traditional Starter Cultures of the Eastern Himalayas"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervision of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Tadong, Sikkim University, is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok. No part thereof has been submitted for any degree or diploma in any University/Institution.

P. Pradhan

.....

Pooja Pradhan
Registration No: 14/PhD/MCB/08
Research Scholar
Department of Microbiology
School of Life Sciences
Sikkim University (www.cus.ac.in)
Gangtok

Date: 24th June 2020
Place: Tadong, Gangtok

माइल, सामदुर, तादोंग - 737102
गटोक, सिक्किम, भारत
ोन-03592-251212, 251415, 251656
लीफैक्स - 251067
बसाइट - www.cus.ac.in

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

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

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

CERTIFICATE

This is to certify that the PhD thesis entitled **"Study of Bacterial Diversity in Traditional Starter Cultures of the Eastern Himalayas"** submitted to **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Ms. Pooja Pradhan** for the award of PhD Degree in Microbiology, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. It is a record of bonafide investigation carried out and completed by her under our supervisions and guidances. She has followed the rules and regulations laid down the University. The results are original and have not been submitted anywhere else for any other degree or diploma.

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

SUPERVISOR

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

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

HEAD

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

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

Place: Tadong
Date: 24th June 2020

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

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

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

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

Date: 23rd June 2020

PLAGIARISM TEST CERTIFICATE

This is to certify that the plagiarism check has been carried out for the following PhD thesis with the help of **URKUND SOFTWARE** and the result is **2% tolerance rate**, within the permissible limit (below 10% tolerance rate) as per the norm of Sikkim University.

"Study of Bacterial Diversity in Traditional Starter Cultures of the Eastern Himalayas"

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

P. Pradhan

Pooja Pradhan
Signature of the candidate

Professor Dr. Jyoti Prakash Tamang
Supervisor

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

Professor A.S. Chandel
Librarian, Sikkim University

पुस्तकालयाध्यक्ष
Librarian
केन्द्रीय पुस्तकालय Central Library
सिक्किम विश्वविद्यालय
Sikkim University

URKUND

Document Information

Analyzed document	PhD Thesis-Pooja Pradhan-plagiarism.docx (D75493367)
Submitted	6/23/2020 7:28:00 AM
Submitted by	Prof A S Chandel
Submitter email	librarian@cus.ac.in
Similarity	2%
Analysis address	librarian.sikkim@analysis.urkund.com

Sources included in the report

W	URL: https://pearl.plymouth.ac.uk/bitstream/handle/10026.1/13664/2019yomla1042366phd.pd ... Fetched: 6/23/2020 7:30:00 AM	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6848222/ Fetched: 3/16/2020 7:18:47 AM	12
W	URL: https://www.researchgate.net/figure/Traditionally-prepared-amylolytic-starter-cult ... Fetched: 6/22/2020 11:12:32 AM	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4805592/ Fetched: 9/30/2019 6:52:48 PM	1
W	URL: https://cyberleninka.org/article/n/699889 Fetched: 6/23/2020 7:30:00 AM	1
W	URL: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02237/full Fetched: 6/23/2020 7:30:00 AM	1

पुस्तकालयाध्यक्ष
Librarian
केन्द्रीय पुस्तकालय Central Library
सिक्किम विश्वविद्यालय
Sikkim University

ACKNOWLEDGEMENTS

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people and institutions. It gives me an immense pleasure to acknowledge the help and support that I got during my PhD which I am concluding successfully today. Foremost, I would like to first express my heartfelt gratitude to my supervisor Professor Dr. Jyoti Prakash Tamang, Department of Microbiology, School of Life Sciences, Sikkim for all the support and encouragement given by him. His continuous guidance during my PhD has been very constructive not only for my career but for my personal development as well. I am greatly indebted to him for everything. Without his constant and continuous guidance this research would not have been completed. I am highly inspired by his work culture which I will also tried to follow and will always cherish it throughout my life.

I would like to extend my sincere gratitude to Dr. Namrata Thapa, Head, Department of Zoology, Sikkim Government College, Tadong, Gangtok, for her unending support and valuable suggestions during my PhD work.

I would also like to sincerely thank all the faculty members of Department of Microbiology, Dr. Hare Krishna Tiwari, Associate Professor and Head of the department, Dr. Buddhiman Tamang, Dr. Nagendra Thakur, Dr. Bimala Singh and Dr. Anil Kumar Verma, Assistant Professors of Department of Microbiology, Sikkim University for their suggestions and help.

I would like to extend my sincere thanks to Smt. Radha Basnet and Shri Pukar Bishwakarma for their help and support which was much needed and helped a lot in my work.

I would like to express my appreciation to the villagers of the areas of Eastern Himalayas whom I contacted personally for information regarding the samples. They were very helpful and were kind enough to share the details of their valuable and rare

knowledge which I got the opportunity to share it with the world. Without their help and information this study would have been incomplete.

I would like to sincerely thank my seniors Dr. Shankar Prasad Sha, Ms. Ranjita Rai and Mrs Bhoomika Poudyali, my dear friends Mr. Ranjan Kaushal Tirwa, Mrs. Anu Anupma, Ms. Meera O. Bhutia, Ms. Nilu Pradhan, Mr.Sayak Das, Dr. Ashis Kumar Singh and Mr. Lalit Chaurasia, my juniors Mr. Nakibapher Jones Shangpliang, Mr. Pynhun Kharnaio, Ms. Priyambada Pariyar, Mr. Souvik Das, Mr. Tanmoy Pati, Mr. Rupayan Nanda and Mr. Rakesh Mohapatra for their help and suggestions during my PhD.

I would also like to thank the faculty members of department of Botany, Chemistry, Physics and Zoology of Sikkim University. I am also thankful to Dr. Sudhan Pradhan, Dr. Rajen Chettri and Dr. Arun Kumar Rai for their advice, help and support.

I am extremely thankful to Professor Dr. T.B Subba (former Vice-Chancellor of Sikkim University) and Professor Dr. Avinash Khare (present Vice-Chancellor of Sikkim University). I sincerely acknowledge Department of biotechnology, Govt. of India for funding me traineeship in bioinformatics center, Sikkim University and UGC for providing me Non-NET fellowship during the entire period of my PhD.

I would like to thank Prof. A.S. Chandel, Librarian and **Shri Pranab Kumar Sarkar,** Controller of Examinations, Sikkim University for their help and support.

And finally, would also like to say a heartfelt thank you to my Mother, Father, husband and my in-laws who have been by my side throughout this PhD, living every single minute of it, and without whom, I would not have had the courage to embark on this journey in the first place.

Pooja Pradhan

Date: 22nd June 2020, Tadong

CONTENTS

4. RESULTS

INTRODUCTION

History and Culture of Drinking

Ethnic fermented foods and alcoholic beverages are produced by the ethnic people of the world using their indigenous knowledge from locally available raw materials of plant or animal sources either spontaneously or by 'back-slopping' or adding starter culture(s) containing functional microorganisms which modify the substrates biochemically and organoleptically into edible products that are culturally and socially acceptable to the consumers (Tamang 2010a; Taylor et al. 2020). Food fermentation also promote the biological enrichment of nutrients such as vitamins, organic acids and essential amino acids in food (Blandino et al. 2003), probiotics (Rezac et al. 2018) and several health-promoting benefits (Tamang 2015). Alcoholic beverage forms a deep-rooted food culture of every community in this world (Tamang 2010b) where production involves mainly five steps: (i) selection of starchy raw material (ii) processing of the raw material to give a fermentable extract mostly sugar (iii) alcoholic fermentation by yeast, principally by strains of *Saccharomyces cerevisiae* and non-*Saccharomyces* and fungi (iv) distillation of the fermented material to give the distillate product and (v) post-distillation processing (Walker and Stewart 2016; Grumezescu and Holban 2019). Alcoholic beverages and liquors are culturally and socially accepted product for drinking, entertainment, customary practices and religious purposes (Tamang 2010b; Savic et al. 2016). Wine, in Latin means vinum, generally refers to alcoholic fermentation of grape juice without distillation (McGovern et al. 2004), and historically wine was made in the Caucasus and Mesopotamia in modern day Georgia in 6000 BCE (Robinson 1994; Pretorius 2000). Beer-making process was originated in ancient Egypt and Mesopotamia around 4000 BCE (Damerow 2012). One of the oldest alcoholic beverages of Latin America known as *pulque* was inherited from the Aztecs 1000 years ago (Escalante et al. 2016).

Traditional alcoholic beverages and liquors have specific sensory properties with strong ritualistic importance among the ethnic people in Asia and Africa where drinking of appreciable quantities of alcohol is a part of dietary culture and social obligation (Orji et al. 2003; Faria-Oliveira et al. 2015; Gou et al. 2015; Tamang et al. 2020). In Asia, traditionally prepared dry amylolytic starters are used to convert starches to sugars (Aidoo and Nout 2010; Tamang 2010a) to prepare alcoholic beverages from cereals (Tamang et al. 2016a). Historically and culturally, wine, whisky, rum, brandy and beer were not traditional beverages/alcoholic drinks of Asian people (Tamang et al. 2020). However, due to the Europeans during colonial rules in many Asian and African countries, such alcoholic drinks have become popular in Asia and Africa (Hutkins 2019).

Drinking of alcoholic beverages and drinks in India first appeared in Indus Valley civilization around 6000 BCE (Sarkar et al. 2016), based on findings of clay pots items from excavations sites (Mahdihassan 1979), mostly through fermentation (Singh et al. 2010), crude alcoholic distillation (Achaya 1991). Historically, liquor God *Soma* was worshipped by *soma rus,* a refreshing sweet juice prepared from leaf-less vine (*Sarcostemma acidum*) during the *Vedic* period (Somasundaram et al. 2016), which had medicinal values (Sarma 1939). High alcoholic distilled liquor prepared by fermenting millets called *sura* and *parisrut*, an alcoholic product fermented from flowers were the earliest alcoholic beverages of India around 1500 BCE (Mahdihassan 1981). There has been no historical evidence of wine making, beer-brewing and malting for alcoholic beverages in India since Indus Valley civilization (Tamang 2020). Wine, rum, brandy, whisky and beer were introduced to modern Indian by British during colonial rule (Goodman 2018). Historically and traditionally in India, alcoholic beverages are fermented either by using traditionally prepared dry amylase

and alcohol-producing cereal-based starters (through back-slopping method) or natural fermentation of plant (such as *toddy, tari*) or cereals, and production of distilled liquor (Tamang 2020).

The Eastern Himalayas

The Himalayas are the centre of rich bio-diversity of culture, ethnicity, natural resources and water resources (Tamang 2010a). The Himalayan arc extends between latitudes 26°20' and 35°40' North and between longitudes 74°50' and 95°40' East (Ives 2006) and based on the geo-morphology and demography, the Himalayas are divided into the Western, Central and the Eastern Himalayas (Nandy et al. 2006). The geographical extend of the Eastern Himalayas stretch from the eastern Nepal, North East India (Darjeeling hills/Gorkhaland Territorial Administration or GTA, Sikkim and Arunachal Pradesh) and Bhutan to Tibet Autonomous Regions in China (Saha 2013) (Figure A). The ethnic people of **Nepal** are Newar, Magar, Tamang, Rai, Limboo, Gurung, Bahun, Chettri, Dewan, Sanyasi, Bhujel, Sunwar, Khagatey, Sherpa, Kami, Damai, Sarki, Yadav, Taru, Mahji, Kumhal, Urau, Meche, Dhimal, Satar, Rajbanshi; ethnic people of **Darjeeling Hills/**GTA are Gorkha/Nepali (Rai, Tamang, Gurung, Limboo, Chettri, Magar, Bahun, Pradhan/Newar, Dewan, Sunwar, Bhujel, Khagatey, Sherpa, Sansyasi/Giri, Kami, Damai, Sarki, Maji, Lepcha, Tibetan); **Sikkim** are ethic Nepali (Bahun, Chettri, Sansyasi/Giri, Magar, Tamang, Pradhan/Newar, Rai, Limboo, Gurung, Bhujel, Dewan, Sunwar, Khagatey, Sherpa, Kami, Damai, Sarki, Maji, Lepcha, Bhutia); **Arunachal Pradesh** are Monpa, Sherdukpen, Memba, Khamba, Khampti, Singpho, Adi, Aka, Apatani, Bangni, Nishing, Mishmi, Miji, Tangsa, Nocte, Wancho; and **Bhutan** are Drukpa/Ngalop, Sharchop and ethnic Nepali/Lhotshamp (Tamang 2010a; Gupta 2018).

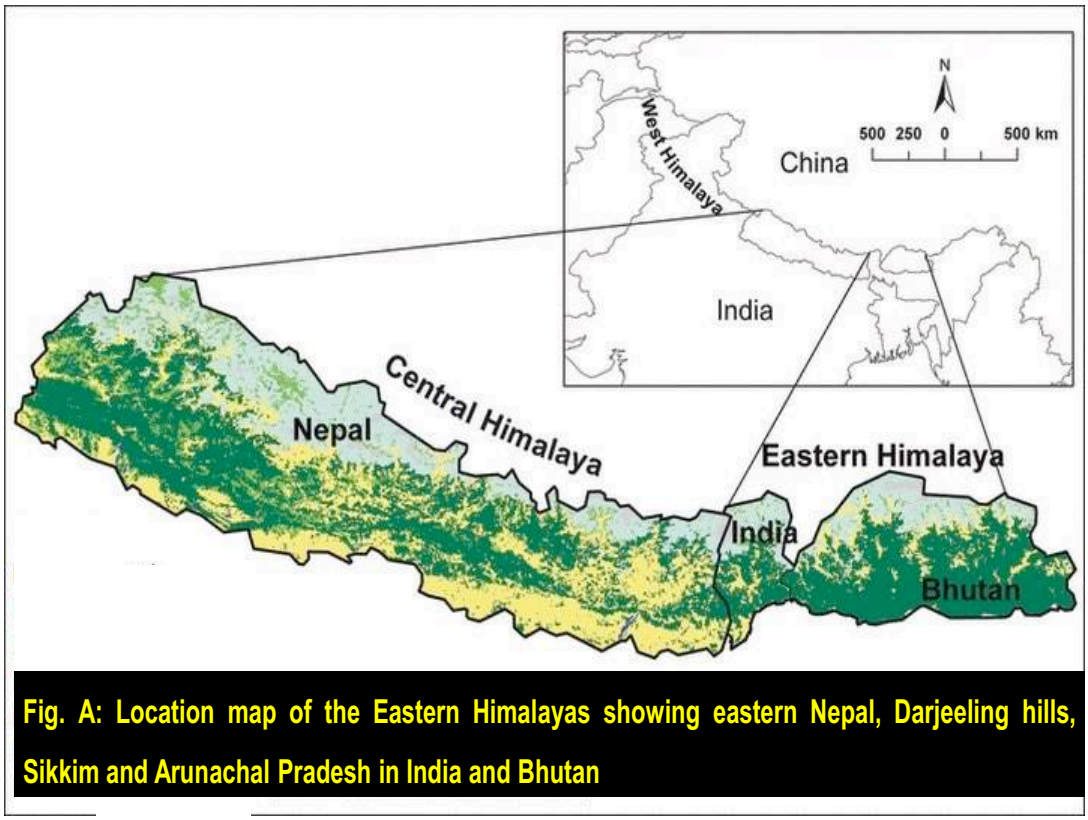


Fig. A: Location map of the Eastern Himalayas showing eastern Nepal, Darjeeling hills, Sikkim and Arunachal Pradesh in India and Bhutan

Topographically, linguistically and culturally the ethnic Nepali or Gorkha of Nepal, Darjeeling hills, Sikkim and south Bhutan have similarities with more than 20 major castes within the community (Preet et al. 2016). The Himalayan people practice agrarian and pastoral types of mountain-farming and animal husbandry system (Sharma et al. 2007; Bhasin 2013). Many region-specific ethnic fermented foods and beverages are traditionally produced from locally available plant and animal-resources which are cooked/fried into varieties of flavoursome and delicious cuisine and consumed as staple diets, side-dish, curry, soup, savoury, condiments and alcoholic drink by ethnic people of the Eastern Himalayas (Tamang 2010a; Tamang et al. 2010; Tamang et al. 2012). Majority of ethnic Himalayan people drink home-made traditional alcoholic beverages and distilled liquor prepared from cereals (rice, finger millets, maize) as a part of their dietary culture and social obligation (Tamang 2010a).

One of the most popular mild-alcoholic (~4% alcohol) beverage called *kodo ko jaanr* (Thapa and Tamang 2004) is prepared from fermented finger millet seeds by dry amylolytic starters, has been recorded during the Kirat dynasty of Nepal in 625 BCE to 100 BCE (Adhikari and Ghimirey 2000). The Newar, one of the ethnic communities of Gorkha, used to ferment alcoholic beverages from rice in 880 BCE during Malla dynasty in Nepal (Khatri 1987). The first record of *chyang*, fermented finger millet alcoholic beverage similar to *kodo ko jaanr* in Sikkim was first mentioned in Gazetteer of Sikkim by Risley (1928) which quoted "*marwa* or *chyang*, is a kind of beer brewed by everyone in Sikkim, and might be called their staple food and drink". Short descriptions of fermented millet beverages as alcoholic drink consumed by the local ethnic people were mentioned in historical documents on Darjeeling and Sikkim (Hooker 1854; Gorer 1938).

Traditional Dry Starters

The ethnic people of the Eastern Himalayan regions of India, Nepal and Bhutan have innovated an art of starter-making traditional technology (Tamang 2005). During preparation of starters, over-night soaked and pounded rice flours are mixed with wild herbs, spices and ~1-2% of previously prepared dry starters in powder forms to make doughs with variable size and shapes are placed in fresh fern leaves and allowed to ferment for 2-3 days at room temperature and the freshly fermented doughs are then sun dried for 2-3 days to get dry starters (Tamang et al. 1996; Thakur et al. 2015; Anupma et al. 2018). Every ethnic community in the Himalayan regions of India, Nepal and Bhutan prepare dry starters with slight variation in use of raw substrates such as rice or wheat, and wrapping materials, which are known as *marcha* in Nepal, Darjeeling hills and Sikkim in India (Thapa and Tamang 2020), *mana* and *manapu* of

Nepal (Nikkuni et al. 1996), *phab* in Bhutan (Tamang 2010a) and varieties of locally prepared starter cultures in North East India such as *chowan* in Tripura*, dawdim* in Mizoram*, humao, modor pitha* in Assam, *hamei* in Manipur*, khekhrii* in Nagaland, and *phut* in Arunachal Pradesh (Anupma et al. 2018). Similar types of dry starters for alcohol production are also traditionally prepared in different countries in South East Asia by ethnic Asian communities such as *benh men* in Vietnam (Dung et al. 2007; Thanh and Tuan 2008), *dombea* or *medombae* in Cambodia (Chim et al. 2015; Ly et al. 2018), *bubod* in Philippines (Kozaki and Uchimura 1990), *nuruk* in Korea (Jung et al. 2012), *ragi* in Indonesia (Surono 2016), *daqu* or *chiu* or *chu* in China (Wang et al. 2008; Chen et al. 2014) and *loogpang* in Thailand (Limtong et al. 2002). The most notable innovation in traditional preparation of starter cultures is practice of "back-slopping" method (terminology in modern food microbiology) for sub-culturing the desirable and essential microbiota by ethnic Asians irrespective of their geographical locations (Tamang et al. 2020).

Filamentous moulds, amylase and alcohol-producing yeasts and bacteria co-exist as consortia in Asian dry starters (Hesseltine and Ray 1988; Steinkraus 1996; Tamang and Fleet 2009; Tamang 2016a). In Asian dry starters, species of filamentous moulds such as *Rhizopus, Mucor, Aspergillus, Penicillium, Amylomyces* (Hesseltine and Ray 1988; Hesseltine 1991; Sha et al. 2019; Anupma and Tamang 2020); yeasts species such as *Saccharomyces, Pichia, Sacharomycopsis, Candida* (Heseltine and Kurtzman 1990; Jeyaram et al. 2008, 2011; Lv et al. 2012; Sha et al. 2016, 2017, 2018, 2019) and bacteria (Hesseltine and Ray 1988; Tamang et al. 2007; Zheng et al. 2012, 2015; Li et al. 2014; Sha et al. 2017) are involved in saccharification (Lee and Lee 2002; Tamang and Thapa 2006) and liquefaction (Pervez et al. 2014), and then alcohol-production (Tsuyoshi et al. 2005; Zheng et al. 2011) in end product (Li et al. 2012; Nile 2015).

Besides saccharifying and alcohol producing ability of mycelia moulds and yeasts, some bacterial species present in starters also contribute in imparting flavour (Yang et al. 2017), antagonism, and acidification of fermenting substrates (Tamang et al. 2007; Huang et al. 2017). Extensive profiling of diversity of yeasts and mycelial moulds in various traditionally prepared dry starters collected from different places of North East India have been reported earlier (Tamang et al. 1988; Tamang and Sarkar 1995; Tsuyoshi et al. 2005; Jeyaram et al. 2008, 2011; Bora et al. 2016; Sha et al. 2017, 2018, 2019; Anupma and Tamang 2020). Samples of *marcha* collected from Darjeeling hills and Sikkim in India were analysed earlier and reported few species of bacteria *Pediococcus pentosaceus* (Tamang and Sarkar 1995), *Pediococcus pentosaceus* and *Lb. brevis* (Tamang et al. 2007), *Acetobacter*, *Fructobacillus, Lactococcus*, *Lactobacillus, Leuconostoc*, *Burkholderia* and *Gluconacetobacter* (Sha et al. 2017).

Experimental Designs to study the Objectives

Sole phenotypic method of identification of bacteria may not be authentic and appropriate, hence application of 16S rRNA gene sequencing method using PCR products is more appropriate and accurate (Clarridge III 2004; Srinivasan et al. 2015). Use of Sanger Sequencing method or Chain-termination DNA (Sanger et al. 1977) modified with automation method to check the sequence of the templates (Heather and Chain 2016) is more popular. Probiotics are one of the functional properties of fermented foods and beverages (Tamang 2015; Tamang et al. 2016b), and some lactic acid bacteria (LAB) are used as health-promoting organisms (Monteagudo-Mera et al. 2012). Some bacteria have been claimed to have probiotics characters in non-dairy fermented beverages (Que et al. 2006; Prado et al. 2008; Giri et al. 2018). Probiotic

cultures should demonstrate the following properties to be recognised as functional food components; such as acid and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes (Shi et al. 2016; Campana et al. 2017; Ghosh et al. 2019). *Lactobacillus plantarum* DM5 isolated from *marcha* of Sikkim showed a very effective antioxidant property along with the ability to produce bioactive γ-aminobutyric acid (GABA) which is a major inhibitory neurotransmitter in mammalian brain (Das and Goyal 2015). *Pediococcus pentosaceus* CIAL-86 isolated from wine have been reported to show anti-adhesion activity against *Escherichia coli* CIAL-153 (Garcia-Ruiz et al. 2014).

No published reports are available on bacterial diversity associated with *marcha* of Nepal and Bhutan, *phab* of Bhutan and *paa, pee* and *phut* of Arunachal Pradesh till date. We collected dry samples of *marcha, pee, paa, phut* and *phab* from different places of the Eastern Himalayan regions of Nepal, India and Bhutan to profile the bacterial diversity, since information on yeasts and mycelial moulds community is already available (Sha et al. 2017, 2018, 2019; Anupma and Tamang 2020). An attempt has also been made in this study to screen bacterial strains from traditionally prepared dry starters for probiotic and functional attributes.

Objectives of the Thesis are:

1. Collection of starter cultures from few places of the Eastern Himalayas (Eastern Nepal, Darjeeling, Sikkim, Arunachal Pradesh and Bhutan).

2. Isolation and enumeration of bacteria from starter cultures.

3. Identification of bacterial isolates by phenotypic and genotypic methods.

4. To determine some functional properties of bacterial isolates.

REVIEW OF LITERATURE

Consumption of fermented foods and beverages has been a long history in the human civilization (Tamang and Samuel 2010). Fermented beverages were believed to have originated around 4000 BCE (Damerow 2012) and archaeological studies have suggested that the practice of fermentation might have even occurred earlier around 6000 BCE (Renfrew 1999; Wilson 1999). Chinese fermented alcoholic beverage which is considered as one of the oldest ferment liquors produced using *daqu*, an amylolytic starter, dated back to Han dynasty (221 BCE to 207 BCE) (Qin 2000; Needham 2000).The concept of solid-state cultivation (SSC) originated in China around 3000-2000 years ago (Murakami 1980; Gomi and Abe 2007). During the *Yayoi* period (10th Century BCE to 3rd Century BCE) this technology of SSC travelled from China to Japan (Murakami 1980). The use of filamentous fungi as inocula in solid state fermentation has been recorded during the period of *Heian and Muromachi* period in Japan (13th-15thcentury) (Murai 1989). This tradition has percolated over the generations and is now available as *koji* which means the material fermented by *Aspergillus oryzae* in SSC and the organism itself (Machida et al. 2008). Though fermentation of food was initially used as a measure to preserve it; fermented foods have been found to have rich nutrient content, better nutrient bioavailability, different functionality and a unique flavor and texture as compared to the raw substrates from which it is prepared (Hesseltine and Wang 1980). The social, cultural, ethnicity, economical and religious factors along with agricultural practices greatly determines the substrates used for preparation of fermented foods and alcoholic beverages (Tamang et al. 2020). In Asia, the fermented food and beverages (*lao-chau, ang-kak, idli, phab, hamei, basi, bhaati jaanr*) are prepared mainly using rice as the primary substrate; in Africa, millets, wheat, maize, sorghum and cassava which are the major food crops of this region are used for food and beverage fermentations (*bantu beer,*

bussa, fufu, gari) (Tamang et al. 2020). In Europe fermented milk, meat and vegetables products are more popular (Tamang et al. 2020). Fermented foods and beverages are a major part of dietary culture of the ethnic people of Asia and Africa (Nout 2009). Maize (*Zea mays*), sorghum (*Sorghum vulgare*), rice (*Oryza sativa*), and millets such as pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracana*) are the cereals commonly used for the preparation of fermented foods, starters and drinks in these regions (Nout 2009). The tropical climate of Asia-Pacific region highly favours the cultivation of rice in these regions and hence due to its abundant availability, most of the alcoholic beverages are produced by fermenting rice (Haard 1999). In Europe and Mediterranean region wine, which is an alcoholic beverage made by fermenting fruits is a popular drink however, in Asia and Africa vinification is rare and alcoholic beverage prepared by fermenting cereals are popular (Berbegal et al. 2017). Fermenting and consuming alcoholic beverage are an ingrained conventional and customary practice amongst the ethnic population of Asia, Africa and Latin America (Ritchie and Roser 2018). A varied range of fermented alcoholic beverages are available in different continents of the world and based on the type and process of production Tamang (2010b) and Tamang et al. (2016a) have broadly classified these alcoholic beverages into following different categories: Non-distilled and unfiltered alcoholic beverages such as *makgeolli* of Korea (Kim et al. 2018) and *bhaati jaanr* of India and Nepal (Tamang and Thapa 2006); Non-distilled and filtered alcoholic beverages such as *sake* of Japan (Koyanagi et al. 2016); Distilled alcoholic beverages such as *soju* of Korea (Bae et al. 2007); Alcoholic beverages produced by using amylase from human saliva such as *chicha* of Peru (Bassi et al. 2020); Alcoholic beverages produced by mono- (single-strain) fermentation such as beer (Thesseling et al. 2019); Alcoholic beverages from honey such as *tej* of Ethiopia (Lemi 2020);

Alcoholic beverages produced using plant parts such as *pulque* of Mexico (Álvarez-Ríos et al. 2020); Alcoholic beverages produced by malting such as *pito* of Nigeria and Ghana (Zaukuu et al. 2016); Alcoholic beverages from fruits (prepared without distillation) such as wine (Sternes et al. 2017); Alcoholic beverages from fruits and cereals (distilled) such as whisky (Tamang 2010a).

The fermented alcoholic beverages are prepared using dry starter cultures which is an innovative back-slopping technique of preserving the native microflora in a form of round to oval shaped cake made up of cereal dough (Tamang et al. 1996; Tamang 2010a). The use of ethnic starter cultures for the fermentation of alcoholic beverages is a common practice as well as a tradition especially in Asian continent (Hesseltine and Kurtzman 1990). The knowledge of preparation of these amylolytic starters and fermented alcoholic beverage is a cultural and technological heritage which has been passed on from generation to generation using this back-slopping method (Anupma et al. 2018; Sha et al. 2017, 2018). These amylolytic starters which consist of consortia of filamentous moulds, yeasts and bacteria, are used for the preparation of fermented alcoholic drinks and distilled liquor (Tamang et al. 1996; Tamang 2010a). In Asia, the amylolytic starters used for alcohol fermentation is broadly categorized into 3 different types (Hesseltine and Ray 1988):

(a) *Koji*: Mixed cultures of *Aspergillus oryzae* and *Aspergillus sojae* used mainly for production of soya sauce and miso (Hesseltine and Ray 1988; Singh et al. 2019).

(b) Amylolytic starter cakes made up of whole wheat flour containing yeasts, *Rhizopus* spp. and *Absidia* spp., which is fermented for about a month and used for the production of alcoholic beverages (Hesseltine and Ray 1988).

(c) Dry, flattened or round balls containing a mixed culture of yeasts, bacteria and fungi, which are used to ferment cereals and cassava to produce fermented alcoholic beverage (Hesseltine and Ray 1988).

Koji is widely used in Japan, Korea and China to produce several alcoholic beverages as well as non-alcoholic beverage's and have been widely studied and industrialized (Kitamura et al. 2016; Kam and Fiona 2018). Jung et al. (2012) have further broadly classified the amylolytic fermentation starters for rice beers in Asia in two categories: (1) Single strain starters: pure culture of *Aspergillus oryzae* such as *koji* of Japan (Kitamura et al. 2016) and (2) Mixed strain starters: starters prepared from mixture of fungi, yeasts and bacteria which is naturally inoculated from the environment into raw cereals dough such as *nuruk* of Korea (Jung et al. 2012; Song et al. 2013) and *ragi* of Indonesia (Sujaya et al. 2001)

Preparation of starter cultures for alcoholic beverages varies from region to region and also among the different ethnic communities within a region (Tamang 2010a). Cereals such as rice, maize, millet, etc. are used as a substrate for preparation of amylolytic starter cultures and in some cases the final product (alcoholic beverage) itself is used as a starter inocula for the next fermentation (Aidoo and Nout 2010). Ethnic starters are known by different vernacular names such as *marcha* in India (Tamang and Sarkar 1995; Tsuyoshi et al. 2005), Nepal and Bhutan, *ragi* in Indonesia, *daqu*/*chiu*/*chu* in China and Taiwan, *loogpang* in Thailand, *nuruk* in Korea, *bubod* in Philippines, and *banh men* in Vietnam (Tamang et al. 1996; Dung et al. 2007). Some starters are also prepared using different herbs along with cereals such as *marcha* of India, Nepal and Bhutan, *phab* of Bhutan, *emao* of Assam (Anupma et al. 2018) and *medomdae* of Cambodia (Chim et al. 2015). The next section discusses some of the earlier reported

amylolytic starter cultures which have been categorized based on the substrates used for its preparation (Table I).

Rice based traditionally prepared dry starters

Medomdae/Mesra

Medomdae is a dried starter culture traditionally prepared by ethnic community-*Khmer* (Tamang 2016a) is used to produce fermented alcoholic beverages such as *Srasor, Srapeang* and *sombai* in Cambodia. Glutinous rice or rice barn is used as the main substrate which is soaked overnight and ground to powdered form. Various plants such as *Albizia myriophyla, Cananga latifolia, Diospyros nitida, Cinnamomum polyadelphum, Amomum krervanh* and *Melaleuca leucadendron* are used during the preparation process (Chim et al. 2015).

Ragi tape

Ragi tape is a traditional dry starter used for fermentation of *tape ketan* (ferment, sweet-sour and mildly alcoholic beverage)*, brem* (Balinese rice wine) and *arak* (distilled alcoholic beverage) in Indonesia (Cronk et al. 1977). *Ragi* is prepared from rice flour and spices which is mixed either with water or with sugarcane juice (Tamang 2016a). *Ragi* is prepared from rice, millet or cassava which is first powdered and mixed with herbs and spices such as pepper, cinnamon, chilly, garlic and galangal (*Alvina galangal*) (Nuraida and Krusong 2014). Water and powder of old *ragi* is then added to the mixture and small balls of fresh *ragi* are prepared. The freshly prepared *ragi* is then kept for incubation on a bamboo tray covered with banana leaves for 72 h in humid environment (Sujaya et al. 2001). After the incubation period the *ragi* balls are sun dried/air dried and used for fermentation of alcoholic beverage (Surono 2016).

Table I: Dry starters of Asia					
Substrate	**Starter Culture**	**Plants used**	**Alcoholic beverage**	**Country/Region**	**References**
Rice	*Medomdae/ mesra*	*Albizia myriophyla, Amomum krervanh,Cananga latifolia, Diospyros nitida, Cinnamomum polyadelphum,* and *Melaleuca leucadendron*	*Srasor/ srapeang/ sombai*	Cambodia	Chim et al. (2015); Tamang (2016a)
	Ragi tape	Pepper, cinnamon, chilly, garlic and galangal (*Alvina galangal* Sw.)	*Tape/ brem/ arak*	Indonesia	Cronk et al. (1977); Nuraida and Krusong (2014); Tamang (2016a)
	Banh men	none	*Ruou*	Vietnam	Dung (2004); Tamang (2010b)
	Khekhrii/ khrie	none	*Zutho*	Nagaland, India	Anupma et al. (2018)
	Marcha	*Plumbago zeylanica, Buddleja asiatica, Vernonia cinerea,* ginger and red dry chili	*Raksi/ Jaanr/ chyang*	Sikkim (India)West Bengal (India), Nepal and Bhutan	Ray et al. (2016); Anupma et al. (2018)
	Loogpang	Powder of *Kha* root, spices	*Lao/ kao-mag*	Thailand	Lotong (1998); Limtong et al. (2002); Tamang (2010b); Tamang (2012)
Wheat	*Nuruk*	none	*Makgeolli/ yakju/ takju*	Korea	Park et al. (1977); Bal et al. (2016)
	Daqu	none	*Moutai/luzhao/ Fen/xifen*	China	Wang et al. (2004); Zheng et al. (2011)
	Mana	Leaves of herb *harrah*	*Ayela*	Nepal	Tamang (2010b); Anupma et al. (2018)
	Malera /treh	none	*Bhatooru /chilra*	Himachal Pradesh, India	Bhalla (2007); Tamang et al. (2016c)

Wheat	*Balam*	*Cinnamomum zeylanicum*, *Piper longum*, *Amomum subulatum*, leaves of wild chillies and the seeds of *Ficus religiosa*	*Jaanr/ daru*	Uttaranchal, India	Roy et al. (2004); Das and Pandey (2007); Kumari et al. (2016)
	Wheat Qu	none	Chinese rice wine	China	Mo et al. (2009); Shang et al. (2012); Ji et al. (2018)
Barley	*Phab*	none	*Chaang*	Ladakh, India	Angmo and Bhalla (2014)
	Keem	Fresh twigs of *Cannabis sativa* and leaves of *Sapindus mukoross,* leaves and tender parts of *Leucas lanata*, *Zanthoxylum armatum*, *Melica azedarach* and *Dicliptera roxburghiana, Cannabis sativa* and *Pinus roxburghi*	*Soor*	Uttaranchal, India	Rana et al. (2004); Sekar and Mariappan (2007) Kumari et al. (2016)
	Dhehli	*Cannabis sativa* (*bhang*), *Trachyspermum copticum* (*ajwain*), *Saussurea* sp. (*bbacha*), *Bupleurum lanceolatum* (*nimla*), *Pistacia integerrima* (*kkakarshinga*), *Solanum xanthocarpum* (*katari*), *Micromeria biflora* (*chharbara*), *Spiranthes australis* (*bakarshingha*), *Viola cinerea* (*banaksa*), *Arisaema hellebore folium* (*chidirichun*), *Fragaria* sp. (*dudlukori*), *Aegel marmelos* (*bhel*), *Drosera lunata* (*oshtori*), *Salvia* sp. (*kotugha*) and *Clitoria ternatea* (*kkayal*)	*Sura*	Himachal Pradesh, India	Thakur and Bhalla (2004)
Sorghum	*Pito*	*Manihot utillissima* pohl	*Pito*	Africa	Orji et al. (2003)
Maize	*Phab/pho*	Flowers of *bhagam* plant	*Ara*	Bhutan	Anupma et al. (2018)
Honey	*Kuri*	*Capsicum frutessens*	*Kuri*	Africa	Roger et al. (2014)
Millet	*Manapu*	Flowers of the plant *manawasha* and black pepper	*Poko*	Nepal	Tamang (2010b)

Banh men

Banh men is an amylolytic starter used to produce the alcoholic drink *ruou* in Vietnam (Dung 2004). Uncooked rice flour is mixed with herbs, spices and water to make dough which is then made into small balls of fresh *banh men* (Thanh and Tuan 2008). The freshly prepared *banh men* is then kept on a bamboo tray and then sprinkled with powdered old *banh men.* The freshly prepared *banh men* is then incubated at room temperature for few days (Dung et al. 2006, 2007).

Khekhrii

Khekhrii also known as *khrie* is an amylolytic starter prepared by the ethnic tribal people of Nagaland, India (Anupma et al. 2018). This starter is used to produce the fermented alcoholic beverage *zutho* by the *Angami* tribe (Anupma et al. 2018). *Khrie* is different compared to other amylolytic starters in two ways: first it is prepared using sprouted unhulled rice and second it is not prepared using the old starter or by back-slopping technique as most of the starters is made (Anupma et al. 2018). During the preparation of *Khrie* the unhulled rice is first soaked in water and allowed to germinate for 2-5 days (Tamang 2010b). Once the rice sprouts it is sun dried, powdered and used as a starter for the preparation of *zutho* (Tamang et al. 2012).

Marcha

Marcha is the amylolytic starter found in India, Nepal and south and eastern part of Bhutan. It is used to prepare the ferment alcoholic drinks such as *raksi* and *jaanr* (Sarkar and Tamang 1995). *Marcha* is prepared by soaking glutinous rice which is then powdered and mixed with different plant parts and herbs such as roots of *guliyo jara* or *chitu* (*Plumbago zeylanica*), leaves of *bheemsen paate* (*Buddleja asiatica*),

flower of sengreknna (*Vernonia cinerea*), ginger and red dry chili (Tamang et al. 1996; Shrestha et al. 2002). This mixture is then kneaded into fresh *marcha* balls and cakes by adding water. These freshly prepared *marcha* is then dusted with old powdered *marcha* and kept on the leaves of fern *Glaphylopteriolopsis erubescens* and incubated at room temperature for 24 h (Tamang 2010b). After the incubation period the *marcha* is sun dried for about 5 days and used (Tsuyoshi et al. 2005).

Loogpang

Loogpang is an amylolytic starter culture for fermented foods, alcoholic beverages and vinegar in Thailand (Kishida et al. 2009). The word "*loogpang"* literally means "Chinese yeast cake" in Thai language (Limtong et al. 2002). The initial substrate used for preparing *loogpang* is rice which is mixed with different spices (Kishida et al. 2009). The microbial inoculum in *loogpang* is either from previously made starter or from the natural environment. The preparation knowledge of *loogpang* is limited probably and it is prepared only in few households in Thailand (Limtong et al. 2002). The final products produced using *loogpang* are *lao* (rice wine), *kao-mag* (alcoholic sweetened rice) and *num somsai chu* (vinegar) (Lotong 1998).

Wheat based Amylolytic starters

Nuruk

Nuruk is an amylolytic starter used for making traditional fermented alcoholic beverages like *makgeolli, yakju* and *takju* in Korea (Bal et al. 2016). *Nuruk* is prepared using wheat as the substrate which is fermented by naturally incorporated microbes (molds, bacteria and yeasts) (Park et al. 1977). During the preparation of *nuruk* the wheat is first moistened and powdered after which it is kneaded into small balls. The

incubation period for *nuruk* fermentation is 17 days after which it is dried for 2 weeks and cured at room temperature for 1-2 months (Park et al. 1977). Originally rice was used as an initial substrate for preparation of *nuruk,* however it has now been replaced by wheat (Lee and Kim 2016).

Daqu

Daqu is a natural fermentation starter for distilled liquor and traditional vinegar and is prepared from wheat, barley and/or pea in China (Wang et al. 2004). In China, famous liquors such as *Moutai liquor, Luzhou liquor, Fen liquor* and *Xifeng liquor* are prepared using *daqu* as fermentation starter (Wang et al. 2004). *Daqu* is quite different from other Asian fermentation starters and is composed of wheat, barley and peas, mixture of fungal and bacterial microflora and microbial enzymes and metabolites (Du et al. 2019). Fermented alcoholic beverage is prepared by adding powdered *daqu* to cooked sorghum which is then fermented in a sealed jar for about a month after which distillation is done to prepare the final product (Tang et al. 2019). *Daqu* not only serves as an inoculum but it also functions as initial fermentation substrate for making the final product. During the preparation of *Fen liquor, daqu* form 10-15% of the total substrate and in *Luzhou liquor* it forms about 20-25% of the total initial substrate (Zheng et al. 2011).

Mana

Mana is a traditionally prepared starter made from wheat flakes of Nepal (Nikkuni et al. 1996). *Mana* is prepared by the Newar/Pradhan community of Nepal and is black in color (Tamang 2010a). Fermented alcoholic product *ayela* is prepared from *mana* (Anupma et al. 2018). Wheat is soaked in water overnight, steamed and then spread on

straw mat for about a week (Tamang 2010a). Dried leaves of herb *harrah* and powdered old *mana* are also added to the wheat before incubation. Green moulds grow on the steamed rice during this period (Nikkuni et al. 1996). This molded rice is then sun dried and used as a starter (Nikkuni et al. 1996).

Balam

Balam is a wheat-based starter prepared by the *Bhotiya* community of Uttaranchal Himalayas (Kumari et al. 2016). *Balam* is used to *prepare the fermented alcoholic beverage jaanr* and *daru* (Das and Pandey 2007). During the preparation of *balam,* wheat is first washed, sun dried and grounded into a fine powder form (Das and Pandey 2007; Roy et al. 2004). The powdered wheat is then roasted and mixed with powdered *Cinnamomum zeylanicum*, *Piper longum*, *Amomum subulatum*, leaves of wild chillies and the seeds of *Ficus religiosa* (Das and Pandey 2007; Roy et al. 2004). Powdered old *balam* is then added to the above mixture as an inoculums (Das and Pandey 2007; Roy et al. 2004). The mixture is then kneaded into fine small balls by adding required amount of water. These fresh *balam* balls are then kept for drying in a shade. After drying the fresh *balam* balls can be used for preparing the alcoholic beverage or stored for future (Das and Pandey 2007*;* Roy et al. 2004).

Wheat Qu

Wheat Qu is a traditionally prepared dry starter culture used for the preparation of famous Chinese rice wine in China (Ji et al. 2018). *Wheat Qu* is of two types; raw *Wheat Qu* and cooked *Wheat Qu* (Ji et al. 2018). The raw *Wheat Qu* is prepared by first washing the wheat and the moist wheat is then grounded to form fine powder which is then mixed with water to form a fine paste (Shang et al. 2012; Mo et al.

2009). The wheat paste is then moulded into brick shaped blocks which are kept in non sterile environment for a period of 15 days-2 months which favours the natural inoculation of moulds, yeast and bacteria from the environment (Shang et al. 2012; Mo et al. 2009). In case of cooked *wheat Qu* the wheat is first steamed and then grounded to make the brick shaped blocks for incubation. The cooked *wheat Qu* is categorised into two types based on the source of inoculums: naturally cooked *wheat Qu* (prepared in natural environment like raw *wheat Qu*) and pure *wheat Qu* (prepared by inoculating pure species of fungi such as *Aspergillus flavus*) (Ji et al. 2018).

Malera/treh

Malera/treh is traditionally prepared dry starter culture in Himachal Pradesh which is used to prepare fermented foods such as *bhatooru/chilra* (Tamang et al. 2016c). It is prepared from wheat/buckwheat flour dough or slurry which is fermented with the addition of *malera* which mainly consists of lactic acid bacteria and yeasts (Bhalla 2007).

Barley based dry starters

Phab

Phab is a traditional dry starter culture prepared by the *Bhotoy* community in Ladakh, India (Angmo and Bhalla 2014). Fermented alcoholic beverage *chhang* is prepared using *phab. Phab* is prepared from husked barley which is first roasted till it turns brown in color (Angmo and Bhalla 2014). The roasted barley is then grounded into coarse flour with the help of water driven wooden wheel locally known as *rantak* (Angmo and Bhalla 2014). The flour is then mixed with lukewarm water and kneaded into small balls. Old *phab* (one ball per kg of flour) is then grounded and sprinkled

over the freshly prepared *phab* as inoculums (Angmo and Bhalla 2014). Wheat flour is then sprinkled over this inoculated freshly prepared *phab* balls. The *phab* balls are the placed over and covered with the shrub *Artemisia* sp. (Angmo and Bhalla 2014). The shrub is further covered with sack or a warm cloth and incubated in a dark room for 2-3 days after which the balls are allowed to dry for 5-7 days in shade (Angmo and Bhalla 2014). The shelf life of *phab* is more than a year and usually old *phab* balls are preferred over freshly prepared for the production of *chhang* (Angmo and Bhalla 2014).

Keem

Keem, a dry starter culture prepared by the ethnic community residing in the Tons valley of Uttaranchal (Kumari et al. 2016). This starter is used for the preparation of fermented alcoholic beverage *soor* (Rana et al. 2004; Sekar and Mariappan 2007). A large number of herbal plants are used in the preparation of *keem* (Rana et al. 2004; Sekar and Mariappan 2007). 40 different herbal plants along with fresh twigs of *Cannabis sativa* and leaves of *Sapindus mukoross* are dried and powdered (Rana et al. 2004; Sekar and Mariappan 2007). The powdered plants are then mixed with barley flour and *jayaras* (an extract prepared from leaves and tender parts of *Leucas lanata*, *Zanthoxylum armatum*, *Melica azedarach* and *Dicliptera roxburghiana*) (Rana et al. 2004; Sekar and Mariappan 2007). The mixture is left overnight in a container and small round cakes are made from it. The fresh *keem* cakes are then alternately layered on shoots of *Cannabis sativa* and *Pinus roxburghi* (Rana et al. 2004; Sekar and Mariappan 2007). The cakes are then incubated in a close room for 24 days. The cakes are further incubated for 12 days after turning it upside down. After the incubation the

keem cakes are sun dried or air dried and stored for further use (Rana et al. 2004; Sekar and Mariappan 2007).

Dhehli

Dhehli is a dry starter used to prepare the fermented alcoholic beverage *sura* in Himachal Pradesh. Preparation of *dhehli* has a ritualistic importance and is prepared only during the month of September (Bhadrapada month) (Thakur and Bhalla 2004). Thirty six different kinds of herbs are used for the preparation of *dhehli*; *Cannabis sativa* (*bhang*), *Trachyspermum copticum* (*ajwain*), *Saussurea* sp. (*bbacha*), *Bupleurum lanceolatum* (*nimla*), *Pistacia integerrima* (*kkakarshinga*), *Solanum xanthocarpum* (*katari*), *Micromeria biflora* (*chharbara*), *Spiranthes australis* (*bakarshingha*), *Viola cinerea* (*banaksa*), *Arisaema hellebore folium* (*chidirichun*), *Fragaria* sp. (*dudlukori*), *Aegel marmelos* (*bhel*), *Drosera lunata* (*oshtori*), *Salvia* sp. (*kotugha*) and *Clitoria ternatea* (*kkayal*) are some of the important herbs used (Thakur and Bhalla 2004). These herbs are crushed and added to the fine flour of roasted barley. The mixture is then kneaded and a wooden vessel is used to make brick shaped starters which are then dried and used (Thakur and Bhalla 2004; Savitri and Bhalla 2007).

Sorghum based amylolytic starter

Pito

Some fermented alcoholic beverage such as *pito* is prepared using previously brewed *pito.* In this case the final product itself is used as the starter culture unlike in the case of other alcoholic fermented beverages discussed earlier (Orji et al. 2003). *Pito* is a fermented alcoholic beverage consumed in Africa, mainly in the west coast regions

such as in Nigeria (Zaukuu et al. 2016). Sorghum and maize is used as the substrate for the production of *pito* (Orji et al. 2003). The traditionally method of preparation of *pito* involves the germination of sorghum grains which is sun dried and then powdered. *Garri,* which is a powdered tuber of cassava (*Manihot utillissima* Pohl) is added as an adjunct to the ground sorghum malt (Orji et al. 2003). Water is added to this mixture and allowed to ferment for 2 days after which it is boiled and allowed to cool. The product is then filtered and small quantity of previous brew is added to it and allowed to ferment for 2 days more (Orji et al. 2003; Zaukuu et al. 2016).

Honey based starter culture

Kuri

Kuri is a liquid starter culture like *pito* of Cameroon, where the extract from the final product (alcoholic beverage) acts as a starter for the next fermentation. *Kuri* is a unique alcoholic beverage prepared by fermenting honey in Cameroon, Africa (Roger et al. 2014). A *kuri* must is first prepared during the preparation of *kuri* (Roger et al. 2014). It is a mixture of unfermented honey; warm water and red pepper (*Capsicum frutessens*) extract (Roger et al. 2014). The mixture is filtered through a traditional filter made up of wood and leaves (Roger et al. 2014). The unfermented clear dark coloured filtrate is also consumed after jarring and cooling and is known as *MbiiNdong* (Roger et al. 2014). *MbiiNdong* is then heated to a temperature of 35-40°C and *kuri* starter is added in step wise manner to ferment the *MbiiNdong*. In the first step 1-2 grams of *kuri* starter is added to 100 ml of *MbiiNdong* and incubated for 12-18 h (Roger et al. 2014). The resultant mixture is again added to 1 litre of fresh *MbiiNdong* and incubated for another 12 h after which it is again added to 10 litres of fresh *MbiiNdong* and further incubated for 12 h (Roger et al. 2014). The final mixture is

then left to ferment in a dark and warm room for 48-72 h. The final fermented liquid (fresh *kuri*) is then filtered and matured for 1-2 weeks after which it is used (Roger et al. 2014). The viscous liquid settled at the bottom of vessel during the fermentation is collected and the upper aqueous layer is decanted to obtain a thick paste (Roger et al. 2014). The paste is then pressed in a muslin cloth to remove remaining liquid and a muddy coloured powder is obtained which is sun dried to form a dark coloured *kuri* starter powder (Roger et al. 2014).

Maize based amylolytic starter

Phab/Pho

Phab is a dark brown colored starter prepared by the ethnic *Drukpa* community of Bhutan (Anupma et al. 2018) and also of Ladakh in India (Angmo et al. 2016). *Phab* is used to prepare the fermented alcoholic beverage *ara* (Anupma et al. 2018). During the preparation of *phab,* maize is powdered and mixed with rice husk and flowers of *bhagam* plant at a ratio of 2:4:3 respectively (Angmo et al. 2016; Anupma et al. 2018). Water is added to the mixture to form a thick paste which is cooked for few minutes. Small balls of *phab* are prepared on which powdered old *phab* is sprinkled. The freshly prepared *phab* is then placed in a bamboo basket and fermented for 3 days after which it is sun dried for 5 days and used (Angmo et al. 2016; Anupma et al. 2018).

Millet based traditional dry starters

Manapu

Manapu is traditionally prepared dry starters found in Nepal and are used to prepare the alcoholic drink *poko* by the ethnic Nepali community (Shrestha et al. 2002). *Manapu* is prepared using millets as the substrate however it can also be prepared

using rice flour (Tamang 2010b). During the preparation of *manapu* the millets are first milled to get fine flour. Flowers of the plant *manawasha*, black pepper and about 20% of old powdered *manapu* is then added to the millet flour and the mixture is kneaded into a fine dough using water (Tamang 2010b). Small cakes are then made from this dough which is kept on a straw mat and covered with straw and fermented for 5-7 days in 30°C-33°C temperature (Tamang 201b). After the fermentation period the freshly prepared *manapu* cakes are sun dried and ready to be used.

Microbial diversity of traditionally prepared dry starters

The microbial profile of these traditionally prepared dry starters is highly diverse. The microflora of the starter cultures is represented by different genera and species of bacteria (Heseltine and Ray 1988; Tamang et al. 2007; Sha et al. 2017), yeasts (Heseltine and Kurtzman 1990; Jeyaram et al. 2008, 2011; Sha et al. 2016, 2017, 2018, 2019) and filamentous moulds (Tamang et al. 1988; Heseltine et al. 1988; Sha et al. 2019), for saccharification (Lee and Lee 2002; Thapa and Tamang 2004), liquefaction (Pervez et al. 2014) and ethanol production (Tsuyoshi et al. 2005; Zheng et al. 2011) and plays an important role in the fermentation of the final product (alcoholic beverages). Studies on microbial community of *medomdea* revealed the dominance of lactic acid bacteria such as *Weisella, Lactobacillus, Pediococcus, Streptococcus* and *Lactococcus* (Ly et al. 2018); moulds such as *Mucor* sp. and *Rhizopus oryzae* and yeasts *Candida tropicalis* and *Saccharomyces cerevisiae* (Chay et al. 2017; Chim et al. 2015). Shrestha and Rati (2002) have reported the presence of mould species: *Saccharomyces cerevisiae, Candida versatilis* and *Rhizopus* spp. and lactic acid bacteria: *Pediococcus pentosaceus* in *manapu.* Mold was reported to be the dominant microflora of *mana* by Shrestha et al. (2002). Moulds such as *Aspergillus oryzae* and

Rhizopus spp. was reported to be present in *mana* by Nikkuni et al. (1996). Coliforms such as *E.coli* and *Staphylococcus* spp have also been reported in *mana* (Shrestha et al. 2002). Studies based on 16S rRNA, 26S rDNA and ITS region of *daqu* for *Fen liquor* have reported *Bacillus licheniformis* and *Bacillus subtilis* as the dominant bacterial species along with *Brevibacterium* sp., lactic acid bacterial species; *Enterococcus faecalis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus;* fungal strains of *Absidia corymbifera, Aspergillus flavus, Mucor circinelloides, Penicillium commune, Rhizomucor pusillus, Rhizomucor variabilis* var. *regularior, Rhizopus stolonifera* and yeast strains of *Pichia kudriavzevii, Saccharomyces cerevisiae, Saccharomycopsis fibuligera,* and *Wickerhamomyces anomalus* (Zheng et al. 2012). *Bacillus* spp., *Lactobacillus* spp. and *Weissella* spp. were reported to be the dominant bacterial species and *W. anomalus*, *S. fibuligera*, *P. kudriavzevii* and *Debaryomyces hansenii* were the four species of fungi reported by PCR-DGGE analysis of *fen-daqu* (Zheng et al. 2012). A study based on 16SrRNA sequencing revealed bacterial species *Bacillus amyloliquefaciens* and *Bacillus subtilis* along with various species of lactic acid bacteria such as *Enterococcus faecium, Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weissella paramesenteroides*, and *W. cibaria*; fungi species including *Aspergillus flavus, A. fumigatus, A. niger, A. versicolor, Eurotium amstelodami, E. rubrum, Lichtheimia corymbifera, L. ramose, Mucor circinelloides, M. indicus, Penicillium chermesinum, P. chrysogenum, P. sumatrense, Rhizomucor pusillus, R. tauricus, R. variabilis,* and *Rhizopus oryzae* and yeast including *Pichia jadinii* and *Saccharomyces cerevisiae* in *nuruk* samples (Song et al. 2013). A pyrosequencing result on fungal diversity of *nuruk* showed the dominance of phylum Ascomycota and Zygomycota and dominance of *Aspergillus, Mucorales,* and *Rhizomucor* at the genus level in *nuruk* (Bal et al.

2016). Microorganisms reported from *loogpang* are moulds: *Amylomyces, Rhizopus, Aspergillus, Mucor*, and *Absidia* (Pichyangkura and Kulprecha 1977) and yeasts: *Saccharomycopsis fibuligera, Hansenula, Saccharomyces* and LAB: *Pediococcus* (Dhamcharee 1982; Uchimura et al. 1991). Tsuyoshi et al. (2005) have reported *Saccharomyces bayanus*, *Candida glabrata*, *Pichia anomala* and *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis*, and *Pichia burtonii* in *marcha* of Sikkim. Lactic acid bacteria *Lactobacillus plantarum* and *Pediococcus pentosaceus* were reported in *marcha* (Tamang et al. 2007). Sha et al. (2016) by PCR-DGGE analysis have reported the dominance of *Wickerhamomyces anomalus* and *Pichia anomalus* in *marcha*. Many studies have been conducted to study the microbial community structure in *ragi, Enterococcus faecalis, Lactobacillus plantarum, Pediococcus pentosaceus* were reported (Ishimaru and Nakano 1960). Ardhana and Fleet (1989) have reported *Bacillus coagulans, B. brevis, B. stearothermophilus and Acetobacter* sp. in *ragi.* Yeast species: *Pichia anomala, Saccharomyces cerevisiae, Candida parapsilosis, C. melinii, C. lactose, Hansenula subpelliculosa, H.anomala, H. malanga* were reported by Dwidjoseputro and Wolf (1970) in *ragi*. Culture dependent study on *banh men* have revealed the occurrence of molds such as *Amylomyces rouxii* and *Rhizopus oligosporous* and *Rhizopus oryzae* and yeast such as *Saccharomyces cerevisiae, Saccharomycopsis fibuliger, Pichia anomala, Candida glabrata* and *Hyphopichia burtonii* (Dung et al. 2006; Dung et al. 2007). Microbial diversity study of *banh men* based on PCR-mediated DDGE analysis revealed the occurrence of fungal species: *Rhizopus oryzae*, *R. microsporus*, *Absidia corymbifera* and *Amylomyces* sp.; yeast species: *Saccharomyces cerevisiae*, *Issatchenkia* sp., *Pediococcus anomala*, *Candida tropicalis*, *Pichia ranongensis* and *Clavispora lusitaniae* and bacterial species: *Bacillus subtilis*, *B. circulans*, *B. amyloliquefaciens*,

B. sporothermodurans, Acetobacter orientalis , *A. pasteurianus* (acetic acid bacteria), *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *L. brevis*, *Weisella confusa*, *W. paramesenteroides* (lactic acid bacteria), *Burkholderia ubonensis* and *Ralstonia solanacearum* and *Pelomonas puraquae* (environmental contaminants) (Thanh and Tuan 2008). *Aspergillus* and *Candida* species and species of bacteria *Weissella* and *Staphylococcus* were reported to be the dominant fungal and bacterial species respectively in Chinese *koji* (Yan et al. 2013).

Culture dependent and culture independent techniques based on molecular fingerprinting is a reliable, appropriate and authentic tool for microbial identification present in the traditionally prepared dry starters (Dolci et al. 2015). Culture-dependent methods include conventional phenotypic identification of microbes which may not be authentic and appropriate and genotypic identification based on 16S rRNA gene sequencing using PCR products which is more reliable and accurate (Clarridge III 2004; Srinivasan et al. 2015). The culture dependent techniques have limitations as it cannot detect the non culturable microbes in the samples and thus is not efficient to study the entire microbial community as compared to culture-independent techniques in which the entire microbial community profile can be obtained from small amount (<1 g) samples (Puerari et al. 2015). Culture-independent techniques such as PCR-denaturing gradient gel electrophoresis (DGGE) analysis, pyrosequencing and next generation sequencing (high-throughput metagenomic amplicon sequencing) serve as an accurate and highly sensitive tools to study the microbial ecology of fermented foods and dry starter cultures (Ercolini 2004; Tamang et al. 2016a; Alegría et al. 2012; Chen et al. 2014; Puerari et al. 2015; Shangpliang et al. 2018; Tamang et al. 2020).

Probiotics

Probiotics, as per the definition of FAO/WHO, are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hill et al. 2014). Probiotics are considered as one of the functional properties of fermented foods (Tamang 2015), and some lactic acid bacteria (LAB) are used as health-promoting bacteria to consumers (Hwanhlem et al. 2010; Monteagudo-Mera et al. 2012). To claim as probiotic candidates, organisms should demonstrate the following properties to be considered as functional food components such as low acid- and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes (Ouwehand et al. 1999; Shi et al. 2016; Campana et al. 2017; Ghosh et al. 2019). Bacterial strains belonging to *Lactobacillus* and *Bifidobacterium* are mostly claimed as probiotics (Prasad et al. 1998; Vlasova et al. 2016). However, some species belonging to the genera *Lactococcus, Enterococcus, Pediococcus, Leuconostoc*, *Bacillus* and yeast *Saccharomyces* are also considered as probiotic microorganisms (Sanders and in't Veld 1999; Fijan 2014; Elshaghabee et al. 2017; Sofyan et al. 2019; Goel et al. 2020). Non-LAB *Propionibacterium freudenreichii* has been claimed as probiotic with functionality (Holzapfel etal. 2001, 1998; Huang et al. 2019). Probiotics cultures are mostly *Lactobacillus acidophilus, Lb. amylovorus, Lb. casei, Lb. crispatus*, *Lb. delbrueckii* subsp. *bulgaricus, Lb. gallinarum, Lb. gasseri, Lb. johnsonii, Lb. paracasei, Lb. plantarum, Lb. reuteri, Lb. rhamnosus*, *Enterococcus faecalis, E. faecium, Lactococcus. lactis, Leuconostoc mesenteroides, Pediococcus acidilactici, Sporolactobacillus inulinus, Streptococcus*

thermophilus and non-LAB- *Bacillus cereus* var. *toyoi, Escherichia coli* strain *nissle*, *Propionibacterium freudenreichii*; and yeasts- *Saccharomyces cerevisiae, Sacch. boulardii* (Holzapfel et al. 2001; Shah 2007; Vlasova et al. 2016; Elshaghabee et al. 2017; Garcia-Gonzalez et al. 2018; Huang et al. 2019; Sofyan et al. 2019; Goel et al. 2020). Fermented foods are especially considered as ideal vehicle for delivering probiotic bacteria to the human gastrointestinal tract (Shah 2007; Rezac et al. 2018). Incorporation of probiotic cultures in fermented foods are mostly practised (a) by addition of probiotics together with the starter cultures; (b) production of two batches separately, one containing the probiotic microorganism in milk with high concentration of viable cells and another with starter cultures, when the fermentation stages are completed the batches are then mixed; and (c) the use of a probiotic microorganism as a starter culture (Soccol et. al. 2010; Moslemy et al. 2015; Fenster et al. 2019). Probiotic attributes have been reported in few alcoholic beverages (Kumari et al. 2016; Giri et al. 2018; Paula et al. 2019; Somashekaraiah et al. 2019).

The bacterial species present in dry starters mostly impart flavour, cause acidification of fermenting substrates, to make a mild acidic and sour taste in the final alcoholic products which are preferred by consumers (Tamang and Thapa 2006; Tamang et al. 2007, 2016b; Huang et al. 2017). Earlier reports have shown that species of *Weissella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Enterococcus* are known for flavour development, production of organic acids and antimicrobial activities in *daqu* (Gou et al. 2015). Lactic acid bacteria such as *Enterococcus* sp. has been reported to produce enterocins which play a major role in preventing the growth of food borne and spoilage causing pathogens (Javed et al. 2011). Some of the bacterial species such as *Bacillus* sp. reported in *daqu* of China (Wang et al. 2008; Zheng et al. 2012) and *banh men* of Vietnam (Thanh and Tuan 2008) are important sources of

amylase and protease enzymes and are involved in saccharification and flavour production (Beaumont 2002). *Staphylococcus* spp. has also been reported in Chinese *daqu* (Yang et al. 2017), which secrete amylase (Li et al. 2014), protease and also produce lipases for production of esters for flavour (Talon et al. 1996). Lactic acid bacteria produce flavour compounds, desirable acids and peptides with antimicrobial activity (Faria-Oliveira et al. 2015) and have probiotic and nutraceutical properties (Grosu-Tudor and Zamfir 2012). Lactic acid bacteria such as *Enterococcus* sp. has been reported to produce enterocins which play a major role in preventing the growth of food borne and spoilage causing pathogens (Javed et al. 2011). Many species of LAB isolated from fermented drinks have been reported to have an efficient cholesterol lowering ability (Taranto et al. 2004; Khare and Gaur 2020). *Lactobacillus pentosus* strain IR32 isolated from palm wine (*Raffia mambillensis*) has been reported to exhibit a good *in-vitro* cholesterol removing ability of 76.43 % (Ngongang et al. 2016). *Lactobacillus plantarum* strain HLX37 also showed a significant decrease in total serum and liver cholesterol content and the atherogenic index of hyperlipidemia rats (Guan et al. 2017). *Hong Qu,* the dry starter for the famous Chinese yellow rice wine have been reported to possess antioxidant properties and cause significant reduction in total cholesterol, LDL cholesterol and total triacylglycerol concentrations (Heber et al. 1999; Taira et al. 2002; Erdogrul and Azirak 2004; Que et al. 2006; Yang et al. 2006). Lactic acid bacteria have also been reported to possess natural antioxidative properties such as the radical scavenging activity of *Lactobacillus plantarum* (Li et al. 2012). Many studies have reported the ability of lactic acid bacteria and bifidobacteria to synthesize B vitamins (LeBlanc et al. 2017; Albuquerque et al. 2020) such as folate (B9) (Albuquerque et al. 2016), riboflavin (B2) (Yepez et al. 2019) and cobalamin (B12) (Piwowarek et al. 2018).

Probiotic marker gene detection approach has enabled the rapid screening of strains with potent probiotic characteristics and thus possibility of finding a novel probiotic strain is possible (Devi et al. 2015; Abriouel et al. 2017). This method of rapid probiotic gene detection is faster, sensitive, cost effective and reliable (Kapse et al. 2019). Goel et al. (2020) have reported that the detection of the targeted probiotic gene in the tested lactic acid bacterial strains correlated with the in vitro probiotic attributes that the strains exhibited. Thus, probiotic gene detection technique can be used as an efficient tool for the identification of potent and a novel probiotic strain (Turpin et al. 2011).

MATERIALS

AND

METHODS

MATERIALS AND METHODS

MEDIA USED

1. MRS Agar (M641, HiMedia, Mumbai)
2. MRS Broth (M369, HiMedia, Mumbai)
3. Nutrient agar (MM012, HiMedia, Mumbai)
4. Nutrient Broth (MM002, HiMedia, Mumbai)
5. M 17 agar (M929, HiMedia, Mumbai)
6. Violet Red Bile Green Agar (M1058, HiMedia, Mumbai)
7. Starch Agar (M107, HiMedia, Mumbai)
8. Carbohydrate fermentation media (Schillinger and Lucke 1987)

Peptone	10 g
Yeast extract	5g
Potassium phosphate	2g
Tri-sodium phosphate	2g
Carbohydrate	0.5%
Tween 80	1mL
Sodium acetate	5g
Magnesium sulphate	0.58g
Manganese sulphate	0.28g
Phenol red	0.004%
Distilled water	1000ml

9. Esculine hydrolysis media (Cappuccino and Sherman 2008)

Protease peptone	5g
Beef extract	3g
Ox gall	40g

Esculine	1g
Ferric citrate	0.5g
Agar	15g
Distilled water	1000 ml
10. Simmon citrate agar	(Cappuccino and Sherman 2008)
Ammonium dihydrogen phosphate	1g
Dipotassium phosphate	1g
Sodium chloride	5g
Sodium citrate	2g
Magnesium sulfate	0.2g
Agar	15g
Bromocresol blue	0.08g
Distilled water	1000 ml
11. SIM Agar	(M411, HiMedia, Mumbai)
12. MR-VP Broth	(LQ082, HiMedia, Mumbai)
13. Urease broth	(M1828, HiMedia, Mumbai)
14. Blood agar	(Cappuccino and Sherman 2008)
Beef heart infusion	0.3g
Tryptose	0.5g
Sodium chloride	0.5g
Agar	1.5g
Defibrinated Sheep blood	5%
Distilled water	1000 ml
pH (7.3±0.2)	
15. Nitrate Broth	(RM 107,HiMedia, Mumbai)

CHEMICALS USED

1. Dipotassium hydrogen phosphate (GRM1045, HiMedia, Mumbai)
2. Glycerol (RM101,HiMedia, Mumbai)
3. Ethidium bromide (RM813,HiMedia, Mumbai)
4. Sorbitol (MB066, HiMedia, Mumbai)
5. Rhamnose (RM062,HiMedia, Mumbai)
6. Raffinose (RM107,HiMedia, Mumbai)
7. Sucrose (RM201,HiMedia, Mumbai)
8. Mellibiose (RM106,HiMedia, Mumbai)
9. Arabinose (RM045,HiMedia, Mumbai)
10. Xylose (RM111,HiMedia, Mumbai)
11. Cellobiose (RM098,HiMedia, Mumbai)
12. Lactose (RM565,HiMedia, Mumbai)
13. Ribose (GRM197, HiMedia, Mumbai)
14. Mannose (DD007, HiMedia, Mumbai)
15. Mellizitose (RM1285, HiMedia, Mumbai)
16. Salicin (DD011, HiMedia, Mumbai)
17. Fructose (DD017, HiMedia, Mumbai)
18. Maltose (RM018,HiMedia, Mumbai)
19. Mannitol (PT0604,HiMedia, Mumbai)
20. Dulcitol (RM100, HiMedia, Mumbai)
21. Inositol (RM102,HiMedia, Mumbai)
22. 1X TE buffer (ML016, HiMedia, Mumbai)
23. Lysozyme (MB098, HiMedia, Mumbai)
24. RNase (A7973, Promega, US)

25. Sodium Dodecyl Sulfate (SDS) (MB010, Himedia, Mumbai)

26. Proteinase K (V3021,Promega, US)

27. Phenol (MB082, HiMedia, Mumbai)

28. Chloroform (AS039,HiMedia, Mumbai)

29. Isoamyl alcohol (MB091,HiMedia, Mumbai)

30. Sodium acetate (MB048, HiMedia, Mumbai)

31. Absolute ethanol (MB106,HiMedia, Mumbai)

32. 50X TAE buffer (ML016, HiMedia, Mumbai)

33. Go green master mix (M7122, Promega, US)

34. DNA extraction kit (M050, ProMega, USA)

35. Agarose (V3125, Promega, US)

36. Primer (27F and 1492 R) (ILS,Delhi)

37. Gel loading dye (G1881, Promega, US)

38. 100bp DNA ladder (MBT049, HiMedia, Mumbai)

39. 1kb DNA ladder (MBT051, HiMedia, Mumbai)

40. PEG (RM400, HiMedia, Mumbai)

41. Sodium chloride (GRM031, HiMedia, Mumbai)

42. Nuclease free water (P1193, Promega, USA)

43. Ethidium bromide (RM813, Himedia, Mumbai)

44. MOPS (ML031, Himedia, Mumbai)

45. Sodium Phytate (GRM6226, Himedia, Mumbai)

46. Para-orthopthalaldehyde (RM1143, Himedia, Mumbai)

47. Cholesterol (TC101, Himedia, Mumbai)

48. Hexane (AS097, Himedia, Mumbai)

49. N-hexadecane (RM 2238 HiMedia, Mumbai)

50. X-Gal (MB069, Himedia, Mumbai)

51. IPTG (G147, Himedia, Mumbai)

52. Sodium deoxytaurocholate (RM9822, Himedia, Mumbai)

53. Sodium Cholate (RM202, Himedia, Mumbai)

54. Calcium chloride (GRM710, Himedia, Mumbai)

55. Calcium carbonate (GRM397, Himedia, Mumbai)

56. Urea (208884,Sigma-Aldrich, US)

57. Ringer solution (M525, HiMedia, Mumbai)

58. Sodium Hydroxide Solution (MF8D, Merck Millipore, US)

REAGENTS USED

1. Solution A (nitrate reduction test) (Cappuccino and Sherman 2008)
   Sulphanilic acid 0.8g
   5N Acetic acid 100ml
2. Solution B (nitrate reduction test) (Cappuccino and Sherman 2008)
   α-Napthylamine 100ml
   5N Acetic acid 100ml
3. Safranin (RM1315, HiMedia, Mumbai)
4. Malachite green (S020, HiMedia, Mumbai)
5. Iodine solution (M425, HiMedia, Mumbai)
6. Gram's crystal violet (S012, HiMedia, Mumbai)
7. Gram's iodine (S019, HiMedia, Mumbai)
8. Solution A (Voges-Proskauer reagent) (Cappuccino and Sherman 2008)
   Alpha-naphthol 5g
   Absolute ethanol 100mL

Solution B (Voges-Proskauer reagent) (Cappuccino and Sherman 2008)

Potassium hydroxide 4g

Distilled water 100mL

9. Methyl red solution (Cappuccino and Sherman 2008)

   Methyl red (I007, HiMedia, Mumbai) 0.1g

   Ethyl alcohol (95%) 300mL

10. Kovacs reagent (R008, HiMedia, Mumbai)

11. Hydrogen peroxide (88597, Merck, New Jersey)

SOFTWARE USED	
Sequence Scanner	(Applied Biosystems-V1.0, USA)
ChromasPro	(Technelysium-V1.34, Australia)
MEGA 7	(Pennsylvania State University Vl.7.0.26, USA)
PAST	(Palaeontological Association-V4.0, Norway)
QIIME 1.8v	(University of Colorado- V2-2019.10, USA)
GRAPHPAD PRISM	(GraphPad software, Inc-V8.0.2, USA)
Sequence Scanner	(Applied Biosystems-V1.0, USA)
SEQMANN software	(DNASTAR, 4462914, USA)

REFERENCE STRAINS	**ACCESSION NO.**
Lactobacillus plantarum	MTCC 1407(T)
Lactococcus lactis subsp. *lactis*	MTCC 440
Leuconostoc mesenteroides subsp. *mesenteroides*	MTCC 867
Lactobacillus plantarum	MCC 2034
Lactobacillus plantarum subsp. *plantarum*	MTCC 2974
Lactobacillus fermentum	MTCC 2760
Lactobacillus brevis	MTCC 2198(T)
Enterococcus faecium	MTCC 2763

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

METHODOLOGY

Survey

The traditional preparation process of dry starter cultures found in various regions of the Eastern Himalayas was documented using structured questionnaire (Table A) and interviews with the local people of different ethnic communities who practiced the preparation of these starter cultures. We surveyed and collected samples from Nepal (Dhankutta, Dharan, Hattikharka, and Hiley districts), Bhutan (Dhonakha, Thimpu and Gedumari) and India (Basilakha, Recabe, Pakyong and Gangtok of Sikkim, Darjeeling and Kalimpong districts of West Bengal, Lower Subansiri, Upper Subansisri and Ziro valley of Arunachal Pradesh). Various parameters were taken into consideration while documenting, which included the substrate used for the preparation, use of herbs, final products (fermented beverage) prepared from different starter cultures, incubation period during the production, shelf life and socioeconomic importance and impact.

Collection of samples

A total of 35 dry starter cultures were collected from various regions of the Eastern Himalayas including Nepal, Bhutan and Sikkim, West Bengal and Arunachal Pradesh of India. Fresh samples of *marcha* (Nepal, Sikkim, West Bengal and Bhutan)*, paa, pee, phut* (Arunachal Pradesh) and *phab* (Bhutan) were collected in sterile sample bags which were then transported to the laboratory and stored in a desiccator at room temperature (traditionally sun-dried starters can be stored in dry place for more than a year) (Tamang et al. 1996).

Table A: Questionnaire for documentation of traditional knowledge of preparation of dry starters in the Eastern Himalayas

I. General information **Date**:

1. Name of the Informant:

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

II. Information on products:

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

Remarks:

Name and signature of investigator:

Proximate analysis of the samples

For the proximate analysis moisture content and pH of the samples were measured. The estimation of moisture content was done using moisture analyzer (OHAUS/MB-45, USA). For the measurement of pH, 1 gram of each of the samples were first homogenized in 10 ml of distilled water and the pH readings of this homogenized mixture were taken using digital pH-meter (Orion 910003, Thermo Fisher Scientific, USA).

CULTURE DEPENDENT ANALYSIS

Microbial analysis

Isolation of bacteria from amylolytic starter cultures was done using pour plate technique. First the samples were homogenized in stomacher lab blender 40 (Seward, United Kingdom) for 2 min. 10 gram of sample were diluted in 90ml of sterile physiological saline (0.85% w/v) NaCl. The homogenate was then serially diluted. One ml of the appropriate suspension was used in pour plating in MRS Agar and M17 Agar used for isolation of lactic acid bacteria, VRBG agar used for isolation of Gram-negative bacteria and Nutrient Agar used for isolation of aerobic mesophilic bacteria. The MRS and M17 agar plates were then incubated at 30°C and VRBGA and Nutrient agar plates at 37°C for 24-48 h (Tamang and Sarkar 1995). Purity of the isolates were maintained by re-streaking in fresh agar plates and further confirmed through microscopic examinations. The isolates were then preserved in 50% glycerol at -20°C for further analysis.

Analysis of Microbial load in the dry starter samples

Determination of microbial load of the amylolytic starter samples was done using plate count technique in which 1mL of the homogenized suspension of the samples were serially diluted and pour plating was done with appropriate dilution in specific medias such as VRBGA, MRS agar, M17 agar and nutrient agar (Puerari et al. 2015). The colonies that appeared after 24 h of incubation period were counted as colony forming units per gram (CFU/g) and were taken as the measure of number of cells present in particular dilution. Thus, the number of colonies per gram of the sample was enumerated.

Gram's staining

The bacterial isolates were first morphologically characterized using Gram's staining technique which broadly classifies bacteria into Gram positive and Gram negative groups (Holt et al. 1994). Smear of 24 h old bacterial suspension was made on a clean grease free slide. The slide was air dried, heat fixed and flooded with crystal violet for 1 min. The slide was then washed with water and flooded with Gram's iodine for 1 min. The slide was again washed with water and then with 95% ethanol. The slide was then counterstained with safranin for 45 sec. The slide was again washed with water, air dried and observed under oil immersion objective of compound microscope (Cappuccino and Sherman 2008).

Potassium hydroxide (KOH) test

KOH test was done to determine the Gram reaction of the bacterial isolates. The 3% aqueous KOH was prepared and a drop of it was placed on a clean grease free slide. A loop full of bacterial test colony was transferred to the slide and was mixed with the

drop of 3% KOH for about 30 sec. Formation of viscoid solution and a mucoid sting when the loop was pulled determined that the isolates were Gram negative. Absence of viscoid solution and a mucoid sting determined that the test isolate were Gram positive (Mulaw et al. 2019)**.**

Catalase test

The catalase test was done as recommended in Bergey's manual of determinative bacteriology to broadly group the bacterial isolates into catalase positive and negative strains (Holt et al. 1994). The 0.3% hydrogen peroxide was used for this test and formation of bubbles on exposure of bacterial colonies to 0.3% H_2O_2 was considered as a positive result.

Sporulation test

Endospore staining was performed in order to differentiate the bacterial isolates into sporulating and non sporulating groups (Holt et al. 1994). Schaeffer-Fulton method of endospore staining was used for this test. In this method malachite green dye was used to stain the endospores (which appeared green due to the stain) and safranin was used as a counter stain for the vegetative cells (which was stained pink due to safranin).

Carbohydrate fermentation tests

The ability of the bacterial isolates to utilize different sugars was tested using carbohydrate fermentation broth medium. Fresh bacterial culture was inoculated in carbohydrate fermentation broth medium containing 1% (w/v) of specific carbohydrate and was incubated at 37°C for 24 h. The fermentation broth was supplemented with 0.25% phenol red indicator which turned yellow in case of a drop in pH indicating a

positive result and turned red if the isolate was unable to utilize the carbohydrate and thus indicating a negative result. A total of 13 carbohydrates (cellobiose, raffinose, sorbitol, arabinose, mellibiose, xylose, lactose, ribose, melizitose, glucose, sucrose, mannitol and rhamnose) were tested for the preliminary identification of the bacterial isolates (Teuber 1993; Gonzalez et al. 2000; Hammes and Hertel 2003).

Indole test

Indole test was performed for Gram negative group of bacterial isolates. This test was done to screen the ability of bacteria to synthesize tryptophanase enzyme which degrades the tryptophan in the media and produces indole. SIM agar was used for this test and Kovac's reagent was used as an indicator which formed a cherry red band upon binding with indole. The isolates were stabbed in SIM agar and incubated at 37°C for 24 h after which Kovac's reagent was added to check the indole production in the media (Cappuccino and Sherman 2008).

Methyl red test

This test was also performed only for Gram negative bacterial isolates. This test was done in order to check the ability of bacterial isolates to performed mixed acid fermentation. The bacterial isolates were inoculated in MR-VP broth which consists of glucose, peptone and phosphate buffer. The inoculated broth was then incubated at 37°C for 24 h after which methyl red indicator was added and the results were noted. Methyl red indicator gives red color at a pH below 4.4 (which happens when mixed acid fermentation has taken place) and was considered a positive result. At a pH above 6 the methyl red indicator turns yellow (no mixed acid fermentation has occurred) and was considered as a negative result (Cappuccino and Sherman 2008).

Voges- Proskauer (VP) test

VP test was done for Gram negative group of isolates. This test was done to evaluate the ability of isolates to degrade glucose and produce acetoin. The MR-VP broth was inoculated with bacterial isolates and incubated at 37°C for 24 h. After the incubation period alpha-naphthol and potassium hydroxide was added to the broth. Alpha-naphthol and potassium hydroxide react with acetoin giving a cherry red colour which was considered a positive result. If acetoin was not produced in the medium a yellow-brown color was formed on addition of these chemical which was considered a negative result (Cappuccino and Sherman 2008).

Citrate utilization test

This test was done to screen the ability of bacterial isolates to utilize citrate as the sole carbon source. Simmon's citrate agar containing sodium citrate as the only carbon source and bromothymol blue as the indicator was used for this test. Simmon citrate agar slants were prepared and streaked with the bacterial isolates. The slants were then incubated at 37°C for 24 h after which the results were noted. If the isolate was able to utilize citrate as the carbon source, sodium carbonate was formed which increased the pH of the media changing the color of bromothymol blue from green to blue indicating a positive culture. No change in the color of the media was considered a negative result (Cappuccino and Sherman 2008).

Nitrate reduction test

This test was done for the presumptive identification of Gram negative group of bacterial isolates as many Gram negative bacteria use nitrate as the final electron acceptor during anaerobic metabolism. This test screens the ability of isolates to

produce the enzyme nitrate reductase and hence reduce nitrate to nitrite and nitrogenous gases. Nitrate broth was inoculated with the test isolates and incubated at 37°C for 24 h. After the incubation period 6 drops of nitrate reagent A followed by 6 drops of nitrate reagent B was added to the broth. Appearance of red color indicated that nitrate has been reduced by the isolates to nitrites hence a positive result. Zinc powder was then added to the broth tubes with no color change. Appearance of red color in the broth after the addition of zinc indicates a negative result as zinc catalyses the reduction of nitrate to nitrites. However no color change even after addition of zinc was taken as a positive result as it indicates that the nitrate in the broth was reduced by the isolates beyond nitrites (Cappuccino and Sherman 2008).

Urease test

This test was done to screen the ability of bacterial isolates to produce urease enzyme. Urea broth containing 2% urea and phenol red indicator was inoculated with the test isolates and incubated 37°C for 24 h. Appearance of magenta pink color (due to increase in pH after the production of ammonia and carbon dioxide as the byproduct of urea hydrolysis) after the incubation period was taken as a positive result and no color change as the negative result (Cappuccino and Sherman 2008).

Gas production from glucose

This test was done for the determination of homofermentative and heterofermentative nature of Gram-positive isolates. MRS broth containing 1% glucose was prepared and inverted durham's tubes were added to the broth. These MRS broth tubes were then inoculated with test isolates and incubated at 37°C for 48 h. Formation of gas bubbles inside the Durham's tubes indicated that CO_2 production has taken place and that the

isolate was heterofermentative. Absence of gas bubbles in the Durham's tube indicates that the isolate was homofermentative (Mulaw et al. 2019).

PHYSIOLOGICAL TESTS

Growth at different pH

The ability of test isolates to grow in different physiological pH was determined according to the method of Tamang et al. (2007). MRS broth with pH 3.6, 9.6 and 10.6 were prepared and inoculated with test isolates. The inoculated broth was then incubated at 30°C for 24 h after which the growth of the isolates were checked and recorded.

Growth at different NaCl concentrations.

The ability of bacterial isolates to grow at different osmotic concentrations was screened following the method of Tamang et al. (2007). MRS broth containing 5% and 10% NaCL were prepared and inoculated with the test isolates. After the incubation at 30°C for 24 h the growth of the test isolates in different osmotic concentrations were checked and recorded.

Growth at different temperature

Freshly grown bacterial isolates were inoculated in MRS broth and incubated at 3 different temperatures i.e. 10°C, 15°C and 45°C for 24 h to check the growth pattern of the isolates (Harrigan and McCance 1976; Schillinger and Lucke 1987; Tamang et al. 2007).

Tentative Identification

The bacterial isolates were tentatively identified up to genus level on the basis of phenotypic and biochemical tests following the taxonomical keys of Bergey's manual of bacteriological classification (Holt et al. 1994).

MOLECULAR IDENTIFICATION OF BACTERIAL ISOLATES

Isolation of genomic DNA from the bacteria isolates

Exaction of genomic DNA from the bacterial isolates was done using the standard Phenol/chloroform method with slight modifications (Cheng and Jiang 2006). Test cultures were grown overnight in MRS broth at 30°C and harvested by centrifugation at 8000 rpm for 10 min. The supernatant was removed and the pellets were washed twice with 1 X PBS (phosphate buffer saline, pH 7). The pellets were re-suspended in 400µl 1X TE buffer (10 mM NaCl, 10 mM Tris/HCL,1 mM EDTA, pH 8), 15µl lysozyme and 15µl RNase enzyme and kept for incubation in water bath at 37°C for 3 h. After the incubation 15µl of 20% SDS and 15µl of Proteinase K was added to the centrifuge tubes and kept for incubation at 55°C for 3 h. Equal volume of phenol and chloroform (49:48) was added and centrifuged at 10,000 rpm for 15 min at 4°C to separate the aqueous phase from organic phase. The upper aqueous phase was collected in a fresh 1.5 ml centrifuge tubes, to which equal volume of chloroform and Isoamyl alcohol (48:1) was added and centrifuged at 10,000 rpm for 15 min at 4°C. The upper aqueous phase was again collected in a fresh 1.5ml centrifuge tube and the above process was repeated until the white interface was no longer visible. Precipitation of DNA in this upper aqueous phase was done by adding 15µl of 3M sodium acetate and 400µl of cold absolute alcohol and incubating it at -20°C for at least an hour. The aqueous phase containing the precipitated DNA was then

centrifuged at 10,000 rpm for 30 min and the pellets were collected. The pellets were washed with 70% ethanol followed by centrifugation at 10,000 rpm for 30 min. The supernatant was discarded and the pellets were air dried, suspended in 30µl of 1 X TE buffer and stored in -20°C until further analysis. Electrophoresis in 0.8% agarose gel was done to check the quality of the extracted DNA and quantified using Nano-Drop ND-1000 spectrophotometer (Nano drop technologies, Willington, USA) (Kumbhare et al. 2015).

PCR amplification of the 16S rRNA gene

The PCR amplification of 16S rRNA gene from the isolated genomic DNA was done using a thermal cycler (Applied biosystems-2720, USA) (Lane 1991). Universal oligonucleotide primers p27F 5'-AGAGTTTGATC[A,C]TGGCTCAG- 3' and p1492R 5'-GG[C,T] TACCTTGTTACGACTT- 3' was used for the amplification (Park et al. 2010). Each reaction mixture contained 12.5µl GoTaq Green (M7122) master mix (reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$), each primer at a concentration of 1µl, 8.5µl nuclease free water and 2µl of template DNA, making a final volume of 25µl. The PCR conditions were as follows: (i) denaturation of DNA at 95°C for 10 min, (ii) 35 cycles of denaturation at 95°C for 30 sec (iii) annealing at 65°C for 30 sec (iv) extension at 72°C for 1 min followed by final extension at 72°C for 10 min (Chagnaud et al. 2001). The PCR products were detected by electrophoresis using 1% agarose gel. The bands were stained with 7µl/100mL ethidium bromide (RM813, HiMedia, Mumbai) and visualized using UV source Gel-Doc 1000 (Bio-Rad, 97-0186-02, US). For the verification of amplicon size standard 100 base pair DNA ladder was used.

Purification of amplified PCR products

Purification of genomic DNA was done using the standard method of PEG-NaCl (Poly ethylene glycol-sodium chloride) precipitation with slight modification (Schmitz and Riesner 2006). The 0.6 volume of PEG-NaCl (20% w/v of PEG, 2.5 M NaCl) was added to the final volume of PCR product and incubated at 37°C for 30 min. The mixture was then centrifuged at 12,000 rpm for 30 min at 4°C after which the tubes were decanted. 100 µl of 70% ethanol was added to the tubes and centrifuged at 12,000 rpm for 30 min after which the tubes were decanted. The above step was repeated twice. The decanted tubes were allowed to air dry and 20 µl of nuclease free water was added to it. The final product was incubated for 30 min, mixed well and run in 1 % agarose gel to check the integrity of the purified PCR product.

Sequencing of the 16S rRNA PCR products

5µl volume of PCR products were set up for single primer amplification with the same universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Lane 1991) for separate reactions for each primer. The PCR reaction was set as follows: denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, elongation at 60°C for 2 min and a stop reaction at 4°C. Precipitation of the amplicons was then carried out with 1µl sodium acetate (3M, pH 5.2) and 24µl of absolute alcohol. The precipitated amplicons were then mixed briefly in vortex and incubated at room temperature for 15 min, centrifuged at 12,000 rpm for 20 min and washed with 70% ethanol. The amplicon were then air-dried and suspended in 10µl formamide. The amplicons were sequenced using Sanger Sequencing method or Chain-termination DNA sequencing method (Heather and

Chain 2016) which was carried out in an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The quality of the sequence was checked by Sequence Scanner v.1.0 (Applied Biosystems, Foster City, CA, USA). After screening the sequence quality, the sequences were assembled using Chromas Pro 1.5 (McCarty 1998). Orientation checker v.1.0 was used for checking the orientation of the assembled sequences. The bacterial isolates were assigned their identity number by comparing their DNA sequences of with those available in the GenBank NCBI (National Center for Biotechnology Information) database using BLAST (basic local alignment search tool) 2.0 program (Altschul et al. 1990). Alignment of the sequences were then done by pairwise alignment using clustal W, and construction of phylogenetic tree was done using MEGA7.0 (Pennsylvania State University, Vl.7.0.26, USA) software by neighbour joining method (Gascuel and Steel 2006; Kumar et al. 2016). A comprehensive statistics package used in many fields of life sciences, economics, earth science, engineering and also by palaeontologists, PAST (PAleontological STatistics) (Palaeontological Association-V4.0, Norway) was used for calculating the diversity indices (Hammer et al. 2001). Chao 1 value for species richness was calculated using the method of Chao and Chiu (2016).

CULTURE INDEPENDENT ANALYSIS

Sample collection

Fresh samples of *marcha* were collected from Gangtok, Sikkim in a sterile sealed container and stored in a desiccator at room temperature until further analysis.

Community DNA extraction

The sample DNA was extracted using ProMega DNA kit (ProMega). One gram of dry starter sample was suspended in lysis solution. The solution was then incubated at 65°C for 15 min. RNase solution was then added and incubated at 35°C for 15 min which lysed the RNA. Protein precipitation solution was then added and centrifuged at maximum speed to remove the residual proteins. Final precipitation of the DNA was done by adding isopropanol and the precipitated DNA was purified with two washes of 70% ethanol. The quality of DNA was checked on 0.8% agarose gel and quantified using Nano-DropND-1000 spectrophotometer (Nano Drop technologies, Willington, USA) (Kumbhare et al. 2015). The purified DNA was then stored at -20°C for further processing.

Amplicon sequencing of community DNA

In this study we have targeted the V4 hyper-variable region (Bartram et al. 2011) to investigate bacterial diversity of *marcha* of Sikkim. The amplification of V4 hyper-variable region was done using universal 16S rRNA gene primer F515 and 806R (Caporaso et al. 2010). The 16S rRNA library was prepared according to the protocol of Illumina (USA). The amplicon library was further processed using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) which was further screened with the LabChipGX (Perkin Elmer, Waltham, MA, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Finally the 16S rRNA gene library was sequenced on the Illumina MiSeq platform using 2x250bp chemistry. The sequences retrieved from high throughput sequence analysis were submitted to National Center for Biotechnology Information (NCBI) and is available under BioProject ID PRJNA376467.

Bioinformatics analysis

FLASH tool (Fast Length Adjustment of Short reads) a Paired end assembler for DNA sequences (Masella et al. 2012) was used to assemble the raw sequences generated from MiSeq platform. The quality filtering of the assembled reads were done using Quantitative Insights into Microbial Ecology (QIIME) (University of Colorado, V2-2019.10, USA) 1.8 (Caporaso et al. 2010). SILVA reference database was then used to assigned bacterial operational taxonomic units (OTUs) to the sequence reads by a closed reference-based OTU picking approach. The UCLUST method with similarity threshold of 97% (Edgar 2010) was used for picking OTUs. RDP naive Bayesian classifier was used to perform the taxonomic assignments (Wang et al. 2007). Alpha diversity indices like Chao, Shannon and Simpson were calculated in QIIME after the rarefaction of all samples to the same sequencing depth.

SCREENING OF PROBIOTIC ACTIVITY

Hydrophobicity assay

Cultures of the strains were harvested in the stationary phase by centrifugation at 12000 g for five min at 5°C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. The cell suspension was adjusted to an A560 nm value of approximately 1.0 with the buffer and 3 ml of the bacterial suspensions were put in contact with 0.6 ml of n-hexadecane and vortexed for 120 sec. The two phases were allowed to separate for 0 h at 37°C. The aqueous phase is carefully removed and the absorbance at 560 nm was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula:

$H\% = [(A_0 - A)/A_0]100$

Where A0 and A are the absorbance before and after extraction with n-hexadecane, respectively (Vinderola and Reinheimer 2003).

Acid tolerance test

Freshly grown cultures were grown in MRS broth for 18-24 h for the test of tolerance of bacterial isolates in low pH. The cells were then harvested by centrifugation at 8000 rpm for 15 min at 4°C. The cell pellets were then washed with PBS (pH 7.2) after which it was resuspended in PBS (pH 3) and incubated at 37°C. The viability of the bacteria was tested by plating the cultures in MRS agar after 3 h and was expressed as colony forming units per milliliter (CFU/ml) (Nithya and Halami 2013). The rate of survival of the bacterial isolates in low pH was calculated using the formula (Fuller 1989):

$$\text{Survival } \% = \frac{\text{Log number of cells survived } \left(\frac{\text{CFU}}{\text{ml}}\right) \times 100}{\text{Log number of initial cells inoculated } \left(\frac{\text{CFU}}{\text{ml}}\right)}$$

Bile tolerance test

The bile tolerance was tested according to the methods followed by Devi et al. (2015) with slight modifications. MRS broth inoculated with test cultures were incubated at 30°C for 24 h. MRS broth containing 0.3% ox gall was prepared and inoculated with 1% (v/v) of 24 h old test cultures. MRS broth without ox gall was used as control. After inoculation test cultures were withdrawn at 3 h of incubation and the viability of the cells were checked by inoculation in MRS agar followed by incubation at 30°C for 24 h (Devi et al. 2015).

Deconjugation of bile salts

Deconjugation of bile salts by the bacterial isolates was screened using the method of Taranto et al. (1995). Modified MRS agar containing 0.5% sodium salts of taurocholic acid (TC), taurodeoxycholic (TDC) acid and 0.37g/litre of $CaCl_2$ was prepared (Xu et al. 2016). Bacterial isolates were streaked on these modified MRS agar plates and incubated at 37°C for 72 h in anaerobic gas jars. The appearance of precipitation around the colonies after the incubation was taken as positive result.

β-Galactosidase activity

β-Galactosidase activity of the bacterial isolates were screened using plate assay method (Angmo et al. 2016). MRS agar plates containing 10 ml of IPTG (iso-propyl-thio β-D galactopyranoside) as inducer and 60 ml X-gal (5-bromo-4-chloro-3- indolyl-b-D-galactopyranoside) were prepared and streaked with 24 h old bacterial culture. The plates were then incubated at 37°C for 42 h. Appearance of blue colored colonies after 42 h indicates a positive result and transparent colonies as negative result.

Cholesterol assimilation

The ability of test isolates to assimilate cholesterol was determined following the method described by Shehata et al. (2016). MRS broth supplement with 0.3% ox gall (bile salt) and water soluble cholesterol (100µg/ ml) was prepared for this test. The test isolates were inoculated in this modified MRS broth at a concentration of 1% and was incubated at 37°C for 24 h. After incubation the cells were removed by centrifugation at 9000 rpm for 15 min and the amount of cholesterol remaining in the broth was determined calorimetrically using o-phthalaldehyde method (Rudel and Morris 1973). 1 ml of KOH (33% w/v) and 2ml of absolute ethanol was added to 1ml of cell free broth obtained after centrifugation. The mixture was vortexed for 1 min and incubated at 37°C for 15 min after which 2ml of distilled water and 3ml of hexane was added to it and again vortexed for a min. 1 ml of the upper hexane layer was then transferred to a fresh glass tube and evaporated in a water bath (65°C). The residue obtained was dissolved in o-phthalaldehyde reagent and 0.5ml concentrated sulphuric acid was added to it and vortexed. After 10 min the absorbance was measured spectrophotometrically at 550 nm.

SCREENING FOR FUNCTIONAL ATTRIBUTES

Phytase activity (Anti-nutritive factor degrading property)

Plate screening assay for the qualitative determination of phytase activity of the test isolates were done following the method of Fischer et al. (2014) with slight modifications. Modified MRS agar containing 1% casein peptone, 0.4% beef extract, 1% glucose, 0.2% yeast extract, 0.82% sodium acetate trihydrate, 0.02% magnesium sulphate heptahydrate, 0.2% ammonium citrate dibasic, 0.0025% manganese sulphate, 0.2% calcium chloride, 2% MOPS, 1.5% agar and 0.25% sodium phytate as the sole

phosphate source was prepared for the screening of phytase activity. The test isolates were streaked on the surface of modified MRS agar and incubated at 35°C for 72 h. Formation of clear zones (due to degradation of phytic acid) around the colonies after 72 h of incubation was recorded as a positive result.

Amylolytic activity

Starch agar media was used for the detection of amylolytic activity of the test cultures. 24 h old test cultures were streaked on the surface of starch agar plates which were then incubated at 30°C for 48 h. After the incubation the plates were flooded with iodine solution for about 15-20 min. Appearance of clear zone around and underneath (after the growth is scrapped off) the grown culture was taken as positive for amylolytic activity (Tamang et al. 2007).

Ethanol tolerance test

Tolerance of test isolates at different concentration of ethanol was performed following the method of Gold et al. (1992) with slight modification. Overnight grown test cultures were inoculated in MRS broth containing 4%, 5%, 6%, 7%, 8%, 9% and 10% absolute ethanol. MRS broth without ethanol was inoculated with bacterial isolates and was kept as control. The inoculated media were then kept in incubation at 35°C after which OD readings at 600nm were recorded after 24 h (Gold et al. 1992).

SCREENING FOR SAFETY EVALUATION

Haemolytic activity

For determining the safety aspect of the lactic acid bacterial isolates hemolytic activity of the isolates were screening following the method described by Somashekaraiah et al. (2019). Blood agar plates containing 5% (v/v) sheep blood were prepared and streaked with the test isolates. The blood agar plates were then incubated at 37°C for 48 h after which the plates were observed for zone of hemolysis (α hemolysis, β hemolysis and γ hemolysis) (Somashekaraiah et al. 2019).

Detection of virulence factor

The isolates were screened for the presence of virulence gene *cyl A* that codes for cytolysin which is a haemolytic toxin produced by some strains of *Enterococcus fecalis* and causes the lysis of RBCs (Coburn and Gilmore 2003). PCR amplification of gene *cyl A* (cytolysin activator encoding gene) was done. The primer used was F 5'ACTCGGGGATTGATAGGC3' and R 5'GCTGCTAAAGCTGCGCTT3' for the screening of *cyl A* (Creti et al. 2004).

SCREENING OF PROBIOTIC AND FUNCTIONAL GENE

Several genes responsible for various probiotic and functional characteristics were analyzed for all lactic acid bacterial isolates. Primers which were reported in earlier literature were used for this study (Table B). Positive controls were selected after testing the primers on the DNA of the reference strains containing the targeted genes For the PCR amplification of the probiotic and functional genes, each reaction mixture contained 10µl GoTaq Green (M7122) master mix (reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$), each primer at a

concentration of 0.8µl, 6.8µl nuclease free water and 1.6µl of template DNA, making a final volume of 20µl. After the amplification the amplicons were run in 1% agarose gel stained with ethidium bromide to check the presence of desired amplicons.

Table B: Primers used for the detection of Probiotic structural genes						
Target gene	**Function**		**Primer**	**Annealing temperature (˚C)**	**Amplicon size (bp)**	**References**
Rib A	riboflavin biosynthesis	3,4-Dihydroxy-2-butanone 4-phosphate synthase/ GTP cyclohydrolase	F 5'TTTACGGGCGATGTTTTAGG3' R 5'CGACCCTCTTGCCGTAAATA3'	60	121	Turpin et al. 2011
Fol P	folate biosynthesis	dihydropteroate synthase/ dihydropteroate pyrophosphorylase	F 5'CCASGRCSGCTTGCATGAC3' R 5'TKACGCCGGACTCCTTTTWY3'	59.5	261	Turpin et al. 2011
clp L	survival in low pH and bile salt	ATPase	F 5'GCTGCCTTYAAAACATCATCTGG3' R 5'AATACAATTTTGAARAACGCAGCTT3'	56	158	Turpin et al. 2011
bsh	bile salt	bile salt acid hydrolase	F 5'ATTGAAGGCGGAACSGGMTA3' R 5'ATWACCGGWCGGAAAGCTG3'	58	155	Turpin et al. 2011
Ir1584	bile salt	Major facilitator superfamily	5'TAYGCCRTTCGGWTGTTTGG3' R 5'TCAWRATGGCRGTCCCAATG3'	55	151	Turpin et al. 2011
Ir1516	survival in low pH and bile salt	putative esterase	F 5'TRACCACTYTCWCCATTCAACAA3' R5'CCACTAGCRATGACYAATACKGGT3''	56.5	143	Turpin et al. 2011
LBA1446	bile salt	Multidrug resistance protein	F 5'GCTGGAGCCACACCGATAAC3' R 5'CAACGGGATTATGATTCCCATTAGT3'	58	275	Turpin et al. 2011
hdc	survival in low pH	Histidine decarboxylase	F 5'AGATGGTATTGTTTCTTATG3' R 5'AGACCATACACCATAACCTT3'	52	367	Costantini et al. 2006
tdc	survival in low pH	Tyrosine decarboxylase	F 5'CCACTGCTGCATCTGTTTG3' R5'CCRTARTCNGGNATAGCRAARTCNGTRTG3'	50	370	Diaz-Ruiz et al. 2003; Eom et al. 2009
odc	survival in low pH	Ornithine decarboxylase	F 5'TMTWCCAACHGATCGWAATGC3' R 5'CRCCCCAWGCACARTCRAA3'	58	245	Turpin et al. 2011

agu A	survival in low pH	Agmatine deiminase	F 5'GAACGACTAGCAGCTAGTTAT3' R 5'CCAATAGCCGATACTACCTTG3'	60	542	Fang et al. 2009
mub	adhesion	mucin binding protein	F5'GAGCAGAAGATGGGCCAAC3' R5'CTTCTGCGTCAACAACTTCG3'	63	922	Archer and Halami 2015
fbp	adhesion	fibronectin binding protein	F 5'AGTGCTGAAATYATGGGAAGA3' R 5'AATTGTCCACCTTGTTGCTG3'	60	835	Archer and Halami 2015
sor	adhesion	sortase	F 5'CCACCTTGTACTGGTTAGTG3' R 5'GACCATTCGTGTACTTGCCG3'	55	672	Archer and Halami 2015
sbp	adhesion	ATP binding substrate protein	F 5'CAGTTCTTAGCCACAGTTTG3' R 5'GGTTCGCCGCTAATAGTAAG3'	55	805	Archer and Halami 2015
msa	adhesion	mannose specific adhesion	F 5'GCGATTAGGGGTGTGCAAG3' R 5' GCAGTTGGTGACGTAGGCA3'	55	319	Archer and Halami 2015
apf	adhesion	aggregation promoting factor	F 5'YAGCAACACGTTCTTGGTTAGCA3' R 5' GAATCTGGTGGTTCATAYWCAGC3'	53	112	Turpin et al. 2011
map A	adhesion	mucus adhesion promoting protein	5'GGATTCTGC TTGAGGTAAG3' R 5'GACTAGTAATAACGCGACCG3'	50	156	Ramiah et al. 2007
mub1	adhesion	mucus binding protein	F 5'GTAGTTACTCAGTGACGATCAATG3' R 5'TAATTGTAAAGGTATAATCGGAGG3'	50	150	Ramiah et al. 2007
ped A	bacteriocin	pediocin	F 5'AAAATATCTAACTAATACTTG3' R 5'TAAAAAGATATTTGACCAAAA3'	44	600	Rodriguez et al. 1997
cyl A	bacteriocin	cytolysin activator encoding gene	F 5'ACTCGGGGATTGATAGGC3' R 5'GCTGCTAAAGCTGCGCTT3'	54	688	Creti et al. 2004
ent A	bacteriocin	enterocin	F 5'GGT ACC ACT CAT AGT GGA AA3' R 5'CCC TGG AAT TGC TCC ACC TAA3'	55	138	Özdemir et al. 2011

ent B	bacteriocin	enterocin	F 5'CAA AAT GTA AAA GAA TTA AGT ACG3' R 5'AGA GTA TAC ATT TGC TAA CCC3'	56	201	De Vuyst et al. 2003
ent P	bacteriocin	enterocin	F 5'GCT ACG CGT TCA TAT GGT AAT3' R 5'TCCTGCAATATTCTCTTTAGC3'	55	87	Özdemir et al.2011
ped B	bacteriocin	pediocin	F5'ATGAATAAGACTAAGTCGGAACATATT3' R5'CTATTGGCTAGGCCACGTATTG3'	57	339	El-Arabi et al. 2018
Bgl	β -glucosidase	β –glucosidase enzyme	F5'GTGACTATGGTAGAGTTTCC3' R5'TCAAAACCCATTCCGTTCCCCA 3'	50	1392	Spano et al. 2005
cyl A	haemolysin	cytolysin activator encoding gene	F 5'ACTCGGGGATTGATAGGC3' R 5'GCTGCTAAAGCTGCGCTT3'	54	688	Creti et al. 2004

Detection of genes involved in biosynthesis of riboflavin and folate

The ability of bacterial isolates to synthesize folate and riboflavin were analyzed by screening the presence of two genes *fol P* and *rib A* gene respectively. *Fol P* gene encodes for dihydropteroate synthase/ dihydropteroate pyrophosphorylase (De Crécy-Lagard et al. 2007) and Rib A gene encodes for 3,4-Dihydroxy-2-butanone 4-phosphate synthase/ GTP cyclohydrolase (Burgess et al. 2004), enzymes involved in biosynthesis of folate and riboflavin respectively. The PCR amplification of *rib A* and *fol P* genes were done using the primer F 5'-TTTACGGGCGATGTTTTAGG-3' and R 5'-CGACCCTCTTGCCGTAAATA-3' for *rib A* gene and F 5'-CCASGRCSGCTTGCATGAC-3' and R 5'- TKACGCCGGACTCCTTTTWY-3' for *fol P* gene (Turpin et al. 2011). The PCR conditions were as follows: 1 cycle at 95°C for 5 min; 40 cycles of 95°C for 30 sec, an annealing temperature for 10 sec (depending on the primer ; listed in Table B), and 72°C for 15 sec; and 1 cycle at 72°C for 5 min. The expected amplicon size of *rib A* and *fol P* genes were 121 and 261 bp, respectively.

Detection of genes involved in bile salt survival

Screening of *clp L, bsh, Ir1584, Ir1516* and *LBA 1446* genes which are involved in survival of bacteria in presence of bile salts were done by amplifying the above genes using specific primers. *clp L* encodes for ATPase, *Ir1516* (Wall et al. 2007) encodes for putative esterase, *bsh* encodes for conjugated bile salt acid hydrolase (McAuliffe et al. 2005; Fang et al. 2009; Denou et al. 2008; Lambert et al. 2008), *Ir1584* encodes for Major facilitator superfamily permease (Whitehead et al. 2008) and *LBA 1446* encodes for Multidrug resistance protein (Pfeiler et al. 2009). All of these genes encode enzymes and protein which facilitate the survival of bacteria in presence of bile salts.

The primers used were F 5'GCTGCCTTYAAAACATCATCTGG3' and R 5'AATACAATTTTGAARAACGCAGCTT3' for *clpL* gene (Turpin et al. 2011), F 5'TRACCACTYTCWCCATTCAACAA3' and R 5'CCACTAGCRATGACY AATACKGGTT3' for *Ir1516* gene (Turpin et al. 2011), F 5'ATTGAAGGCGGAACSGGMTA3' and R 5'ATWACCGGWCGGAAAGCTG3' for *bsh* gene (Turpin et al. 2011), F 5'TAYGCCRTTCGGWTGTTTGG3' and R 5'TCAWRATGGCRGTCCCAATG3' for *Ir1584* gene (Turpin et al. 2011) and F 5'GCTGGAGCCACACCGATAAC3' and R 5'CAACGGGATTATGATTCCC ATTAGT3' for *LBA1446* gene (Turpin et al. 2011). The PCR conditions were as follows: 1 cycle at 95°C for 5 min; 40 cycles of 95°C for 30 sec, an annealing temperature for 10 sec (depending on the primer; listed in Table B) and 72°C for 15 sec; and 1 cycle at 72°C for 5 min.

Detection of genes involved in survival at low pH

The ability of the bacterial isolates to survive and proliferate at low pH conditions were analyzed by screening the presence or absence of *hdc* (Histidine decarboxylase), *odc* (Ornithine decarboxylase), *tdc* (Tyrosine decarboxylase), *agu A* (Agmatine deiminase), *clp L* and *Ir1516* genes (Turpin et al. 2011). The primers used for the amplification of the genes were: F 5'-AGATGGTATTGTTTCTTATG-3' and R 5'AGACCATACACCATAACCTT3' for *hdc* gene (Costantini et al. 2006), F 5'CCACTGCTGCATCTGTTTG3' and R 5'CCRTARTCNGGNATAGCRAAR TCNGTRTG3' for *tdc* gene (Diaz-Ruiz et al. 2003; Eom et al. 2009), F 5'TMTWCCAACHGATCGWAATGC3' and R 5'CRCCCCAWGCACARTCRAA3' for *odc* gene (Turpin et al. 2011) and F 5'GAACGACTAGCAGCTAGTTAT3' and R 5'CCAATAGCCGATACTACCTTG3' for *agu A* gene (Fang et al. 2009). The PCR

conditions were as follows: 1 cycle at 95°C for 5 min; 40 cycles of 95˚C for 30 sec, an annealing temperature for 10 sec (depending on the primer ; listed in Table B), and 72°C for 15 sec; and 1 cycle at 72°C for 5 min.

Detection of genes involved in adhesion

Mub, fbp, sor, sbp, msa, apf, map A and *mub1* genes were screened to check the ability of bacterial isolates to adhere to the mucoid membrane. The primers used for this study were F 5'GAGCAGAAGATGGGCCAAC3' and R5'CTTCTGCGTCAA CAACTTCG3' for *mub* (mucin binding protein) gene, F 5'AGTGCTGAAATYATGGGAAGA3' and R 5'AATTGTCCACCTTGTTGCTG3' for *fbp* (fibronectin binding protein) gene, F 5'CCACCTTGTACTGGTTAGTG3' and R 5'GACCATTCGTGTACTTGCCG3' for *sor* (sortase) gene, F 5'CAGTTCTTAGCCACAGTTTG3' and R 5'GGTTCGCCGCTAATAGTAAG3' for *sbp* (ATP binding substrate protein), F 5'GCGATTAGGGGTGTGCAAG3' and R 5'GCAGTTGGTGACGTAGGCA3' for *msa* (mannose specific adhesion) gene (Archer and Halami 2015), F 5'YAGCAACACGTTCTTGGTTAGCA3' and R 5' GAATCTGGTGGTTCATAYWCAGC3' for *apf* (aggregation promoting factor) gene (Turpin et al. 2011), F 5'GGATTCTGCTTGAGGTAAG3' and R 5'GACTAGTAATAACGCGACCG3' for *map A* (mucus adhesion promoting protein) gene and F 5'GTAGTTACTCAGTGACGATCAATG3' and R 5'TAATTGTAAAGGTATAATCGGAGG3' for *mub1* (mucus binding protein) gene (Ramiah et al. 2007). The conditions for PCR reaction in a thermal cycler (Applied biosystems-2720, USA) were as follows: 95°C for 5 min; 34 cycles of 95°C for 1 min, annealing temperature (Table B) for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min (Archer and Halami 2015).

Detection of bacteriocin genes

Bacteriocin genes ped *A* (pediocin)*, ped B, ent A* ("enterococin)*, entB, ent P* and *cyl A* (cytolysin activator encoding gene) were screened to analyze the antimicrobial ability of the bacterial isolates. The primers used for this study were: F 5'GGTACCACTCATAGTGGAAA3' and R 5'CCCTGGAATTG CTCCACCTAA3' for *ent A* gene (Özdemir et al. 2011), F 5'CAAAAT GTAAAAGAATTAAGTACG3' and R 5'AGAGTATACATTTGCTAACCC3' for *ent B* (De Vuyst et al. 2003)*,* F 5'GCTACGCGTTCATATGGTAAT3' and R 5'TCCTGCAATATTCTCTTTAGC3' for *ent P* (Özdemir et al. 2011), F 5'AAAATATCTAACTAATACTTG3' and R 5'TAAAAAGATATTTGACCAAAA3' for *ped A* (Rodrıguez et al. 1997), F 5'ATGAATAAGACTAAGTCGGAACATATT3' and R 5'CTATTGGCTAGG CCACGTATTG3' for *ped B* (El-Arabi et al. 2018) and F 5'ACTCGGGG ATTGATAGGC3' and R 5'GCTGCTAAAGCTGCGCTT3' for *cyl A* (Creti et al. 2004).

Data availability of 16S rRNA sequencing

The partial sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-National Center for Biotechnology Information (NCBI) under the nucleotide accession number: MK748250-MK748278, MK202997-MK203032, and MK752675-MK752677.

Data availability of High-throughput sequencing

The sequence data of high-throughput amplicon sequencing has been uploaded at MG-RAST server with the MG-RAST ID number Bio Project ID PRJNA376467.

RESULTS

DOCUMENTATION OF DRY STARTERS

This research was mainly focused on the study of traditionally prepared dry starters of the Eastern Himalayan regions of Nepal, India and Bhutan (Figure 1). Hence we visited the eastern part of Nepal, Darjeeling, Sikkim and Arunachal Pradesh in India, and Bhutan for the purpose of documentation and sample collection. The documentation and sample collection was carried for a period of 12 months i.e. from December 2015 till November 2016. The data collection for documentation was conducted using structured questionnaire (Table A), personally visiting the villages where the starters were prepared and analyzing the preparation procedure, interviewing the local people who prepare these starters, collecting the plants used in the preparation of the starters. Table 1 briefs the area of sample collection and documentation, its coordinates, samples collected, community that prepares the samples and the substrates used for the preparation of the starter samples. We visited different places in the Eastern Himalayan regions of Nepal, India and Bhutan to document the traditional knowledge of preparation of dry starters and collection of samples. These places are Dharan, Dhankutta, Hattikharka and Hiley districts of eastern Nepal, Darjeeling and Kalimpong districts of West Bengal, Pakyong, Gangtok, Recab and Basilakha village of Sikkim, lower Subansiri, upper Subansiri and Ziro valley of Arunachal Pradesh and Gedumari, Thimpu and Dhonakha of Bhutan (Table 1).

Figure 1: Map showing the sample collection and documentation sites of different regions of the Eastern Himalayas.

Table 1: Traditionally prepared dry starters of the Eastern Himalayas							
Sample	**Substrate used**	**Dominant Ethnic Community as Starter maker**	**Country**	**Collection Site**	**Altitude (Meter)**	**Latitude**	**Longitude**
Marcha	Rice, Herbs (*chittu, chabo)*	Rai, Limboo, Newar	Nepal (District Dhankuta, Sunsari)	Dharan	371	26°48' N	87°17' E
				Dhankuta	1154	26°53' N	87°8 ' E
				Hiley	857	27°02' N	87°24' E
				Hatikharka	1394	27°01' N	87°32' E
Marcha	Rice, roots of *Plumbago zeylanica*, leaves of *Buddleja asiatica*, flowers of *Vernonia cinerea*, ginger and dry red chilies	Rai, Limboo	India (West Bengal)	Darjeeling	2059	27°04' N	88°26' E
				Kalimpong	1176	27°07' N	88°47' E
Marcha	Rice, roots of *Plumbago zeylanica*, leaves of *Buddleja asiatica*, flowers of *Vernonia cinerea*, ginger and dry red chilies	Rai, Limboo	India (Sikkim)	Pakyong	1341	27°24' N	88°59' E
				Gangtok	1637	27°32' N	88°61' E
				Recab	1072	27°21' N	88°50' E
				Basilakha	906	27°22' N	88°60' E
Marcha	Rice, roots of *Plumbago zeylanica*, leaves of *Buddleja asiatica*, flowers of *Vernonia cinerea*, ginger and dry red	Nepali	Bhutan (district Thimpu)	Gedumari	1045	26°90' N	89°39' E
				Thimpu	2401	27°47' N	89°62' E
Paa	Rice, Leaves of *Ctuepatti* (*Cissampelos pareira Linn.)* and *Khanoba (Clerodenderon viscosum)*	Nyshing	India (Arunachal Pradesh)	Lower Subansiri	661	27°8' N	93°6' E
Pee	Rice	Apatani		Ziro valley	1576	27°53' N	93°81' E
Phut	Rice, leaves of *Cinnamomum glanduliferum*	Mongpa		Upper Subansiri	1816	28°3' N	94° E
Phab	Maize, rice husk, flowers of *bagham* plant	Drukpa	Bhutan (Punakha)	Dhonakha	2311	27°66' N	89°70' E

MARCHA

Marcha is a dry round and flattened creamy white starter traditionally prepared in Nepal, India and Bhutan (Figure 2A-D). The preparation process of *marcha* is almost same in these regions except for the plants that are used during the preparation. *Marcha* is used to prepare the fermented alcoholic beverage locally known as *raksi* and *jaanr*.

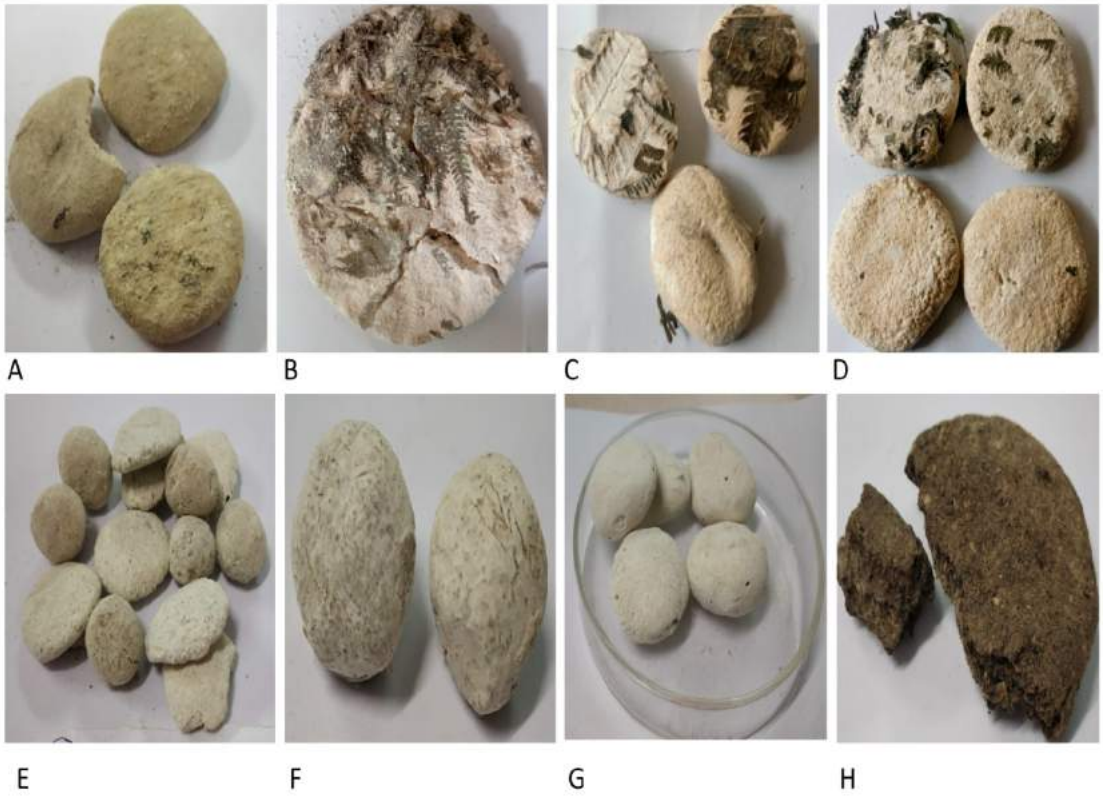


Figure 2: Traditional starter cultures: A; *Marcha* of Nepal, B; *Marcha* of Darjeeling, C; *Marcha* of Sikkim, D; *Marcha* of Bhutan, E; *Paa* of Arunachal Pradesh, F; *Pee* of Arunachal Pradesh, G; *Phut* of Arunachal Pradesh, H; *Phab* of Bhutan.

Traditional method of preparation of *marcha* of Nepal

The substrate used for the preparation of *marcha* (Figure 2A) is rice which is soaked overnight and then beaten to a fine powdered form. Leaves, flowers and roots of plant *chittu* and whole plant of *chabo* are powdered and added to the powdered rice in equal amounts. Both the ingredients are mixed properly after which water is added to it to make fine dough. Bowl sized *marcha* balls are then prepared from this dough. The freshly prepared *marcha* is then sprinkled with the old inoculums (old *marcha* which has been powdered). The fresh *marcha* is then placed on straw mats and incubated for 48 h during winters and 24 h during summers after which it is sun dried for 2-3 days and sold in the market (Figure 2A and 3).

Figure 3: Traditional method of preparation of *marcha* in Nepal.

Traditional method of preparation of *marcha* of Darjeeling, Sikkim and Bhutan

Preparation of *marcha* in Darjeeling hills and Sikkim in India (Figure 2B, C) and Bhutan (Figure 2D) is slightly different from *marcha* of Nepal specifically in the herbs that are used during the preparation. The initial substrate used is rice which is soaked overnight and then grounded into a fine powdered form. This powdered rice is mixed with roots of *Plumbago zeylanica*, leaves of *Buddleja asiatica*, flowers of *Vernonia cinerea*, ginger and dry red chilies and powdered along with the rice. Water is then added to the above mixture and kneaded into fine dough. Small *marcha* balls are then prepared from the dough which is sprinkled with inoculums (old *marcha* which is powdered). The freshly prepared *marcha* balls are kept on fern *Glaphylopteriolopsis erubeseens* and covered with it and incubated for 24 h. After the incubation period the *marcha* balls are sun dried for 3-5 days and sold in the market (Figure 4).

Rice is the common substrate used for the preparation of *marcha* in all the three countries i.e., in Nepal, India and Bhutan however, *marcha* in India and Bhutan are prepared by using *Plumbago zeylanica, Buddleja asiatica, Vernonia cinerea,* ginger and dry red chilies and the fern *Glaphylopteriolopsis erubeseens* is used to store the product for incubation whereas, *marcha* in Nepal is prepared using *chabo* and *chittu* plant and straw mats are used instead of ferns for storing the product during incubation period.

Figure 4: Traditional method of preparation of *marcha* in Darjeeling hills, Sikkim in India and South Bhutan.

PAA

Paa (Figure 2E) is a round; creamy white, dry starter balls prepared by *Nyshing* community of Arunachal Pradesh.

Traditional method of preparation of *paa* of Arunachal Pradesh

The initial substrate used for the preparation of *paa* is rice which is soaked in water for 4-5 h after which is powdered. Leaves of *Ctuepatti* (*Cissampelos pareira* Linn.) and *Khanoba* (*Clerodenderon viscosum* Vent.) is boiled in water and ground to paste. The grounded rice and herbs are then mixed with 50-60 gram of old starter culture powder and water to make a thick paste. Small balls of *paa* is prepared from this paste which is then placed on a bamboo basket kept over a fire place and allowed to dry for 8-16 days (Figure 5). The dried cakes are then used to prepare fermented alcoholic drinks. The shelf life of *paa* is about 1 year.

PAA

Rice is soaked in water for about 4-6 hours and ground into paste.

↓

About 20 gram leaves of *Ctuepatti* and *Khanoba* is boiled in water and ground to paste.

↓

The rice and leaves are mixed in a vessel and 50-60 grams of old starter culture powder is added to make a thick paste

↓

This paste is kneaded into small cakes.

↓

These cakes are then allowed to drying for 8-16 days by keeping them on a bamboo basket over the fire.

↓

The dried cakes are stored in a cool place for further use.

↓

The storability of this amylolytic starter culture is about 1 year.

↓

PAA

Figure 5: Traditional method of preparation of *paa* in Arunachal Pradesh.

PEE

Pee (Figure 2F) is a dry starter for fermented alcoholic beverage prepared by the *Apatani* community of Arunachal Pradesh.

Traditional method of preparation of *pee* of Arunachal Pradesh

During the preparation of pee, rice is first soaked in water for 2 h after which the excess water is drained and the rice is dried. The rice is then crushed in heavy wooden mortar and pestle (locally called *Yaper* and *Unyi*) into fine powdered form. Water is added to this powdered rice flour to make a thick paste. Small round cakes of different shape and size is kneaded from this paste which is sprinkle with old starter culture. The freshly prepared *pee* balls are then placed on bamboo strips over the fire place for about 1-2 days to dry. After the incubation, these *pee* balls are sun dried for 3-5 days and are stored in dry place for further use (Figure 6). The shelf life of *pee* is about 1 year. *Pee* differs from other dry starters of Arunachal Pradesh as no plants or herbs are used for its preparation.

PEE

Rice

↓

Soaked in water

↓

Crushed in wooden motar and pestel

↓

Rice flour is mixed with water to form a thick paste

↓

Small balls of *pee* is prepared from the above paste

↓

Freshly prepared *pee* balls is then sprinkled with powdered old starter culture

↓

These fresh *pee* balls are kept on bamboo strips over a fire place for about 1-2 days to dry

↓

Sun dried for 3-5 days

↓

PEE

Figure 6: Traditional method of preparation of *pee* in Arunachal Pradesh.

PHUT

The *Mongpa* community of Arunachal Pradesh prepares the dry starter *phut* for fermented alcoholic beverage (Figure 2G).

Traditional method of preparation of *phut* of Arunachal Pradesh

Rice is soaked in water overnight and is powdered with the help of motor pestle. Old *phut* balls along with leaves of *Cinnamomum glanduliferum* is crushed and added to powdered rice and mixed properly. Water is added to the mixture to form dough from which *phut* balls are prepared. The freshly prepared *phut* balls are then wrapped in straw and fermented for about 1-2 days. After the fermentation the *phut* balls are then sun dried for 3-7 days and used (Figure 7).

PHUT

Rice is soaked in water overnight

↓

Rice is powdered with the help of wooden motar pestel.

↓

Old starter culture and leaves of *Cinnamomum glanduliferum* is crushed and added to powdered rice

↓

Dough is made by adding water to the above mixture and *Phut* cakes are made

↓

Phut cakes are wrapped in straw and fermented for 1-2 days

↓

sun dried for 3-7 days

↓

Phut

Figure 7: Traditional method of preparation of *phut* in Arunachal Pradesh.

PHAB

Phab (Figure 2H) also known as *pho* is a dry starters prepared in Bhutan by the *Drukpa* community. It is a flat dark brown coloured starter prepared using maize and rice husk. *Phab* is used to prepare the famous fermented alcoholic beverage *Ara* of Bhutan.

Traditional method of preparation of *phab* of Bhutan

For the preparation of *phab* powdered maize, rice husk and dried flowers of *bhagam* plant (powdered) is taken in a ratio of 2:4:3, respectively. Water is added to the above mixture and cooked for 5-10 min. After the cooking a thick paste is formed which is allowed to cool down to room temperature after which inoculum (powdered old *phab*) is added to the paste and mixed. Fresh *phab* balls are prepared from this paste which is then place on plastic sheets and covered with the same. The covered *phab* balls are then kept on a bamboo basket in a dark room for about 3 days. After the fermentation, *phab* balls are sun dried for about 5 days (Figure 8) and are used to ferment alcoholic beverages. The shelf life of *phab* is for a year or more.

Phab of Bhutan

Powdered maize, rice husk and powdered bhagam flower is mixed

↓

Water is added to the above mixture and boiled for about 5-10 minutes.

↓

A thick paste is then formed which is allowed to cool down to room temperature.

↓

Previously prepared *pho*/*phab* is then powdered and added to the above paste as a starter culture

↓

Small balls of *pho* is then made from the above mixture and placed on top of a plastic sheet and covered on top with a plastic sheet

↓

It is then kept in a bamboo basket (*chachu*) in a dark room for 3 days

↓

After the incubation the *pho* balls are sun dried for 5 days and then used for preparation of ara.

↓

PHAB

Figure 8: Traditional method of preparation of *phab* in Bhutan.

COLLECTION OF SAMPLES

A total of 35 different traditionally prepared dry starters were collected from various regions of the Eastern Himalayan regions of Nepal, India and Bhutan (Figure 1) which include 8 samples of *marcha* from Nepal, 5 samples of *marcha* from Darjeeling hills, 8 samples of *marcha* from Sikkim, 5 samples of *marcha* from Bhutan, 2 samples of *paa*, 3 samples of *pee* and 2 samples of *phut* from Arunachal Pradesh and 2 samples of *phab* from Bhutan, respectively (Table 2).

Table 2: Moisture, pH and bacterial load in dry starters of the Eastern Himalayas							
Sample	**Country**	**Sample Collection Site**	**Total no. of sample**	**Total isolates**	**Moisture content (%)**	**pH**	**cfu/g (x 10^7)**
Marcha	Nepal	Dharan	2	7	12.5 (9.6-17.0)	5.6 (5.5-5.9)	2.1 (1.1-2.9)
		Dhankuta	2	9			
		Hiley	2	11			
		Hattikharka	2	7			
Marcha	India (Darjeeling hills)	Darjeeling	3	21	13.1 (12.9-13.3)	5.4 (5.2-5.6)	15.3 (11.0-19.6)
		Kalimpong	2	17			
Marcha	India (Sikkim)	Pakyong	2	11	11.8 (10.0- 13.4)	5.7 (5.6-5.9)	18.5 (10.2-26.5)
		Gangtok	2	14			
		Recab	2	11			
		Basilakha	2	13			
Marcha	Bhutan	Gedumari	3	10	13.76 (11.8-15.7)	5.7 (5.5-5.9)	0.01 (0.01-0.02)
		Thimpu	2	8			
Paa	India (Arunachal Pradesh)	Lower Subansiri	2	12	11.7 (11-12)	5.1 (5-5.2)	2.3 (2.0-2.6)
Pee		Ziro valley	3	17	12.1 (11-13)	5.5 (5.2-5.8)	17.6 (16.8-18.4)
Phut		Upper Subansiri	2	11	11.6 (11.4-11.8)	5.2 (5.1-5.3)	11.5 (9.8-13.2)
Phab	Bhutan	Dhonakha	2	22	6.17 (6.13-6.2)	5.2 (5.0-5.4)	0.03 (0.02-0.04)
Total			35	201			

MOISTURE, pH AND BACTERIAL LOAD OF STARTER SAMPLES

The moisture content, pH and the microbial load of the entire dry starter samples were analyzed (Table 2). The moisture contents of all dry starter samples ranged between 6%-15% (Table 2); individually moisture contents (%) in sample-wise, *marcha* of Nepal (9.6-17.0), *marcha* of Darjeeling (12.9-13.3), *marcha* of Sikkim (10.0- 13.4), *marcha* of Bhutan (11.8-15.7), *paa* of Arunachal Pradesh (11-12), *pee* of Arunachal Pradesh (11-13), *phut* of Arunachal Pradesh (11.4-11.8) and *phab* of Bhutan (6.13-6.2) (Table 2). The moisture content of *marcha* of Bhutan was recorded to be the highest amongst the starter samples collected with an average moisture content of 13.76%. Average moisture content of *phab* samples of Bhutan was found to be lowest with the moisture content of 6.17%.

The pH of all dry starter samples was acidic and ranged within 5.0-5.9 (Table 2). The pH range of *marcha* samples of Nepal was 5.5-5.9, *marcha* of Darjeeling was 5.2-5.6, *marcha* of Sikkim was 5.6-5.9, *marcha* of Bhutan was 5.5-5.9, *paa* of Arunachal Pradesh was 5-5.2, *pee* of Arunachal Pradesh was 5.2-5.8, *phut* of Arunachal Pradesh was 5.1-5.3 and *phab* of Bhutan was 5-5.4 (Table 2). The average pH of *marcha* samples of Nepal, Darjeeling, Sikkim and Bhutan was 5.6, 5.4, 5.7 and 5.7 ; the pH of *paa, pee* and *phut* samples of Arunachal Pradesh was recorded as 5.1, 5.5 and 5.2 and pH of *phab* of Bhutan was 5.2 respectively (Table 2).

The microbial load of the traditionally prepared dry starter samples was analyzed using plate count technique in specific media such as VRBGA (violet red bile glucose agar), MRS agar (De Man, Rogosa and Sharpe agar), M17 agar and nutrient agar (Table 2). The bacterial count of all the 35 traditionally prepared dry starter samples ranged from $1.0x10^5$cfu/g to $2.7x10^8$cfu/g. The range of microbial load in *marcha* samples of Nepal, Darjeeling, Sikkim and Bhutan were $1.1\text{-}2.9x10^7$ cfu/g, $11\text{-}19.6x10^7$cfu/g, 10.2-

26.5x10^7 cfu/g and 0.01-0.02x10^7 cfu/g, respectively; 2-2.6x10^7 cfu/g, 16.4-18.4 x10^7 cfu/g, and 9.8-13.2 x10^7 cfu/g in *paa, pee* and *phut* samples of Arunachal Pradesh and 0.02-0.04 x10^7 cfu/g in *phab* samples of Bhutan, respectively (Table 2). The average microbial load in *marcha* samples of Nepal, Darjeeling, Sikkim and Bhutan was 2.1 x10^7 cfu/g, 15.3 x10^7 cfu/g, 18.5 x10^7 cfu/g and 0.01 x10^7 cfu/g, respectively; in *paa, pee* and *phut* samples of Arunachal Pradesh the microbial load was recorded as 2.3 x10^7 cfu/g, 17.6 x10^7 cfu/g and 11.5 x10^7 cfu/g, respectively and microbial load of *phab* of Bhutan was 0.03 x10^7 cfu/g (Table 2).The highest microbial load was recorded in *marcha* samples of Sikkim with an average bacterial load of 1.9 x10^8 cfu/g, whereas the lowest bacterial count was recorded in *marcha* samples of Bhutan with the average bacterial load of 1.0x10^5cfu/g (Table 2).

PHENOTYPIC CHARACTERIZATION

In this study we used specific media such as MRS agar and M17 for lactic acid bacteria, VRBGA (violet red bile glucose agar), for Gram-negative bacteria, and nutrient agar for aerobic mesophilic bacteria. We isolated 201 bacterial isolates from 35 different dry starter samples of the Eastern Himalayas: 34 isolates from *marcha* of Nepal, 38 isolates from *marcha* of Darjeeling hills, 49 isolates from *marcha* of Sikkim, 18 isolates from *marcha* of Bhutan, 12 isolates from *paa*, 17 isolates from *pee,* 11 isolates from *phut* all from Arunachal Pradesh, and 22 isolates from *phab* of Bhutan, respectively (Table 2).

Morphological Characterization

All bacterial isolates were morphologically characterized based on cultural characteristics, cell morphology, Gram's reaction, KOH (potassium hydroxide test) test, catalase test and endospore test (Table 3). Out of 201 isolates, 183 bacterial isolates were Gram-positive and 18 isolates were Gram-negative which was further confirmed by KOH test. A total of 26 isolates showed a positive result for catalase test and 175 isolates were catalase negative (Table 3). According to the endospore test results only 6 of the isolates were found to be spore formers and the rest of 195 isolates were non spore formers (Table 3).

Table 3: Cultural and morphological characteristics of bacteria isolated from traditional dry starters of the Eastern Himalayas

Sample	Isolates	Media	Cultural characteristics	Gram Stain	Shape	KOH test	Catalase	Endospore
Marcha (Nepal)	NMB3	MRS	Small, round shaped, regular edged, bulged, creamy white colonies.	+	Rod	NS	-	-
	NMB7	NA	Small, irregular, flat, transparent colonies.	+	Rod	NS	-	-
	NMB8	MRS	Small, round, regular edge, transparent colonies.	+	Rod	NS	-	-
	NMB10	NA	Large, irregular, flat, thick, white colonies.	+	Rod	NS	+	+
	NMB23	NA	Small, round shaped, regular edged, transparent colonies.	+	Cocci	NS	+	-
	NMB11	NA	Large, round, regular, bulged, white colonies.	+	Rod	NS	+	+
	NMB12	NA	Large, spread growth, thick, yellowish white coloured colonies.	+	Rod	NS	+	-
	NMB13	VRBGA	Large, pink coloured colonies with spread growth, thick and slimy.	+	Rod	NS	+	-
	NMB20	NA	Small, round, regular, bulged, transparent colonies.	+	Cocci	NS	+	-
	NMB22	NA	Large, round shaped, regular edge, bulged surface, creamy white colonies.	+	Cocci	NS	+	-
Marcha (Darjeeling)	DMB4	MRS	Thick, irregular edge, bulged surface, white colonies.	+	Cocci	NS	-	-
	DMB11	M17	Large, flat, irregular, thick, creamy white colonies.	+	Cocci	NS	-	-
	DMB12	MRS	Small, round, transparent, white colonies.	+	Cocci	NS	-	-
	DMB6	MRS	Thick, irregular edge, bulged surface, white colonies.	+	Cocci	NS	-	-
	DMB3	M17	Thick, irregular edge, bulged surface white colonies.	+	Cocci	NS	-	-
	DMB11	MRS	White, small colonies with round edge and bulged surface.	+	Cocci	NS	-	-
	DMB13	MRS	Large, flat, irregular, thick, creamy white colonies.	+	Cocci	NS	-	-
	DMB14	MRS	Very small colonies, white, glistening surface and round edged.	+	Cocci	NS	-	-
	DMB 15	MRS	Small round transparent white colonies.	+	Cocci	NS	-	-
	DMB5	NA	Slightly brownish, transparent very small bulged colonies.	+	Cocci	NS	-	-
	DMB2	MRS	White, round, medium sized bulged colonies.	+	Cocci	NS	-	-

	DMB1	M17	Bulged, milky white, thick, regular edged medium sized colonies.	+	Cocci	NS	-	-
Marcha (Sikkim)	SMB13-7	MRS	Medium sized bulged, milky white, thick, regular edged colonies.	+	Cocci	NS	-	-
	SMB15	MRS	Large round colonies with glistening surface, thick consistency and bulged.	+	Cocci	NS	-	-
	SMB19	NA	Creamy white, thick, bulged, irregular edged colonies.	+	Rod	NS	+	+
	SMB21	M17	Small, round, transparent white, bulged colonies	+	Cocci	NS	-	-
	SMB5	MRS	White, shinny, bulged, round with regular edge colonies.	+	Cocci	NS	-	-
	SMB7	VRBGA	Small, round, regular, bulged, pink colonies.	+	Cocci	NS	-	-
	SMB9	VRBGA	Large colonies, bulged, spreaded growth, red in colour.	+	Rod	NS	-	-
	SMB13-1	MRS	Very small colonies, white, glistening surface and round edged.	+	Cocci	NS	-	-
	SMB22	NA	Medium sized, yellowish white colonies bulged and round shaped colonies.	+	Cocci	NS	+	-
	SMB1	NA	Large, round, regular, bulged, creamy white colonies.	+	Rod	NS	+	+
	SMB8	NA	large, irregular edge, spreaded growth, bulged creamy white colonies.	+	Rod	NS	+	-
	SMB3	MRS	Small, irregular, flat, shinny white colonies.	+	Cocci	NS	-	-
	SMB14	NA	Thick, creamy coloured, large colonies with spreaded growth.	+	Rod	NS	+	+
	SMB11	MRS	Thick, irregular edge, bulged surface, white colonies.	+	Cocci	NS	-	-
Marcha (Bhutan)	BPB1	NA	Small, brownish colonies, shiny and round.	+	Cocci	NS	+	-
	BPB10	NA	Small, brownish colonies, shiny and round.	+	Cocci	NS	+	-
	BPB13	MRS	White, thick, bulged colonies, round in shape.	+	Cocci	NS	-	-
	BPB17	NA	Yellowish, round, small bulged colonies.	+	Cocci	NS	+	-
	BPB3	NA	Yellowish, round, small bulged colonies.	+	Cocci	NS	+	-
	BPB4	M17	Small, regular, bulged, thick, white colonies.	+	Cocci	NS	-	-
	BPB7	NA	Creamy white coloured, thick colonies with filamentous growth.	+	Rod	NS	+	-
	BPB18	MRS	White, round, medium sized, bulged colonies.	+	Cocci	NS	-	-
	BPB11	MRS	White, round, medium sized bulged colonies.	+	Cocci	NS	-	-

	BPB8	NA	Thick, creamy, large colonies with spreaded growth.	+	Rod	NS	+	+
Paa (Arunachal Pradesh)	AOB5	M17	Small, round, regular, bulged, transparent colonies.	+	Cocci	NS	-	-
	AOB9	VRBGA	Yellowish pink, thick, spreaded growth and large colonies.	-	Rod	S	+	-
	AOB2	MRS	Small, round, regular, bulged, transparent colonies.	+	Cocci	NS	-	-
	AOB11	M17	White, shinny, bulged, round with regular edged colonies.	+	Cocci	NS	-	-
	AOB4	MRS	Small, white, round colonies with regular edge.	+	Cocci	NS	-	-
Pee (Arunachal Pradesh)	AOB19	MRS	Small, regular, bulged, thick, white colonies.	+	Cocci	NS	+	-
	AOB20	NA	Creamy white coloured, thick colonies with filamentous growth.	+	Rod	NS	+	-
	AOB14	M17	White, round, medium sized, bulged colonies.	+	Cocci	NS	-	-
	AOB15	MRS	Small, white, round colonies with regular edge and bulged.	+	Cocci	NS	-	-
	AOB18	VRBGA	Very thick, slimy, yellowish pink, large colonies.	-	Rod	S	-	-
	MBV14	MRS	Small, white, round shaped, bulged, regular edged colonies .	+	Cocci	NS	-	-
Phut (Arunachal Pradesh)	AOB24	MRS	Milky white, thick, regular edged, medium sized colonies.	+	Cocci	NS	-	-
	AOB26	MRS	Small, round shaped, regular edged, white colonies.	+	Cocci	NS	-	-
	AOB48	NA	Transparent white, large colonies with irregular edge.	-	Rod	S	+	-
	AOB25	M17	Medium size, regular edge, bulged, creamy white colonies.	+	Cocci	NS	-	-
Phab (Bhutan)	BPB21	MRS	Small, round, transparent white colonies.	+	Cocci	NS	-	-
	BPB26	NA	Small, round shaped, transparent white colonies.	-	Rod	S	+	-
	BPB24	NA	Thick, irregular edge, bulged surface, white colonies.	+	Rod	NS	+	-
	BPB23	VRBGA	Small, pink coloured, round colonies with regular edge.	-	Rod	S	+	-
	BPB27	VRBGA	Small, pink coloured, round colonies with regular edge.	-	Rod	S	+	-
	BPB31	M17	Very small colonies, white, glistening surface and round edged.	+	Cocci	NS	-	-
	BPB33	MRS	White, round, small, bulged colonies.	+	Cocci	NS	-	-

+, positive; -, negative; S, formation of string (Gram-negative); NS, string was not formed (Gram-positive); MRS, De Man, Rogosa and Sharpe agar; VRBGA, violet red bile glucose agar; NA, nutrient agar; M17 agar, growth medium for *Lactococcus* species.

Biochemical and Physiological Characterization

The bacterial isolates were divided into two groups: Gram-positive isolates and Gram-negative isolates based on the morphological characterizations. Sugar fermentation, biochemical and physiological tests were performed separately for both the group of isolates (Table 4-9). Out of 201 isolates 183 bacterial isolates were Gram-positive and 18 isolates were Gram-negative. Based on phenotypic characterisations, 8 bacterial genera were tentatively identified which were 6 genera of Gram-positive bacteria: *Pediococcus, Enterococcus, Leuconostoc, Bacillus, Lactobacillus* and *Staphylococcus* and 2 genera of Gram-negative bacteria: *Enterobacter* and *Citrobacter* following the taxonomical keys of Bergey's manual of bacteriological classification (Holt et al. 1994).

Table 4: Sugar fermentation and growth conditions of bacterial isolates from *marcha* samples of the Eastern Himalayas

Representative Isolates	Sugar fermentation															Growth in/at								Tentative identity
																NaCl		pH			Temperature (°C)			
	Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	5%	10%	3.6	9.6	10.6	15°C	10°C	45°C	
NMB3	+(2)	-(2)	+(2)	-(2)	-(2)	-(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	*Lactobacillus*
NMB8	+(3)	-(1) +(2)	+(3)	-(1) +(2)	-(1) +(2)	-(1) +(2)	+(3)	+(3)	-(3)	+(3)	+(3)	+(3)	+(3)	+(2) -(1)	+(3)	+(3)	+(2) -(1)	+(2) -(1)	v(2) -(1)	-(3)	+(3)	+(2) -(1)	-(1) v(2)	*Lactobacillus*
NMB7	+(5)	-(2) +(3)	+(5)	-(2) +(3)	-(2) +(3)	-(2) +(3)	+(5)	+(5)	-(3) +(2)	+(5)	+(5)	+(5)	+(5)	+(2) -(3)	+(5)	+(5)	+(2) -(3)	-(2) +(3)	v(2) -(3)	-(5)	+(5)	+(2) -(3)	-(2) v(3)	*Lactobacillus*
NMB 12	-(3)	+(3)	-(3)	+(3)	-(3)	+(3)	-(3)	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	v(1) +(2)	-(3)	-(3)	+(3)	+(3)	+(3)	+(3)	-(3)	*Pediococcus*
NMB 13	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	v(1) +(1)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	*Pediococcus*
NMB 10	+(3) -(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(2) +(2)	-(2) +(2)	+(4)	+(3)-(1)	+(1) -(3)	-(3) +(1)	+(3) -(1)	-(2) +(2)	v(1) +(1) -(2)	-(3) v(1)	-(4)	+(4)	+(2) -(2)	+(1) -(3)	+(3) v(1)	-(4)	+(4)	+(3) v(1)	+(4)	*Unidentified*
NMB 11	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	-(2)	+(2)	-(2)	v(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	*Lactobacillus*

NMB 23	-(3) +(1)	-(3) +(1)	-(4)	-(4)	-(4)	-(4)	+(1) -(3)	-(4)	+(1) -(3)	-(4)	+(4)	-(4)	+(1) -(3)	v(1) -(3)	-(4)	+(4)	-(4)	-(4)	+(4)	-(4)	-(3) +(1)	- (4)	+(4)	*Enterococcus*
NMB 20	-(2) +(2)	-(2) +(2)	-(4)	-(3) +(1)	-(4)	-(3) +(1)	-(1) +(3)	-(3) v(1)	-(1) +(3)	-(3) +(1)	+(4)	-(4)	-(1) +(3)	-(3) v(1)	-(4)	+(4)	-(4)	-(4)	+(4)	-(4)	-(3) +(1)	-(3) +(1)	+(3) -(1)	*Pediococcus*
NMB 22	-(3) +(2)	-(3) +(2)	-(4) +(1)	-(3) +(2)	-(4) +(1)	-(3) +(2)	-(3) +(2)	-(4) v(1)	-(3) +(2)	-(4) +(1)	+(5)	-(4) v(1)	-(3) +(2)	-(4) v(1)	-(5)	+(5)	-(4) +(1)	-(5)	+(5)	-(5)	-(3) +(2)	-(4) +(1)	+(3) -(2)	*Leuconostoc*
AKB6	-(3)	-(3)	-(3)	+(1) -(2)	-(3)	-(3)	-(2) +(1)	-(2) +(1)	-(3)	-(2) -(1)	+(3)	+(2) -(1)	+(2) -(1)	-(2) +(1)	-(3)	v(3)	v(1) +(2)	+(3)	v(1) -(2)	-(3)	+(1) -(2)	+(3)	-(3)	*Pediococcus*
DMB4	+(3)	+(2) -(1)	+(1) -(2)	+(2) -(1)	+(3)	-(2) +(1)	+(2) v(1)	+(3)	+(3)	-(3)	+(3)	+(3)	-(3)	+(1) -(2)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	+(2) -(1)	*Leuconostoc*
AKB3	+(3)	-(3)	-(3)	v(1) +(2)	-(3)	v(1) +(2)	v(3)	+(3)	-(3)	+(3)	+(3)	-(3)	-(3)	+(3)	+(3)	+(3)	-(3)	-(3)	-(3)	-(3)	v(1)- (2)	v(1) -(2)	+(3)	*Pediococcus*
DMB 12	+(3)	-(3)	-(3)	v(2) +(1)	-(3)	v(2) +(1)	v(1) +(2)	+(3)	-(3)	+(3)	+(3)	-(2) +(1)	-(3)	+(3)	-(2) +(1)	v(1) +(2)	-(3)	v(1) -(2)	-(3)	-(3)	-(3)	- (3)	+(3)	*Pediococcus*
DMB 14	+(2)	-(2)	-(2)	v(2)	-(2)	+(2)	v(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	v(2)	-(2)	-(2)	v(2)	v(2)	+(2)	*Pediococcus*
DMB 11	+(5)	-(5)	-(5)	v(3) +(2)	-(5)	v(2) +(3)	v(3) +(2)	+(5)	-(5)	+(5)	+(5)	-(3) +(2)	-(5)	+(5)	+(3) -(2)	v(2) +(3)	-(5)	v(2) -(3)	-(2) +(3)	-(5)	v(3) -(2)	v(2) -(3)	v(2) +(3)	*Pediococcus*
DMB 11	-(1) +(3)	+(3) -(1)	-(3) +(1)	-(3) +(1)	+(4)	+(2) -(2)	+(4)	+(4)	+(4)	-(4)	+(4)	+(4)	+(1) -(2)	-(3) +(1)	+(4)	+(4)	-(4)	-(4)	+(3) – (1)	-(4)	-(3) +(1)	+4	+(3) -(1)	*Enterococcus*
DMB6	-(2) +(2)	+(3) -(1)	-(2) +(2)	-(3) +(1)	+(4)	+(3) -(1)	+(4)	+(4)	+(3) v(1)	-(4)	+(4)	+(4)	-(2) +(2)	-(2) +(2)	-(2) +(2)	+(3) v(1)	-(3) v(1)	-(4)	+(2) -(2)	-(4)	-(2) +(2)	+(3) v(1)	+(4)	*Enterococcus*

DMB3	-(3)	+(3)	+(2) -(1)	-(2) +(1)	+(3)	+(1) -(2)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	-(3)	+(2) -(1)	-(3)	+(3)	-(3)	-(3)	+(2) -(1)	-(3)	-(3)	+(3)	+(3)	*Pediococcus*
DMB 13	-(2) +(1)	+(3)	+(2) -(1)	+(3)	+(3)	-(3)	+(3)	+(3)	+(2) v(1)	+(3)	+(3)	+(3)	-(3)	+(2) -(1)	-(3)	+(2) v(1)	-(2) v(1)	-(3)	+(3)	-(3)	-(3)	+(2) v(1)	+(3)	*Pediococcus*
DMB 15	-(3) +(1)	+(3) -(1)	+(3) -(1)	-(3) +(1)	+(4)	+(1) -(3)	+(3) v(1)	+(4)	+(4)	-(2) +(2)	+(4)	+(4)	+(1) -(3)	+(3) -(1)	+(4)	+(4)	-(4)	-(4)	+(4)	-(4)	-(3) +(1)	+(4)	+(2) -(2)	*Enterococcus*
DMB5	-(1) +(1)	-(1) +(1)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	- (2)	+(2)	unidentified
SMB 14	+(3) -(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(2) +(2)	+(3) -(1)	+(3) -(1)	+(3) -(1)	+(3) -(1)	+(2) -(2)	+(3) -(1)	-(3) v(1)	-(4)	+(4)	-(3) +(1)	-(3) +(1)	+(2) v(2)	-(4)	+(4)	+(4)	+(4)	*Bacillus*
SMB1	+(2) -(1)	-(2) +(1)	-(2) +(1)	-(2) +(1)	-(2) +(1)	-(3)	-(1) +(2)	+(2) -(1)	+(2) -(1)	+(2) -(1)	+(2) -(1)	+(2) -(1)	+(2) -(1)	-(3)	-(3)	+(3)	-(2) +(1)	-3	+(2) v(1)	-(3)	+(3)	+(2) v(1)	+(3)	*Bacillus*
SMB8	+(2) -(2)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(3) +(1)	-(1) +(3)	+(2)- (2)	-(3) +(1)	+(3) -(1)	+(3) -(1)	+(2) -(2)	+(2) -(2)	-(4)	-(4)	+(4)	-(3) +(1)	-(3) +(1)	+(3) v(1)	-(4)	+(4)	+(2) v(2)	+(4)	*Bacillus*
SMB 11	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Leuconostoc*
SMB3	-(2) +(2)	+(2) -(2)	+(4)	-(3) +(1)	+(4)	+(1) -(3)	+(4)	+(4)	+(3) v(1)	+(1) -(3)	+(4)	+(4)	-(4)	+(4)	+(2) -(2)	+(3) v(1)	-(3) v(1)	-(4)	+(4)	-(4)	-(4)	+(3) v(1)	+(2) -(2)	*Enterococcus*
SMB 15	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Enterococcus*
SMB 21	-(2) +(1)	+(2) -(1)	+(2) -(1)	-(3)	+(3)	-(2) +(1)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	-(2) +(1)	+(2) -(1)	-(2)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	+(3)	*Staphylococcus*

SMB5	-(2) +(1)	+(3)	+(2) -(1)	-(2) +(1)	+(3)	-(2) +(1)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	-(3)	+(2) -(1)	-(2)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	+(3)	*Pediococcus*
SMB7	-(2) +(1)	+(1) -(2)	-(2) +(1)	-(2) +(1)	+(3)	+(2) -(1)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	-(2) +(1)	-(2) +(1)	-(3)	+(3)	-(3)	-(3)	+(2) -(1)	-(3)	-(2) +(1)	+(3)	+(3)	*Leuconostoc*
SMB 13-1	+(3)	-(3)	-(3)	+(3)	-(3)	v(2) +(1)	v(2) +(1)	+(3)	-(3)	+(3)	+(3)	-(3)	-(3)	+(3)	-(3)	v(1) +(2)	-(3)	v(2) -(1)	-(2) +(1)	-(3)	v(1) -(2)	v(1) - (2)	v(1) +(2)	*Staphylococcus*
SMB9	+(3)	-(3)	-(3)	+(3)	-(3)	+(3)	-(3)	+(3)	+(3)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	+(3)	-(3)	-(3)	-(3)	-(3)	+(3)	+(3)	+(3)	*Unidentified*
SMB 22	-(3)	+(3)	+(3)	+(3)	-(3)	-(3)	-(3)	-(3)	+(3)	+(3)	+(3)	-(3)	-(3)	+(3)	-(3)	+(3)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	+(3)	*Enterococcus*
SMB 19	+(4)	-(4)	-(4)	-(4)	-(4)	+(4)	+(4)	+(4)	-(4)	-(4)	+(4)	-(4)	+(4)	-(4)	+(4)	+(4)	-(4)	-(4)	+(4)	-(4)	+(4)	+(4)	+(4)	*Bacillus*
SMB 13	-(4)	-(2) v(2)	-(4)	+(2) -(2)	-(4)	-(4)	-(3) +(1)	-(3) +(1)	-(4)	-(2) +(2)	+(4)	+(3) -(1)	+(3) -(1)	-(3) +(1)	-(4)	v(4)	v(2) +(2)	+(4)	v(1) -(3)	-(4)	+(1) -(3)	+(4)	+(4)	*Staphylococcus*
BPB18	+(2) - (1)	+(2) -(1)	+(1) -(2)	-(3)	+(3)	+(2) -(1)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	-(3)	+(1) -(2)	+(3)	+(3)	-(3)	-(3)	+(2) - (1)	-(3)	-(3)	+(3)	+(3)	*Enterococcus*
BPB 13	+(3)	-(3)	-(3)	v(2) +(1)	-(3)	v(2) +(1)	v(3)	+(3)	-(3)	+(3)	+(3)	-(3)	-(3)	+(3)	+(2) -(1)	+(3)	-(3)	v(1) -(2)	-(3)	-(3)	v(2) -(1)	v(2) - (1)	v(2) +(1)	*Unidentified*
BPB4	-(2) +(1)	+(2) -(1)	+(2) -(1)	-(3)	+(3)	+(1) -(2)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(1) -(2)	+(2) -(1)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	-3	+(3)	+(3)	*Pediococcus*
BPB7	+(2)	-(2)	-(2)	-(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	*Bacillus*

BPB8	+(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-2	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	*Bacillus*
BPB1	-(1) +(2)	-(1) +(2)	-(2) +(1)	-(2) +(1)	-(2) +(1)	-(2) +(1)	-(1) +(2)	-(3)	-(1) +(2)	-(3)	+(3)	-(2) v(1)	-(1) +(2)	-(1) v(2)	-(3)	+(3)	-(1) +(2)	-(3)	+(3)	-(3)	-(2) +(1)	-(3)	+(2) -(1)	*Staphylococcus*
BPB 10	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	-(2)	+(2)	*Pediococcus*
BPB 17	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	*Leuconostoc*
BPB3	-(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	v(2)	-(2)	+(2)	+(2)	-(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	*Pediococcus*
BPB 11	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(1) +(1)	-(2)	-(2)	+(2)	+(2)	*Pediococcus*
-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.																								

Table 5: Sugar fermentation and growth conditions of bacterial isolates from *paa* samples of Arunachal Pradesh

Representative Isolates	Sugar fermentation															Growth in/at								Tentative identity
																NaCl		pH			Temperature (°C)			
	Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	5%	10%	3.6	9.6	10.6	15°C	10°C	45°C	
AOB5	-(3)	-(1) +(2)	+(2) -(1)	-(2) +(1)	+(3)	+(2) -(1)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(1) -(2)	+(2) -(1)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	+(3)	*Pediococcus*
AOB4	+(3) -(1)	-(1) +(3)	+(3) -(1)	-(3) +(1)	+(4)	+(2) -(2)	+(4)	+(4)	+(2) v(2)	-(4)	+(4)	+(4)	+(1) -(3)	+(3) -(1)	+(4)	+(2) v(2)	-(2) v(2)	-(4)	+(3) -(1)	-(3) v(1)	-(2) +(2)	+(2) v(2)	+(3) -(1)	*Enterococcus*
AOB2	-(2) +(2)	+(3) -(1)	+(3) -(1)	-(3) +(1)	+(4)	+(1) -(3)	+(4)	+(4)	+(4)	+(3) -(1)	+(4)	+(4)	-(4)	+(3) -(1)	-(4)	+(4)	-(4)	-(4)	+(4)	-(3) v(1)	-(3) +(1)	+(4)	+(3) -(1)	unidentified
AOB 11	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Pediococcus*

-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.

Table 6: Sugar fermentation and growth conditions of bacterial isolates from *pee* samples of Arunachal Pradesh

Representative Isolates	Sugar fermentation															Growth in/at								Tentative identity
																NaCl		pH			Temperature (°C)			
	Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	5%	10%	3.6	9.6	10.6	15°C	10°C	45°C	
AOB 14	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(1) v(1)	-(2)	+(2)	+(2)	-(2)	-(2)	-(2)	+(1) v(1)	-(1) v(1)	-(2)	+(2)	-(2)	-(2)	+(1) v(1)	+(2)	*Enterococcus*
AOB 19	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	+(v) (3)	-(3)	-(3)	+(3)	+(3)	+(3)	+(3)	-(3)	Unidentified
MBV 14	-(2)	-(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	-(2)	+(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Enterococcus*
AOB 20	+(2) -(1)	-(3)	-(3)	-(3)	+(3)	-(3)	+(3)	+(2) -(1)	+(2) -(1)	-(3)	+(3)	+(2) -(1)	-(2) v(1)	-(3)	-(3)	+(3)	-(2) +(1)	-(2) +(1)	v(2) +(1)	-(3)	+(3)	+(3)	+(3)	*Lactobacillus*
AOB 15	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(1) v(1)	-(2)	+(2)	+(2)	-(2)	-(2)	-(2)	+(1) v(1)	-(1) v(1)	-(2)	+(2)	-(2)	-(2)	+(1) v(1)	+(2)	*Enterococcus*

-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.

Table 7: Sugar fermentation and growth conditions of bacterial isolates from *phut* samples of Arunachal Pradesh

Representative Isolates	Sugar fermentation														Growth in/at								Tentative identity
															NaCl		pH			Temperature (°C)			
	Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	5%	10%	3.6	9.6	10.6	15°C	10°C	45°C	
AOB 25	+(2) -(1)	+(3)	+(2) -(1)	-(2) +(1)	+(3)	-(2) +(1)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	-(3)	-(2) +(1)	+(3)	+(3)	+(3)	+(3)	+(3)	-(3)	+(3)	+(3)	+(3)	*Enterococcus*
AOB 24	-(2) +(1)	+(1) -(2)	-(2) +(1)	-(2) +(1)	+(3)	-(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	-(1) +(2)	-(2) +(1)	-(3)	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	+(3)	*Pediococcus*
AOB 26	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Pediococcus*

-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.

Table 8: Sugar fermentation and growth conditions of bacterial isolates from *phab* samples of Bhutan.

Representative Isolates	Sugar fermentation															Growth in/at								Tentative identity
																NaCl		pH			Temperature (°C)			
	Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	5%	10%	3.6	9.6	10.6	15°C	10°C	45°C	
BPB 24	+(2)	-(2)	-(2)	-(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	*Bacillus*
BPB 31	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Pediococcus*
BPB 33	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Pediococcus*
BPB 21	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	+(2)	+(2)	-(2)	+(2)	+(2)	+(2)	-(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	*Enterococcus*

-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.

Table 9: Sugar fermentation and IMViC test for tentative identification of Gram-negative bacteria from dry starters of the Eastern Himalayas

Representative Isolates	Starter	Sugar fermentation														IMViC test						Tentative identity
		Cellobiose	Raffinose	Sorbitol	Arabinose	Mellibiose	xylose	Lactose	Ribose	Melizitose	Salicin	Glucose	Sucrose	Mannitol	Rhamnose	Aesculin	Indole	MR	VP	Citrate	Urease	Nitrate	
BPB 23	*Phab* (Bhutan)	+(3)	-(3)	-(3)	+(3)	-(3)	+(3)	-(3)	-(1) +(2)	-(3)	-(1) +(2)	+(3)	+(3)	+(3)	+(3)	-(3)	-(3)	v(3)	+(3)	+(3)	+(3)	+(3)	*Enterobacter*
BPB 27	*Phab* (Bhutan)	+(2)	-(2)	-(2)	+(2)	-(2)	+(2)	-(2)	+(2)	-(2)	-(2)	+(2)	+(2)	+(2)	+(2)	-(2)	-(2)	v(2)	+(2)	+(2)	+(2)	+(2)	*Enterobacter*
BPB 26	*Phab* (Bhutan)	+(3)	-(3)	-(1) +(2)	+(3)	-(3)	+(3)	-(3)	-(1) +(2)	-(3)	-(3)	+(3)	+(3)	+(3)	+(3)	-(3)	-(3)	v(3)	+(3)	+(3)	+(3)	+(3)	*Enterobacter*
AOB 18	*Pee* (Arunachal Pradesh)	+(4)	+(4)	+(4)	-(4)	+(4)	+(4)	+(4)	-(4)	-(4)	-(4)	+(4)	+(4)	+(4)	+(4)	+(4)	-(4)	+(4)	+(4)	-(4)	-(4)	+(4)	*Citrobacter*
AOB 48	*Phut* (Arunachal Pradesh)	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	+(3)	-(3)	-(3)	-(3)	-(3)	-(3)	-(3)	-(3)	-(3)	-(3)	-(3)	*Citrobacter*
AOB 9	*Paa* (Arunachal Pradesh)	+(3)	-(3)	-(3)	-(3)	-(3)	-(3)	+(3)	+(3)	-(3)	-(3)	+(3)	-(3)	v(3)	-(3)	+(3)	-(3)	-(3)	-(3)	+(3)	-(3)	v(3)	*Citrobacter*

-, negative; +, positive. Numbers in parenthesis () indicates the total number of representative isolates.

Distribution of bacteria

Distribution of tentatively identified bacteria in starter-wise based on phenotypic and biochemical tests are summarised:

***Marcha* of Nepal**: *Lactobacillus, Pediococcus, Enterococcus, Leuconostoc* and Unidentified.

***Marcha* of India**: *Pediococcus, Leuconostoc, Enterococcus, Bacillus, Staphylococcus* and Unidentified.

***Marcha* of Bhutan**: *Enterococcus, Pediococcus, Bacillus, Staphylococcus, Leuconostoc* and Unidentified.

***Paa* of Arunachal Pradesh**: *Pediococcus, Enterococcus, Citrobacter* and Unidentified.

***Pee* of Arunachal Pradesh**: *Lactobacillus, Enterococcus, Citrobacter* and Unidentified.

***Phut* of Arunachal Pradesh**: *Enterococcus, Citrobacter* and *Pediococcus.*

***Phab* of Bhutan**: *Pediococcus, Enterococcus, Enterobacter* and *Bacillus.*

Sixty-eight representative strains of bacteria were randomly selected from 201 isolates on the basis of cell morphology, Gram stain, growth at different NaCl concentration, pH, temperatures, sugar fermentations and IMViC test for Gram-negative bacteria (Table 10).

Table 10: Representative strains from dry starters of the Eastern Himalayas			
Starter	**Place**	**Isolate Code (n=201)**	**Representative isolates (n=68)**
Marcha	Nepal	NMB3, NMB1, NMB8, NMB2, NMB4, NMB7, NMB 24, NMB 25, NMB33, NMB36, NMB12, NMB16, NMB17, NMB13, NMB21, NMB10, NMB15, NMB37, NMB38, NMB11, NMB40, NMB23, NMB 30, NMB31, NMB41, NMB19, NMB20, NMB32, NMB50, NMB22, NMB27, NMB28, NMB39, NMB46	NMB3, NMB8, NMB7, NMB12, NMB13, NMB10, NMB11,NMB23, NMB20, NMB22,
Marcha	India (Darjeeling hills)	AKB6, DMB28, DMB4, DMB25, DMB30, AKB3, DMB 1, DMB9, DMB10, DMB12, DMB14, DMB33, DMB17, DMB32, DMB36, DMB11, DMB8, DMB5, DMB2, DMB29, DMB38, DMB6, DMB7, DMB24, DMB31, DMB19, DMB35, DMB3, DMB13, DMB23, DMB26, DMB15, DMB18, DMB21, DMB22, DMB34, DMB27, DMB 16	DMB4, AKB6, AKB3, DMB11, DMB12, DMB14, DMB5, DMB6, DMB3, DMB13, DMB15
Marcha	India (Sikkim)	SMB13, SMB17, SMB33, SMB46, SMB57, SMB20, SMB22, SMB39, SMB17, SMB13-1 , SMB33, SMB9, SMB 24, SMB30, SMB44, SMB56, SMB19, SMB15, SMB23, SMB34, SMB43, SMB21, SMB5, SMB6, SMB35, SMB7, SMB31, SMB38, SMB11, SMB18, SMB3, SMB4, SMB25, SMB45, SMB1, SMB2, SMB49, SMB36, SMB 40, SMB 8, SMB52, SMB14, SMB16, SMB32, SMB42, SMB10, SMB12, SMB26, SMB29	SMB13, SMB22, SMB13-1, SMB9, SMB19, SMB15, SMB21, SMB5, SMB7, SMB11, SMB3, SMB1, SMB 8, SMB14

Marcha	Bhutan	BPB 30, BPB 18, BPB 13, BPB22, BPB4, BPB16, BPB7, BPB34, BPB8, BPB1, BPB6, BPB20, BPB 10, BPB17, BPB 19, BPB3, BPB11, BPB29	BPB 18, BPB 13, BPB4, BPB7, BPB8, BPB1, BPB 10, BPB17, BPB3, BPB11
Paa	India (Arunachal Pradesh)	AOB1, AOB4, AOB6, AOB3, AOB5, AOB8, AOB2, AOB29, AOB11, AOB38, AOB9, AOB12	AOB4, AOB5, AOB2,AOB11, AOB9
Pee	India (Arunachal Pradesh)	AOB39, AOB14, AOB23, AOB15, AOB46, AOB40, AOB30, AOB16, AOB19, MBV14, AOB17, AOB20, AOB28, AOB10, AOB18, AOB33, AOB42	AOB14,AOB15, AOB19,MBV14,AOB20, AOB18
Phut	India (Arunachal Pradesh)	AOB 25, AOB32, AOB36, AOB24, AOB 49, AOB51, AOB26, AOB43, AOB 44, AOB45, AOB48	AOB 25, AOB24, AOB26, AOB48
Phab	Bhutan	BPB31, BPB32, BPB 33, BPB44, BPB21, BPB35, BPB24, BPB36, BPB12, BPB23, BPB 25, BPB 27, BPB28, BPB41, BPB9, BPB26, BPB39, BPB2, BPB43, BPB5, BPB38, BPB40,	BPB31, BPB 33, BPB21, BPB24, BPB23, BPB 27, BPB26

GENOTYPIC CHARACTERIZATION

Molecular Identification of Bacterial Isolates by 16S rRNA Gene Sequencing

Genomic DNA of each isolate from 68 representative bacterial strains was extracted and PCR products were prepared and were amplified. The amplified gene was then sequenced using Sanger sequencing method or Chain-termination DNA sequencing method (Sanger et al. 1977; Heather and Chain 2016). The sequences retrieved from 16S rRNA gene sequencing were assigned the nucleotide accession numbers which were deposited in GeneBank-NCBI. The identification of bacterial isolates were done by comparing the sequences obtained with those available in GeneBank NCBI database using BLAST 2.0 program. Phylogenetic tree was constructed using the Neighbour-joining method with 1000 replicates bootstrap values (Figure 9). Genera with species/sub-species isolated from traditionally prepared dry starters of the Eastern Himalayan regions of Nepal, India and Bhutan were identified by 16S rRNA gene sequence based on Basic Local Alignment Search Tool (BLAST) (Table 11-12).

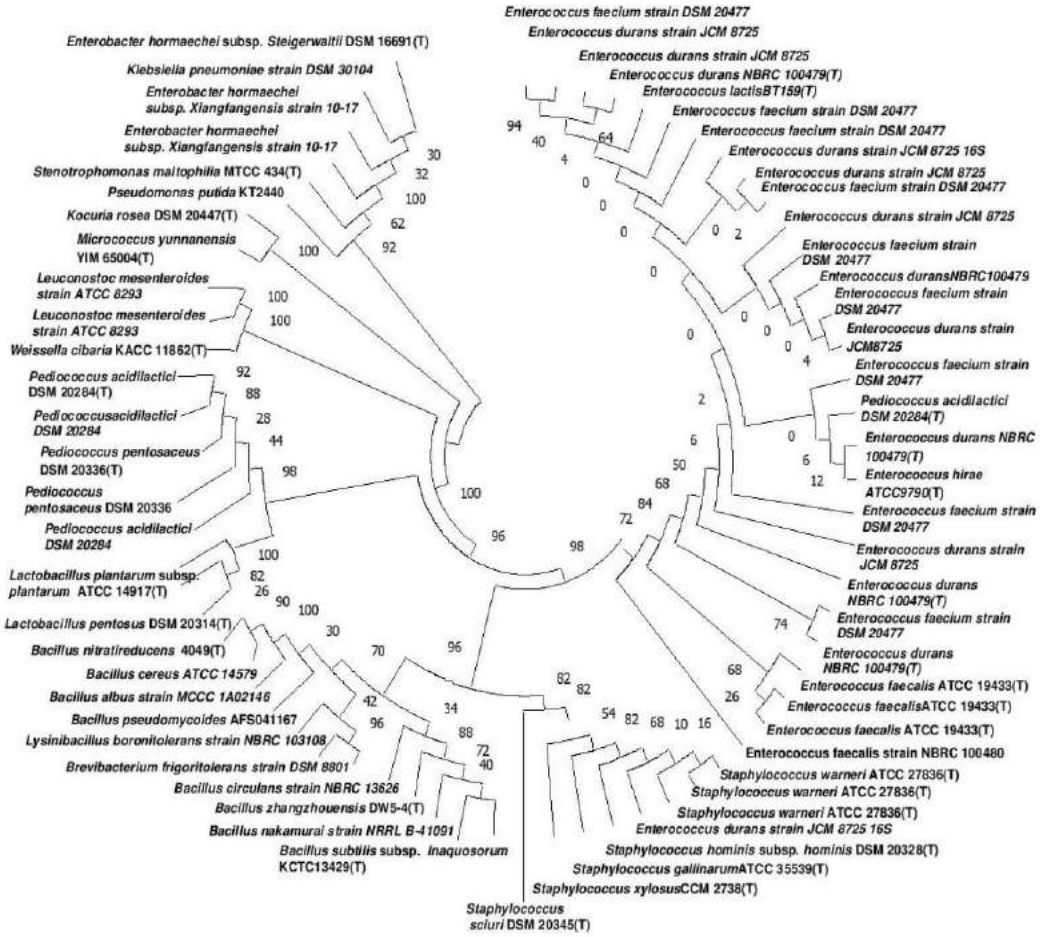


Figure 9: Phylogenetic tree of nucleotide sequences of 68 bacterial isolates from 35 different samples of dry starter of the Eastern Himalayas based on 16S rRNA sequencing. The tree was constructed by the Neighbour-joining method (Gascuel and Steel 2006) with bootstrap values for 1000 replicates shown at the nodes of the tree using MEGA-7 (Kumar et al. 2016). The optimal tree with the sum of branch length = 0.98855936 is shown. The evolutionary distances were computed by the Maximum Composite Likelihood method (Varin et al. 2011) and are in the units of the number of nucleotide substitutions per site. All positions containing gaps and missing data were eliminated. There were 308 total positions in the final dataset.

Table 11: Identification of LAB isolates from dry starters of the Eastern Himalayas based on 16S rRNA genes sequencing					
Starter	**Isolate code**	**Identity**	**Type species (% similarity)**	**GenBank Accession No.**	**Size (base pair)**
Marcha (Darjeeling)	AKB6	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.54%)	MK748250	1315
Marcha (Bhutan)	BPB18	*Enterococcus durans*	*Enterococcus durans* JCM 8725 16S (99.52%)	MK748251	1254
Marcha (Darjeeling)	DMB4	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.55%)	MK748252	1325
Marcha (Sikkim)	SMB13-7	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.55%)	MK748253	1339
Marcha (Darjeeling)	AKB3	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.62%)	MK748254	833
Pee(Arunachal Pradesh)	AOB14	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.62%)	MK748255	1333
Pee (Arunachal Pradesh)	AOB15	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.11%)	MK748256	1430
Phut(Arunachal Pradesh)	AOB25	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.71%)	MK748258	1406
Paa (Arunachal Pradesh)	AOB4	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.38%)	MK748259	1460
Marcha (Bhutan)	BPB11	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.91%)	MK748260	1476
Phab (Bhutan)	BPB31	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.28%)	MK748264	1390
Phab (Bhutan)	BPB33	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.51%)	MK748265	1432
Marcha (Darjeeling)	DMB11	*Enterococcus durans*	*Enterococcus durans* JCM 8725(99.78%)	MK748267	1342

Marcha (Darjeeling)	DMB12	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.59%)	MK748268	1462
Marcha (Darjeeling)	DMB6	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.38%)	MK748269	1443
Pee (Arunachal Pradesh)	MBV14	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.86%)	MK748270	1436
Marcha (Sikkim)	SMB15	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.86%)	MK748274	1447
Marcha (Sikkim)	SMB21	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.64%)	MK748276	1400
Marcha (Sikkim)	SMB5	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.78%)	MK748277	1391
Marcha (Sikkim)	SMB7	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (98.71%)	MK748278	1158
Paa (Arunachal Pradesh)	AOB5	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.86%)	MK202997	1421
Marcha (Bhutan)	BPB13	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.73%)	MK203008	1456
Phab (Bhutan)	BPB21	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.79%)	MK203010	1430
Marcha (Bhutan)	BPB4	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.65%)	MK203013	1437
Marcha (Darjeeling)	DMB3	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203015	1441
Phut (Arunachal Pradesh)	AOB24	*Enterococcus hirae*	*Enterococcus hirae* ATCC 9790(T) (99.86 %.)	MK202998	1411
Marcha (Darjeeling)	DMB13	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.58%)	MK203017	1433
Marcha (Darjeeling)	DMB14	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.52%)	MK203018	1461

Marcha (Darjeeling)	DMB11	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.66%)	MK203019	1456
Marcha (Darjeeling)	DMB15	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203020	1437
Marcha (Nepal)	NMB3	*Lactobacillus pentosus*	*Lactobacillus pentosus* DSM 20314(T) (97.44%)	MK203022	1276
Marcha (Nepal)	NMB8	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) (99.65%)	MK203024	1441
Phut (Arunachal Pradesh)	AOB26	*Enterococcus lactis*	*Enterococcus lactis* BT159 (T) (98.0 %)	MK202999	1398
Marcha (Nepal)	NMB7	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum subsp. plantarum* ATCC 14917(T) (100%)	MK203027	1435
Marcha (Sikkim)	SMB9	*Weissella cibaria*	*Weissella cibaria* KACC 11862(T) (99.66%)	MK203028	1455
Marcha (Sikkim)	SMB13-1	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.79%)	MK203029	1433
Paa (Arunachal Pradesh)	AOB2	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.79%).	MK203002	1420
Paa (Arunachal Pradesh)	AOB11	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.58%).	MK203003	1421
Marcha (Sikkim)	SMB11	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (96.44%)	MK752677	1432
Marcha (Sikkim)	SMB3	*Enterococcus faecalis*	*Enterococcus faecalis* NBRC 100480 (97.42%)	MK752675	1123

ATCC, American Type Cell Culture; JCM, Japan Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen; MCCC, Microbial Culture Collection; NBRC, Biological Resource Centre, NITE; CCM, Czech collection of microorganisms; KACC, Korean Agricultural Culture Collection; YIM, Yunnan Institute of Microbiology; KCTC, Korean Collection for Type Cultures; NRRL, Agricultural Research Service Culture Collection.

Table 12: Identification of non-LAB and Gram-negative bacteria from dry starters of the Eastern Himalayas based on 16S rRNA gene sequencing					
Starter	**Isolate code**	**Identity**	**Type species (% similarity)**	**GenBank Accession No.**	**Size (base pair)**
Phut (Arunachal Pradesh)	AOB 48	*Pseudomonas putida*	*Pseudomonas putida* KT2440 (99.85%)	MK203004	1379
Pee (Arunachal Pradesh)	AOB18	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104 (99.3%)	MK748257	1439
Phab (Bhutan)	BPB 23	*Enterobacter hormaechei* subsp. *xiangfangensis*	*Enterobacter hormaechei subsp. xiangfangensis* 10-17 (99.58%)	MK748261	1431
Phab (Bhutan)	BPB 27	*Enterobacter hormaechei* subsp. *xiangfangensis*	*Enterobacter hormaechei subsp. xiangfangensis* 10-17 (98.88%)	MK748263	1446
Phab (Bhutan)	BPB 26	*Enterobacter hormaechei* subsp. *steigerwaltii*	*Enterobacter hormaechei subsp. steigerwaltii* DSM 16691(T) (99.23%)	MK203011	1422
Paa (Arunachal Pradesh)	AOB 9	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* MTCC 434(T) (99.79%)	MK203000	1416
Marcha (Nepal)	NMB10	*Bacillus zhangzhouensis*	*Bacillus zhangzhouensis* DW5-4(T) (99.58%)	MK203023	1432
Marcha (Nepal)	NMB23	*Staphylococcus xylosus*	*Staphylococcus xylosus* CCM 2738(T) (99.86%)	MK203021	1426
Phab (Bhutan)	BPB24	*Bacillus albus*	*Bacillus albus* MCCC 1A02146 (99.02%)	MK748262	1437

Marcha (Bhutan)	BPB8	*Bacillus circulans*	*Bacillus circulans* NBRC 13626 (98.64%)	MK748266	1412
Marcha (Nepal)	NMB11	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (100%)	MK748271	1460
Marcha (Nepal)	NMB12	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (99.72%)	MK748272	1426
Marcha (Nepal)	NMB13	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (100%)	MK748273	1388
Marcha (Sikkim)	SMB19	*Lysinibacillus boronitolerans*	*Lysinibacillus boronitolerans* NBRC 103108 (99.59%)	MK748275	1220
Marcha (Bhutan)	BPB1	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203006	1437
Marcha (Bhutan)	BPB10	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.79%)	MK203007	1432
Marcha (Bhutan)	BPB17	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203009	1429
Marcha (Bhutan)	BPB3	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (98.92%)	MK203012	1490
Marcha (Bhutan)	BPB7	*Bacillus nitratireducens*	*Bacillus nitratireducens* 4049(T) (99.36%)	MK203014	1404
Marcha (Darjeeling)	DMB5	*Staphylococcus hominis* subsp. *hominis*	*Staphylococcus hominis* subsp. *hominis* DSM 20328 (T) (99.93%)	MK203016	1425

Marcha (Nepal)	NMB20	*Staphylococcus gallinarum*	*Staphylococcus gallinarum* ATCC 35539 (T) (99.86%)	MK203025	1437
Marcha (Nepal)	NMB22	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345 (T) (99.65%)	MK203026	1439
Marcha (Sikkim)	SMB22	*Micrococcus yunnanensis*	*Micrococcus yunnanensis* YIM 65004 (T) (99.64%)	MK203030	1379
Marcha (Sikkim)	SMB1	*Bacillus subtilis* subsp.*inaquosorum*	*Bacillus subtilis subsp. inaquosorum* KCTC 13429 (T) (99.65%)	MK203031	1425
Marcha (Sikkim)	SMB8	*Bacillus pseudomycoides*	*Bacillus pseudomycoides* AFS041167 (99.93%)	MK203032	1407
Pee (Arunachal Pradesh)	AOB19	*Kocuria rosea*	*Kocuria rosea* DSM 20447 (T) (99.79%.)	MK203001	1399
Pee (Arunachal Pradesh)	AOB20	*Bacillus subtilis* subsp.*inaquosorum*	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429 (T) (99.79%)	MK203005	1431
Marcha (Sikkim)	SMB14	*Bacillus nakamurai*	*Bacillus nakamurai* NRRL B-41091 (96.65%)	MK752676	1103

ATCC, American Type Cell Culture; JCM, Japan Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen; MCCC, Microbial Culture Collection; NBRC, Biological Resource Centre, NITE; CCM, Czech collection of microorganisms; KACC, Korean Agricultural Culture Collection; YIM, Yunnan Institute of Microbiology; KCTC, Korean Collection for Type Cultures; NRRL, Agricultural Research Service Culture Collection.

Occurrence of three phyla of bacteria was observed in the samples of *marcha, paa, pee, phut* and *phab* according to the results obtained from 16S rRNA sequencing. Phylum *Firmicutes* (85%) was found to be dominant followed by *Proteobacteria* (9%) and *Actinobacteria* (6%), respectively (Figure 10). In *marcha* samples of Nepal, dominance of phylum *Firmicutes* represented by 80% of the total isolates followed by *Actinobacteria* (20%) was observed. Bacteria belonging to phylum *Proteobacteria* were not recorded in *marcha* of Nepal. 100% dominance of phylum *Firmicutes* was observed in *marcha* samples of Darjeeling and Bhutan. In *marcha* sample of Sikkim, dominance of phylum *Firmicutes* (92%) followed by *Actinobacteria* (8%) was found. A variable distribution pattern of phyla was observed in dry starters of Arunachal Pradesh; phylum *Firmicutes* (80%) and phylum *Proteobacteria* (20%) was observed in *paa* samples, *pee* showed phylum *Firmicutes* (67%), phylum *Proteobacteria* (16%) and phylum *Actinobacteria* (17%), and *phut* samples showed phylum *Firmicutes* (75%) and phylum *Proteobacteria* (25%). Similarly, *Firmicutes* (57%) was the dominant phylum followed by phylum *Proteobacteria* (43%) in *phab* of Bhutan (Figure 11).

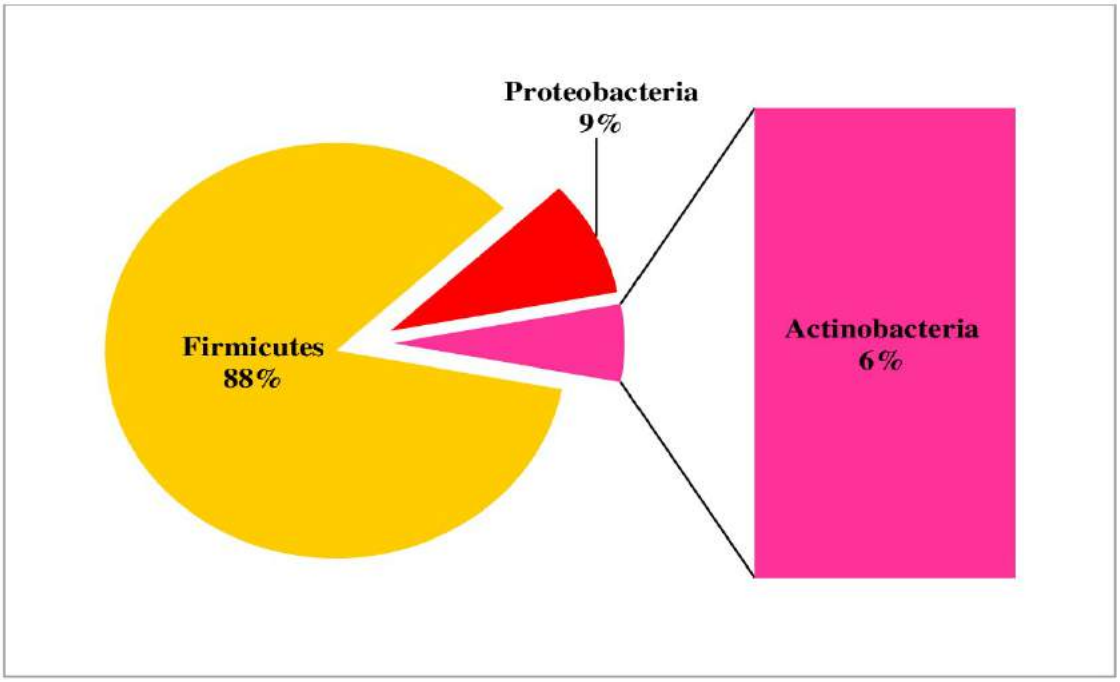


Figure 10: Overview of phyla distribution of bacteria in starters of the Eastern Himalayan.

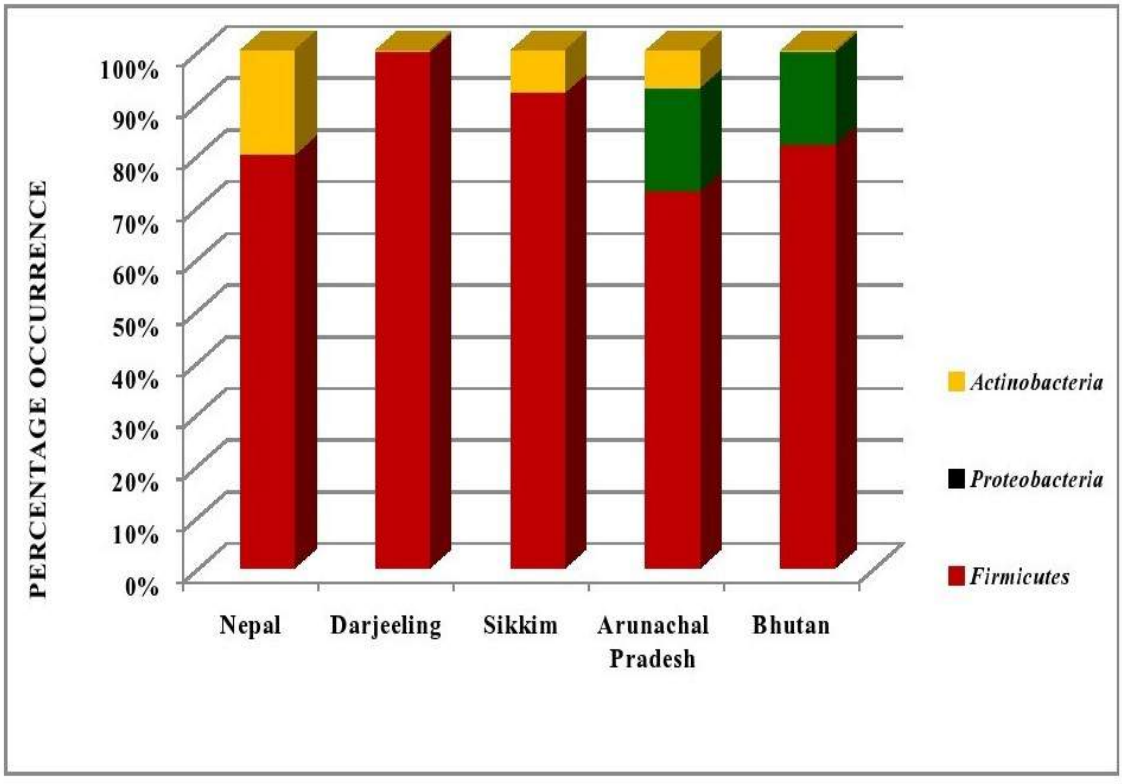


Figure 11: Phyla distribution of bacteria in different starters of the Eastern Himalayan.

In genus and species level, 15 genera with 32 species of bacteria were identified which included *Leuconostoc* (3%), *Enterococcus* (41%), *Bacillus* (13%), *Staphylococcus* (12%), *Lactobacillus* (5%), *Enterobacter* (4%), *Klebsiella* (2%), *Pseudomonas* (2%), *Pediococcus* (9%), *Strenotrophomonas* (2%), *Kocuria* (2%), *Brevibacterium* (3%), *Lysinibacillus* (2%), *Weisella* (2%) and *Micrococcus* (2%) (Figure 12).

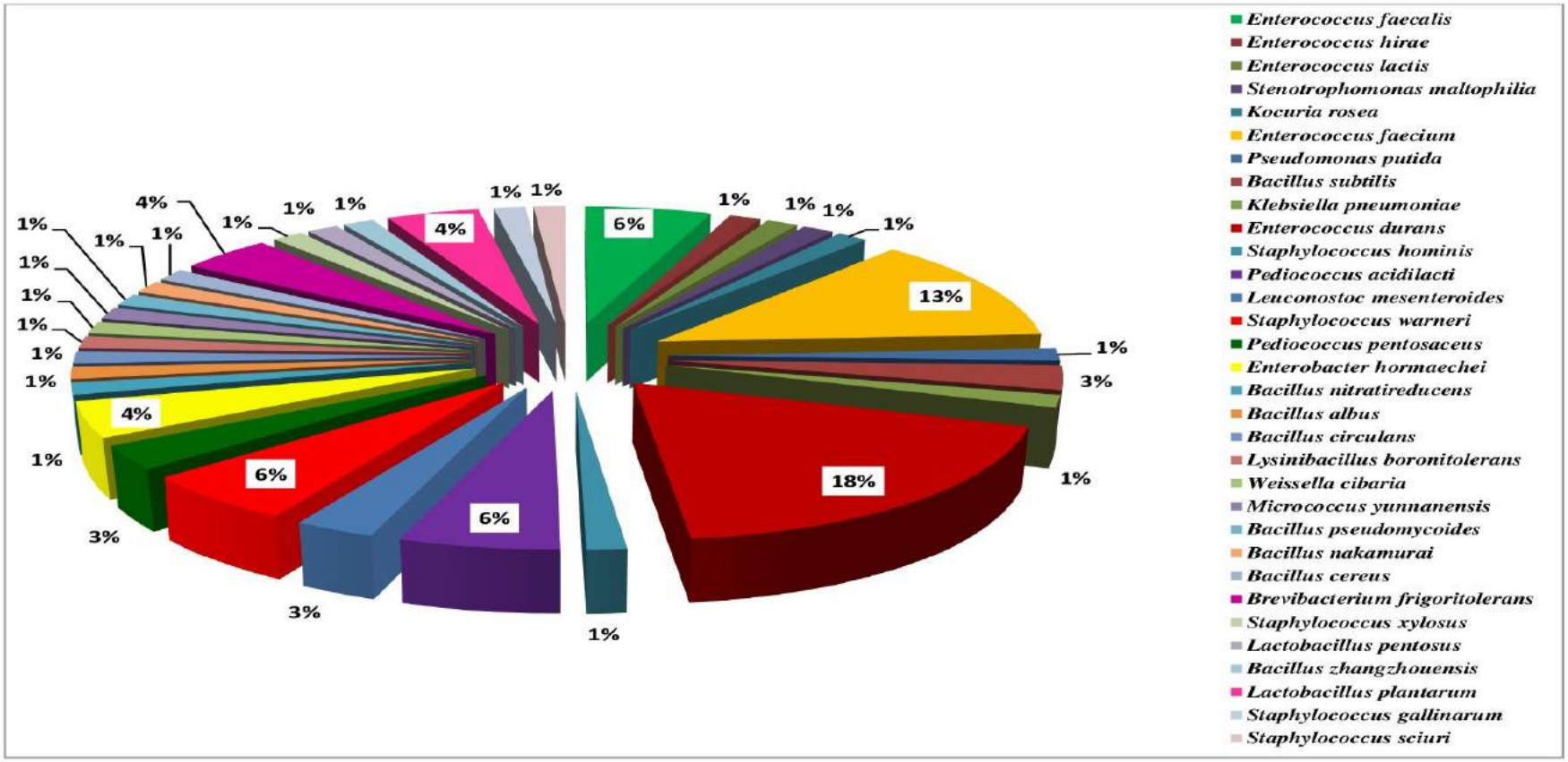


Figure 12: Percentage occurrence of different bacterial species in various starters of the Eastern Himalayas.

In *marcha* samples of Nepal, *Brevibacterium frigoritolerans* (20%) and *Lactobacillus plantarum* (20%) were found to be the dominant bacterial species (Figure 13). In *marcha* samples of Darjeeling and Sikkim *Enterococcus durans* and *Enterococcus faecium* were found to be the dominant species with percentage occurrence of 55% and 22% respectively (Figure 14, 15). In samples of Arunachal Pradesh, *Enterococcus faecium* was found to be the dominant species with percentage occurrence of 20% (Figure 16). *Staphylococcus warneri* represented by 24% of the total isolates was the dominant bacterium in starter sample *marcha* of Bhutan (Figure 17).

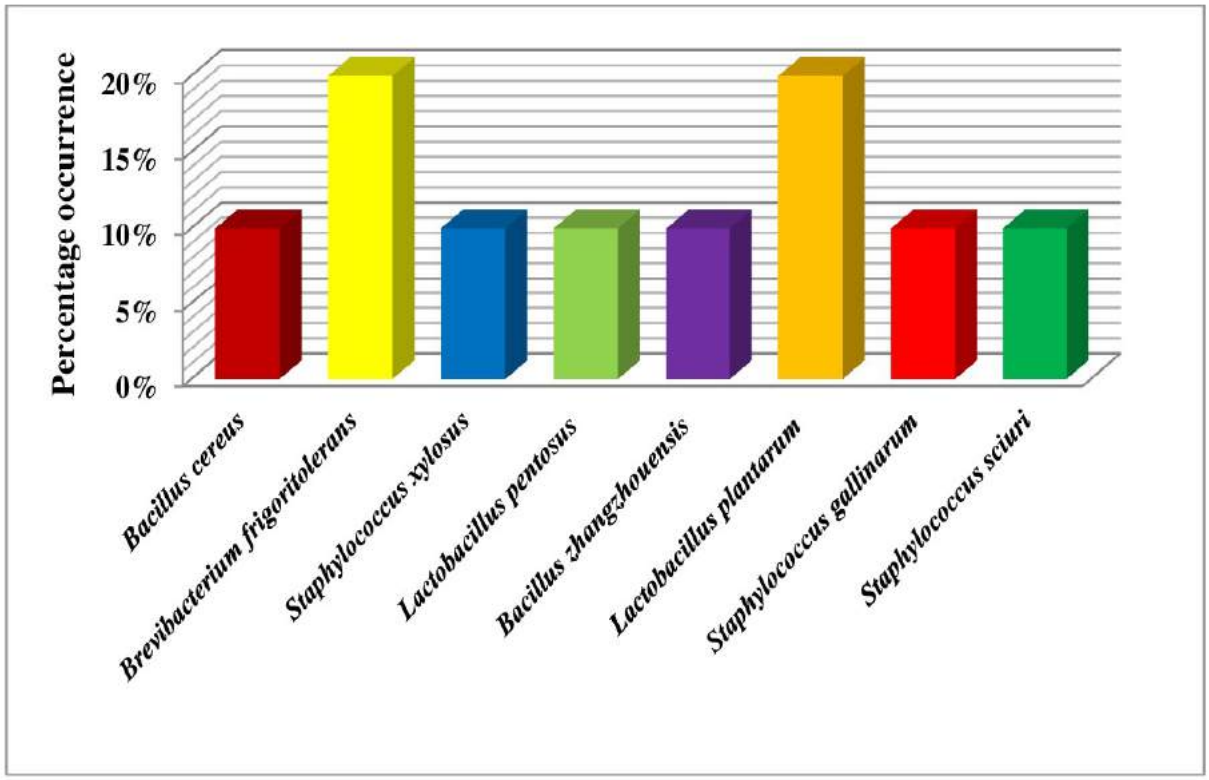


Figure 13: Percentage occurrence of different bacterial species in *marcha* of Nepal.

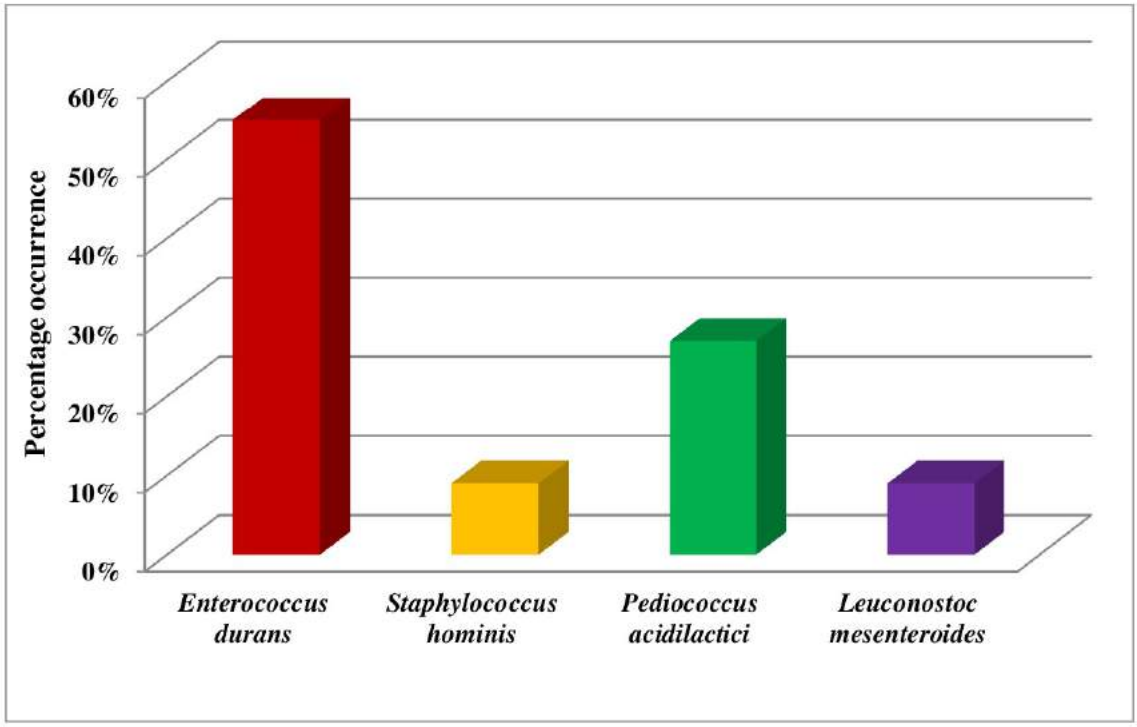


Figure 14: Percentage occurrence of different bacterial species in *marcha* of Darjeeling.

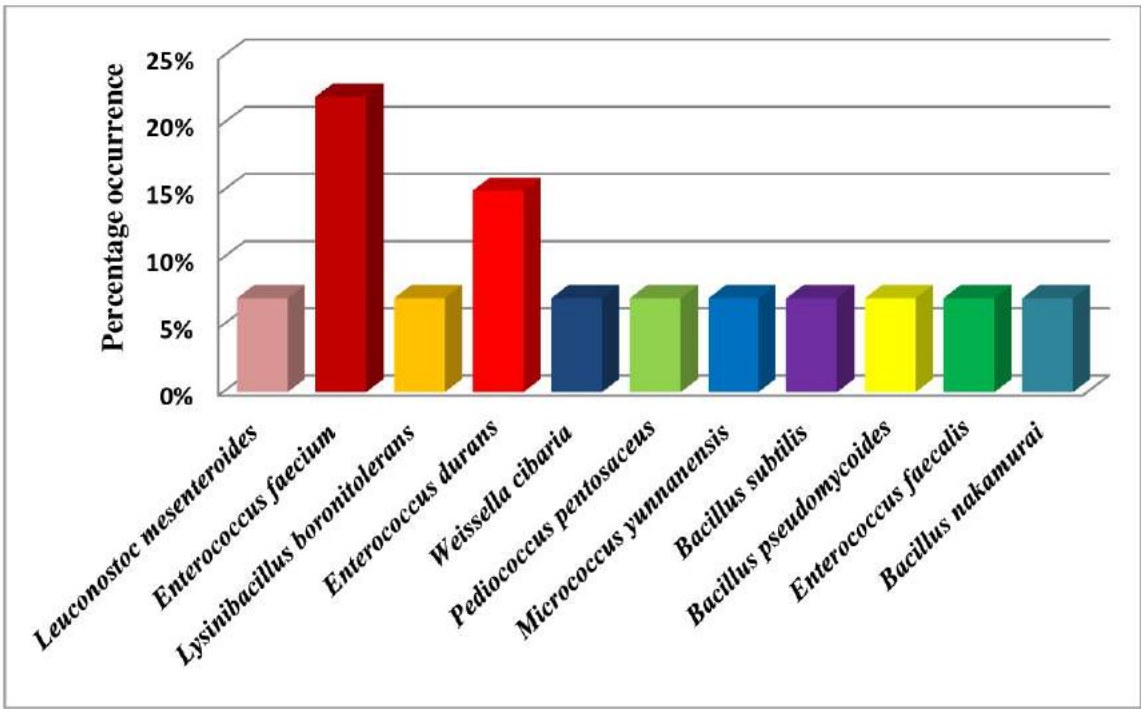


Figure 15: Percentage occurrence of different bacterial species in *marcha* of Sikkim.

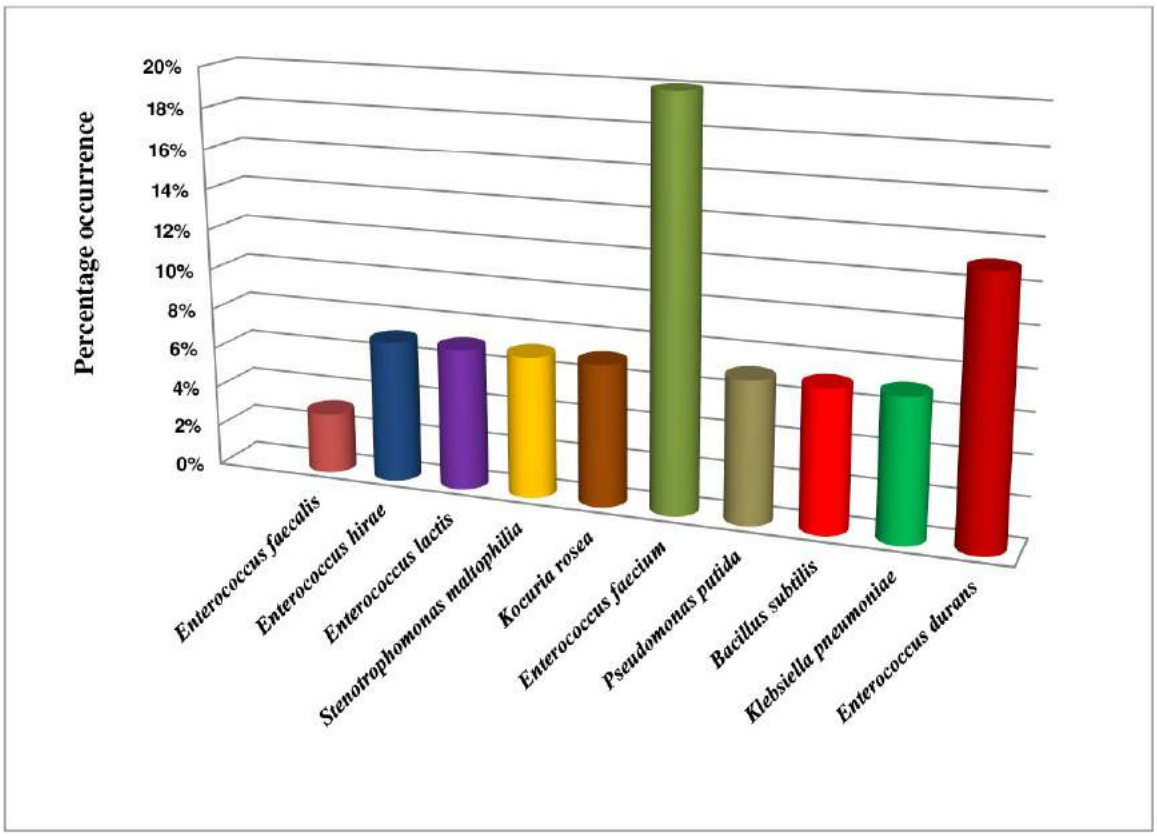


Figure 16: Percentage occurrence of different bacterial species in *paa, pee* and *phut* of Arunachal Pradesh.

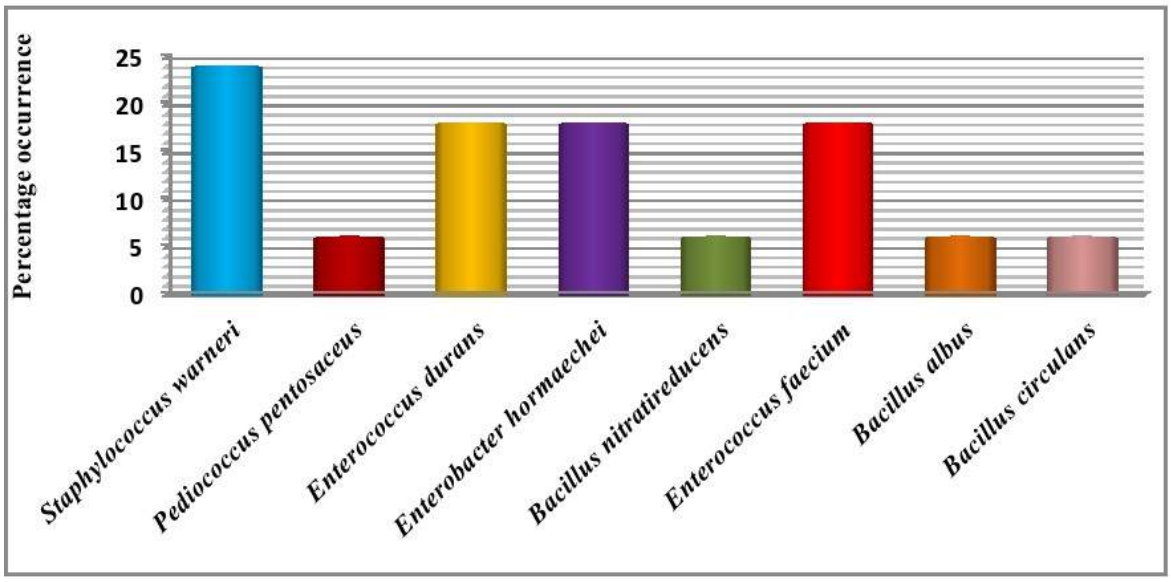


Figure 17: Percentage occurrence of different bacterial species in *marcha* and *phab* of Bhutan.

The lactic acid bacteria (LAB) represented 59% of total bacterial isolates, whereas non-LAB were 31% and Gram-negative bacteria were 9% of the total bacterial isolates (Table 13, Figure 18). Lactic acid bacterial species found in this study were *Enterococcus durans, E. faecium, E. faecalis, E. hirae, E. lactis, Pediococcus acidilacti, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, L. pentosus, Leuconostoc mesenteroides* and *Weissella cibaria*. *Enterococcus durans* was found to be the most dominant species represented by 18% followed by *E. faecium* represented by 13% of total bacterial isolates. *Enterococcus durans* was the most dominant species in *marcha* samples of Darjeeling (54.5%) whereas *Pediococcus pentocaseus* (5.8%) showed the lowest prevalence in *marcha* samples of Bhutan. LAB were found in all samples with the highest occurrence in *marcha* samples of Darjeeling (91%) and lowest in *marcha* of Nepal (30%).

Table 13: Percentage distribution of LAB, non LAB and Gram negative bacteria in various dry starter samples of the Eastern Himalayas

Region	Lactic acid bacteria	Non Lactic acid bacteria	Gram negative bacteria
Nepal	30%	70%	0
Darjeeling	91%	9%	0
Sikkim	64%	36%	0
Arunachal Pradesh	67%	13%	20%
Bhutan	41%	41%	18%

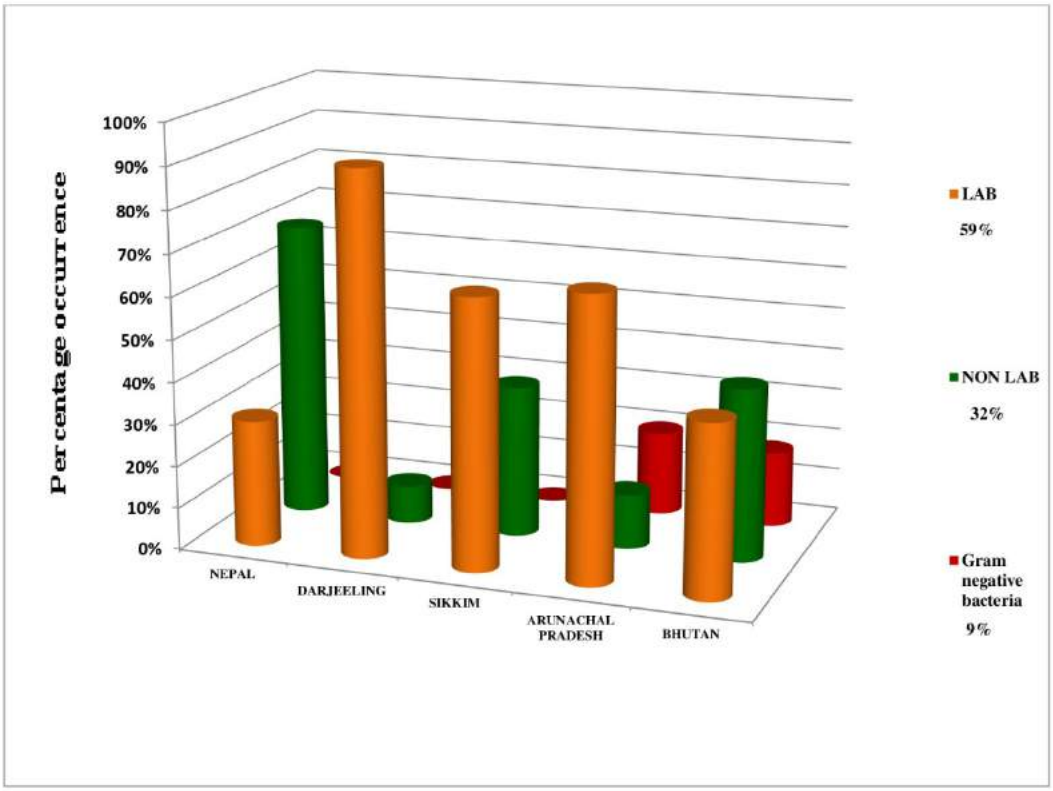


Figure 18: Percentage occurrence of LAB, non-LAB and Gram-negative bacteria in various starters of the Eastern Himalayas.

The non-LAB were represented by *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans* and *Micrococcus yunnanensis* (Table 14). The highest occurrence of non-LAB group of bacteria (70%) was found in *marcha* samples of Nepal and lowest in *marcha* samples of Darjeeling (9%) (Table 14). This group of bacteria was not found in samples of *paa* and *phut* of Arunachal Pradesh and in *marcha* samples of Bhutan (Table 14).

Some Gram negative bacteria (9%) were also found in dry starter samples of Arunachal Pradesh and Bhutan in this study. *Stenotrophomonas maltophilia* was found in *paa* sample, *Klebsiella pneumonia* in *pee* sample, *Pseudomonas putida* in *phut* sample, and *Enterobacter hormaechei* subsp. *xiangfangensis* and *E. hormaechei* subsp. *steigerwaltii* in samples of *phab* of Bhutan (Table 14). Interestingly we could not detect Gram negative isolates in any sample of *marcha* (Table 14, Figure 18).

Table 14: Distribution of LAB, Non-LAB and Gram negative bacteria in the dry starters of the Eastern Himalayas			
Country/ place	**Dry Starter**	**Bacterial species**	
Nepal	*Marcha*	LAB	*Lactobacillus pentosus; Lb. plantarum* subsp. *plantarum*
		Non LAB	*Bacillus cereus; Brevibacterium frigoritolerans; S. sciuri; Staphylococcus xylosus; B. zhangzhouensis; S. gallinarum*
		Gram -ve bacteria	NF
India, Darjeeling	*Marcha*	LAB	*Enterococcus durans; Pediococcus acidilactici; Leuconostoc mesenteroides*
		Non LAB	*Staphylococcus hominis* subsp. *hominis*
		Gram -ve bacteria	NF
India, Sikkim	*Marcha*	LAB:	*Leuconostoc mesenteroides; Enterococcus faecium; E.durans; Weissella cibaria; Pediococcus pentosaceus; E.faecalis; E.durans*
		Non LAB	*Lysinibacillus boronitolerans; Micrococcus yunnanensis; Bacillus subtilis* subsp. *inaquosorum; B.pseudomycoides; B.nakamurai*
		Gram -ve bacteria	NF
India, Arunachal Pradesh	*Paa*	LAB	*Enterococcus faecalis; E. faecium*
		Non LAB	NF
		Gram -ve bacteria	*Stenotrophomonas maltophilia*
India, Arunachal Pradesh	*Pee*	LAB	*Enterococcus durans; E. faecium*
		Non LAB	*Kocuria rosea; Bacillus subtilis* subsp. *inaquosorum*
		Gram -ve bacteria	*Klebsiella pneumoniae*
India, Arunachal Pradesh	*Phut*	LAB	*Enterococcus hirae; E.lactis; E. faecium*
		Non LAB	NF
		Gram -ve bacteria	*Pseudomonas putida*
Bhutan	*Marcha*	LAB	*Pediococcus pentosaceus;Enterococcus durans;E. faecium*
		Non LAB	*Staphylococcus warneri; Bacillus nitratireducens; B.circulans*
		Gram -ve bacteria	NF
	Phab	LAB	*Enterococcus durans; E. faecium*
		Non LAB	*Bacillus albus*
		Gram -ve bacteria	*Enterobacter hormaechei subsp. xiangfangensis, Enterobacter hormaechei* subsp. *steigerwaltii*
LAB, Lactic acid bacteria; -ve, negative; NF, not found.			

DIVERSITY INDICES

Diversity indices of bacteria in dry starters of the Eastern Himalayas which included Shannon diversity index *H*, Simpson's index, Dominance and Chao1 index were calculated (Table 15). The *H* index value was recorded as 2.025, 1.121, 2.305, 2.176 and 1.925 for dry starters of Nepal, Darjeeling, Sikkim, Arunachal Pradesh and Bhutan, respectively (Table 15). The *H* index value was highest in *marcha* of Sikkim (*H*: 2.305) and lowest in *marcha* of Darjeeling (*H*:1.121). Simpson's diversity index (1-*D*) values were recorded as 0.8878, 0.8711, 0.86, and 0.8374 for dry starters of Sikkim, Arunachal Pradesh, Nepal and Bhutan, respectively. Chao 1 index which gives the estimation of species richness based on abundance was found to be highest in *marcha* samples of Sikkim (29) and lowest in *marcha* samples of Darjeeling (5). The dominance D values which is inversely proportional to the species diversity were recorded highest for *marcha* samples of Darjeeling (0.3884) and lowest for *marcha* samples of Sikkim (0.1122) indicating a very rich and diverse ecosystem in *marcha* of Sikkim (Figure 19).

Table 15: Diversity indices of different dry starters of the Eastern Himalayas				
Country/ Region	**Diversity indices**			
	Simpson's index (1-*D*)	**Shannon's index (*H*)**	**Dominance (*D*)**	**Chao-1**
Nepal	0.86	2.025	0.14	13
India (Darjeeling hills)	0.6116	1.121	0.388	5
India (Sikkim)	0.8878	2.305	0.112	29
India (Arunachal Pradesh)	0.8711	2.176	0.1289	20.5
Bhutan	0.8374	1.925	0.1626	14

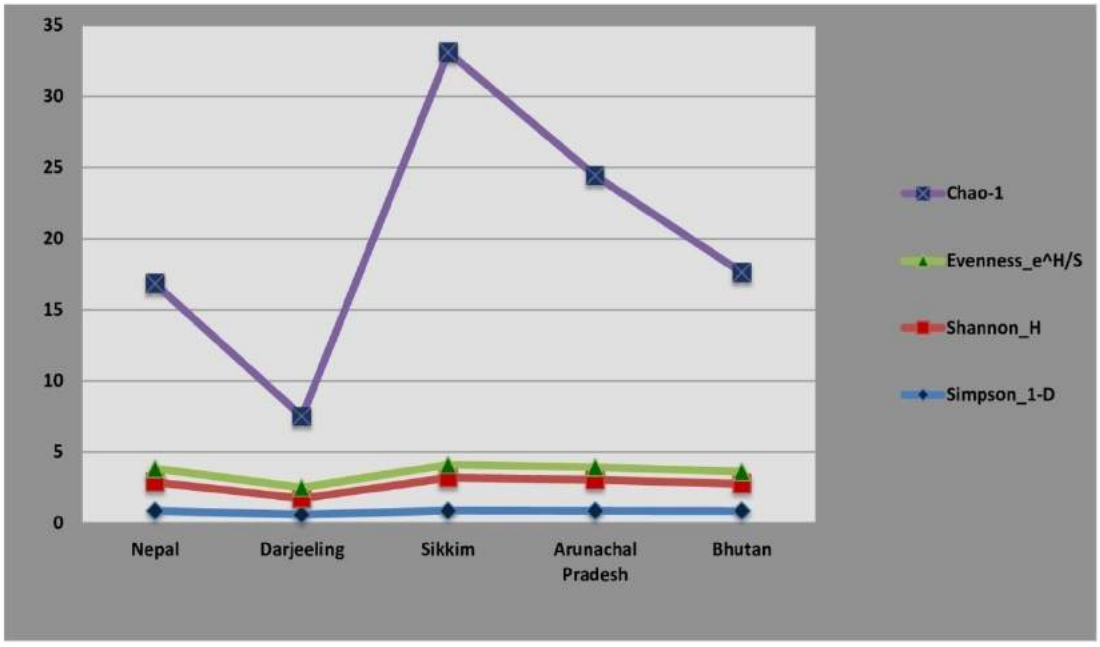


Figure 19: Bacterial diversity indices of starters of the Eastern Himalayas.

HIGH-THROUGHPUT SEQUENCING OF BACTERIAL COMMUNITY

We selected only *marcha* sample of Sikkim for high-throughput sequencing (HTS) to profile the bacterial community, due to cost effective in analysis HTS, and also easy availability of *marcha* samples in Sikkim. Approximately 0.85 million quality reads were obtained in HTS results of *marcha* which were used for further analysis. The 16S rRNA amplicon sequencing results showed the occurrence of 15 bacterial phyla in *marcha* samples of Sikkim (Figure 20). Phylum *Proteobacteria* (53.8%) and *Firmicutes* (45.4%) were major bacterial phyla in *marcha*. The other minor phyla which constituted 0.8% of the total bacterial phyla in *marcha* were *Actinobacteria*, *Deinococcus, Thermus*, *Chloroflexi*, *Synergistetes*, *Cyanobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Fusobacteria*, *Planctomycetes*, *Acidobacteria*, *Saccharibacteria*, *Gemmatimonadetes* and *Armatimonadetes.*

Family level distribution of bacteria in *marcha* samples of Sikkim revealed the occurrence of 6 major bacteria family. Based on the operational Taxonomic Units (OUTs) at >1% abundance family levels were detected. *Acetobacteraceae* (50.6%) was found to be the most dominant family in *marcha* samples followed by *Leuconostocaceae* (25.5%), *Streptococcaceae* (10.5%), *Lactobacillaceae* (8.38%), *Burkholderiaceae* (2.13%) and *Staphylococcaceae* (0.54%) (Figure 20).

At genus level 13 different bacterial genera were detected in *marcha* samples considering OUTs at >1% abundance (Figure 21). *Acetobacter* (52.6%) was found the most dominant genus in *marcha* of Sikkim followed by *Fructobacillus* (21.1%), *Lactococcus* (10.3%), *Lactobacillus* (8.4%), *Leuconostoc* (4.0%), *Burkholderia* (2.1%) and *Gluconacetobacter* (1.4%). Bacterial genera with percentage composition of less than 3.9% such as *Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea* are not shown in the Figure 21.

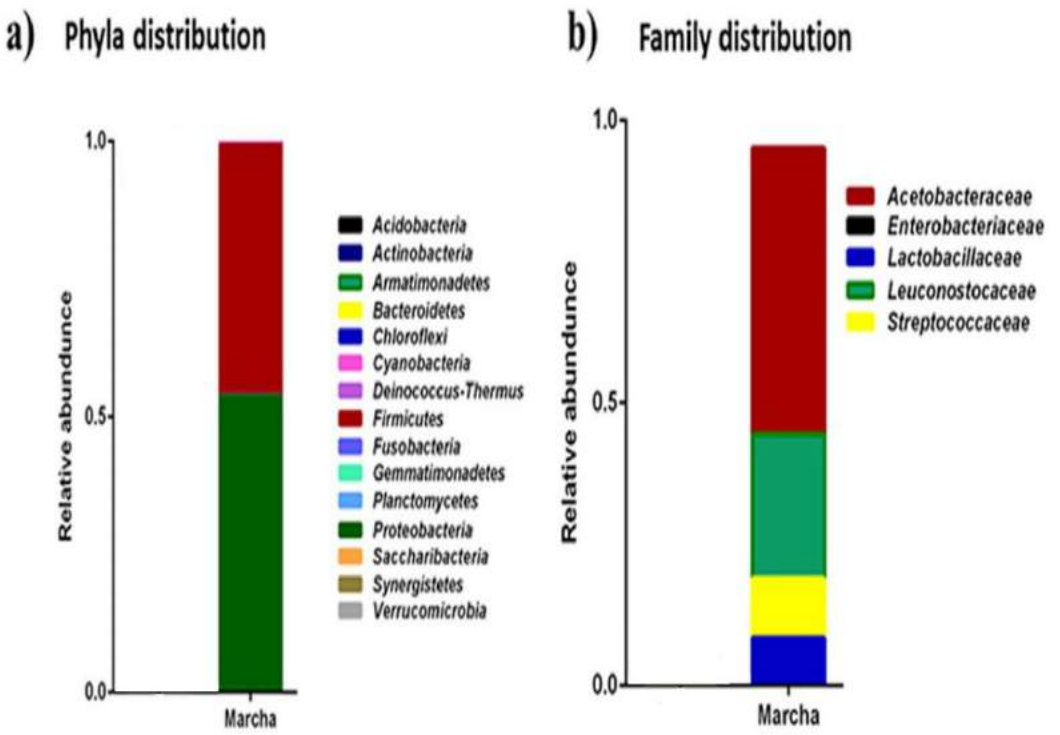


Figure 20: Phyla and family distribution of bacteria in *marcha* of Sikkim according to the High Throughput Sequencing result.

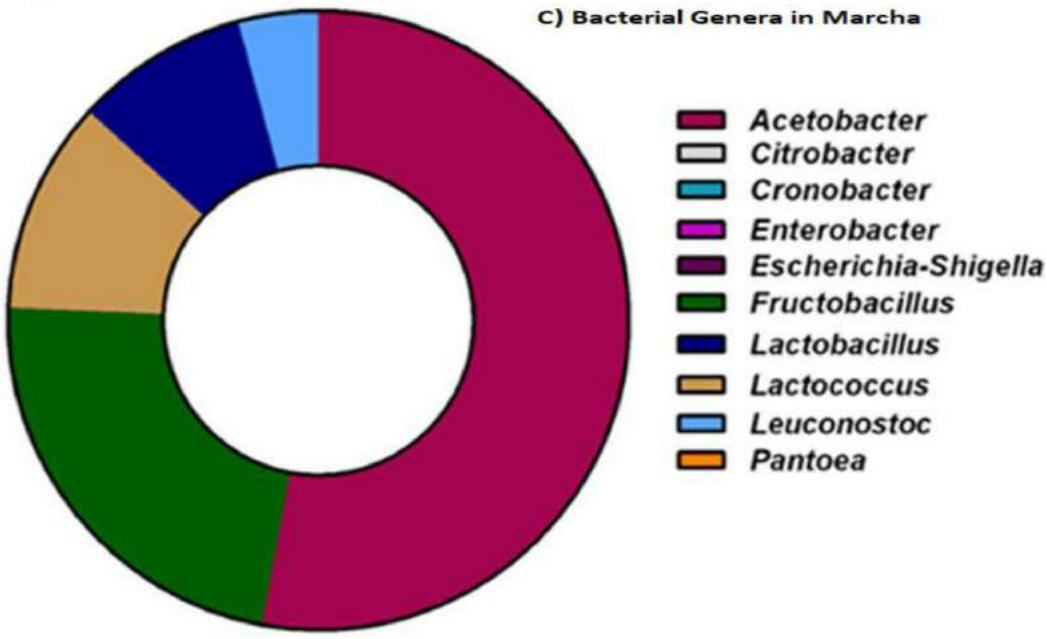


Figure 21: Distribution of bacterial genera in *marcha* of Sikkim.

Alpha Diversity

The alpha diversity indices for bacterial diversity were computed. The average Goods coverage for HTS was 99.08% (mean) ± 0.1% (SD), whereas the Shannon index for bacterial diversity in *marcha* was calculated as 4.01115959 and Simpson's diversity index was recorded as 0.866763863. The Chao1 index which gives the bacterial species richness was recorded to be 1520.925 in the *marcha* samples.

Overall Profile of Bacteria in Dry Starters

Identification of bacteria obtained from results of culture dependent (phenotypic characterizations and 16S rRNA sequencing) and culture independent (high throughput sequencing) was compared (Table 16). In culture-dependent study of bacterial diversity of *marcha* samples of Sikkim we found the dominance of phylum *Firmicutes* represented by 93% of the total bacterial phyla. Phylum *Actinobacteria* was found as a minor phyla represented by 7% of the total bacterial phyla. In the culture-independent study we found the dominance of phylum *Proteobacteria* (53.8%) followed by *Firmicutes* (45.4%) which were the major bacterial phyla of *marcha* and minor phyla (0.8%) *Actinobacteria*, *Deinococcus, Thermus*, *Chloroflexi*, *Synergistetes*, *Cyanobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Fusobacteria*, *Planctomycetes*, *Acidobacteria*, *Saccharibacteria*, *Gemmatimonadetes* and *Armatimonadetes.*

In culture-dependent study of *marcha* samples of Sikkim, 7 genera of bacteria were found; *Leuconostoc, Enterococcus, Bacillus, Pediococcus, Lysinibacillus, Weisella* and *Micrococcus* with the dominance of genus *Enterococcus* (41%) (Table 16). However, 13 genera of bacteria: *Acetobacter, Fructobacillus, Lactococcus, Lactobacillus, Leuconostoc, Burkholderia, Gluconacetobacter, Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea* were recovered by high-throughput

sequencing result of *marcha* of Sikkim with the dominance of genus *Acetobacter* (52.6%) (Table 16). Acetic acid bacteria were not recovered in culture-dependent study however; two genera of acetic acid bacteria *Acetobacter* and *Gluconacetobacter* were found in culture-independent study (Table 16). High occurrence of Gram-negative bacteria such as *Acetobacter, Burkholderia, Gluconacetobacter, Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea* was found in culture-independent study. Gram-negative bacteria were not found in culture-dependent study of *marcha* of Sikkim (Table 16).

Table 16: Profile of bacterial species in traditionally prepared dry starters of the Eastern Himalayan region of Sikkim revealed by Culture-dependent and Culture-independent Methods			
Starters	**Culture-dependent**		**Culture-independent**
	Phenotypic	**Genotypic (16S rRNA gene sequencing)**	**High-throughput Sequencing**
	Bacteria		
Marcha (Nepal)	*Leuconostoc, Lactobacillus, Pediococcus, Enterococcus.*	*Lactobacillus pentosus*, *Lactobacillus plantarum* subsp. *plantarum*, *Lactobacillus plantarum* subsp. *plantarum*, *Bacillus zhangzhouensis*, *Staphylococcus xylosus*, *Bacillus cereus*, *Brevibacterium frigoritolerans*, *Staphylococcus gallinarum*, *Staphylococcus sciuri*.	Not analyzed
Marcha (Darjeeling)	*Pediococcus, Leuconostoc, Enterococcus.*	*Leuconostoc mesenteroides*, *Enterococcus durans*, *Pediococcus acidilactici*, *Pediococcus acidilactici*, *Enterococcus durans*, *Enterococcus durans*, *Enterococcus durans*, *Pediococcus acidilactici*, *Pediococcus acidilactici*, *Enterococcus durans*, *Staphylococcus hominis* subsp. *homini.*	Not analyzed
Marcha (Sikkim)	*Bacillus, Staphylococcus, Leuconostoc, Enterococcus, Pediococcus.*	*Leuconostoc mesenteroides*, *Enterococcus faecium*, *Enterococcus faecium*, *Enterococcus faecium*, *Enterococcus durans*, *Weissella cibaria*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*, *Lysinibacillus boronitolerans*, *Micrococcus yunnanensis*, *Bacillus subtilis* subsp. *inaquosorum*, *Bacillus pseudomycoides*, *Bacillus nakamurai.*	*Acetobacter, Fructobacillus, Lactococcus, Lactobacillus, Leuconostoc, Burkholderia, Gluconacetobacter, Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella*, *Pantoea.*
Marcha (Bhutan)	*Enterococcus, Pediococcus, Bacillus, Staphylococcus, Leuconostoc.*	*Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecium*, *Enterococcus durans*, *Bacillus circulans*, *Staphylococcus warneri*, *Staphylococcus warneri*, *Staphylococcus warneri*, *Staphylococcus warneri*, *Bacillus nitratireducens.*	Not analyzed

Paa (Arunachal Pradesh)	*Pediococcus, Enterococcus, Citrobacter.*	*Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus faecalis*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis*	Not analyzed
Pee (Arunachal Pradesh)	*Enterococcus, Lactobacillus, Citrobacter.*	*Enterococcus durans*, *Klebsiella pneumonia*, *Kocuria rosea*, *Bacillus subtilis* subsp. *inaquosorum, Enterococcus durans*, *Enterococcus faecium*	Not analyzed
Phut (Arunachal Pradesh)	*Enterococcus, Pediococcus, Citrobacter.*	*Enterococcus hirae*, *Enterococcus lactis*, *Pseudomonas putida*, *Enterococcus faecium*.	Not analyzed
Phab (Bhutan)	*Enterococcus, Pediococcus, Bacillus, Enterobacter.*	*Enterobacter hormaechei* subsp. *xiangfangensis, Enterobacter hormaechei* subsp. *xiangfangensis*, *Enterobacter hormaechei* subsp. *steigerwaltii*, *Bacillus albus*, *Enterococcus faecium*, *Enterococcus faecium*, *Enterococcus durans.*	Not analyzed

EVALUATION OF PROBIOTIC ATTRIBUTES

Resistance to Acidic Conditions (pH 3)

Tolerance of bacterial isolates at low acidic pH of 3 was evaluated by taking the total viable count after 24 h of incubation and was expressed as percentage survival rate (Table 17). About 38% of the total bacterial isolates evaluated for probiotic properties showed a survival rate of more than 50% at pH 3. *Leuconostoc mesenteroides* SMB13-7 (*marcha,* Sikkim) showed the highest survival rate of 96% in pH 3 and *Enterococcus durans* DMB15 (*marcha* Darjeeling) showed no growth at pH 3 (Figure 22).

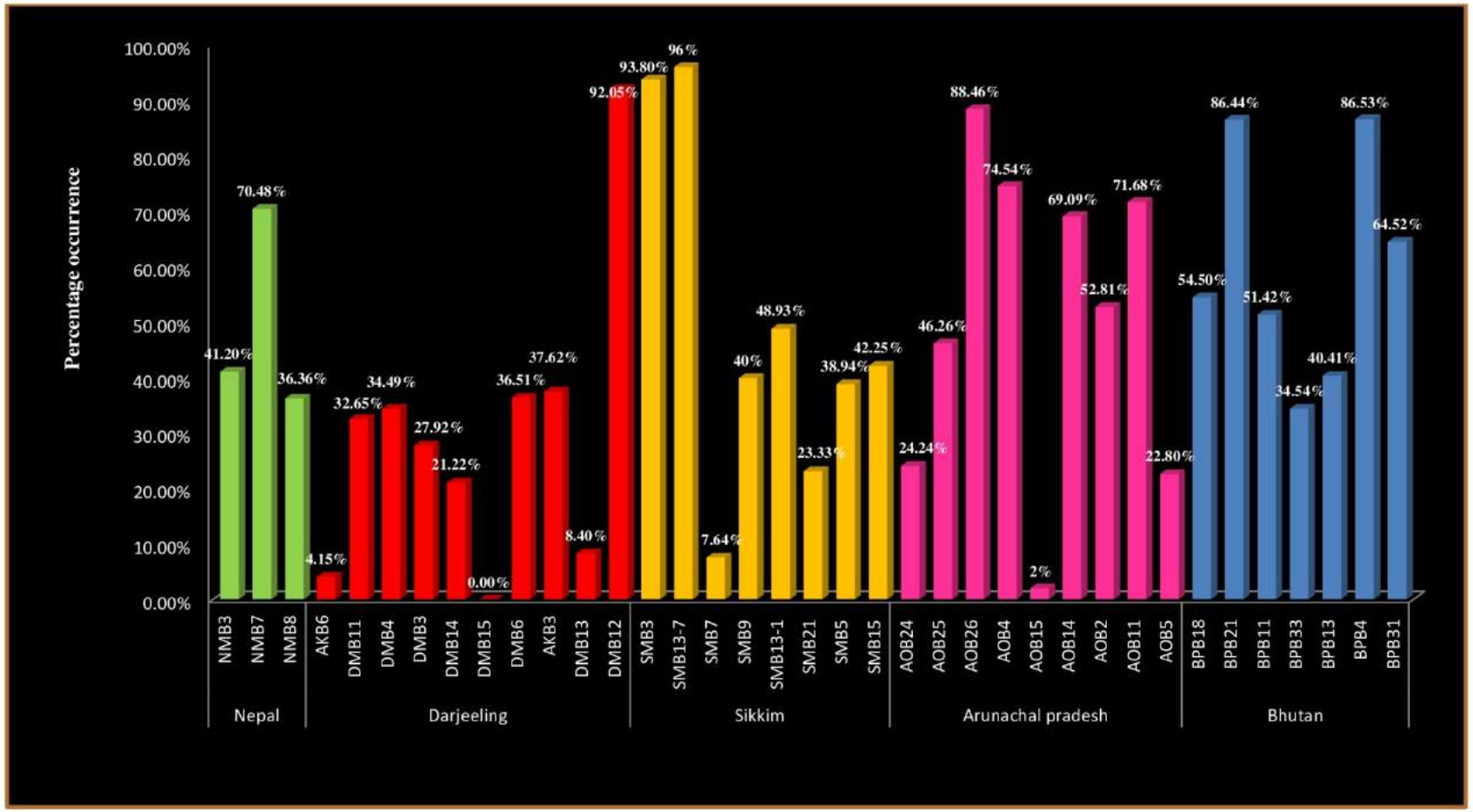


Figure 22: Percentage survival of bacterial isolates from different starters of the Eastern Himalayas in pH 3.

Resistance To 0.3% Bile Concentration

About 38% of the total isolates showed more than 50% of survival rate after 24 h of incubation in 0.3% bile salts (Table 17). *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling) showed a highest survival rate of 97.26% in 0.3% bile salts (Figure 23). *Weisella cibaria* SMB9 (*marcha,* Sikkim) showed no growth and *Enterococcus durans* DMB3 (*marcha,* Darjeeling) showed a very low survival rate of below 1% in the presence of bile salts (0.3%).

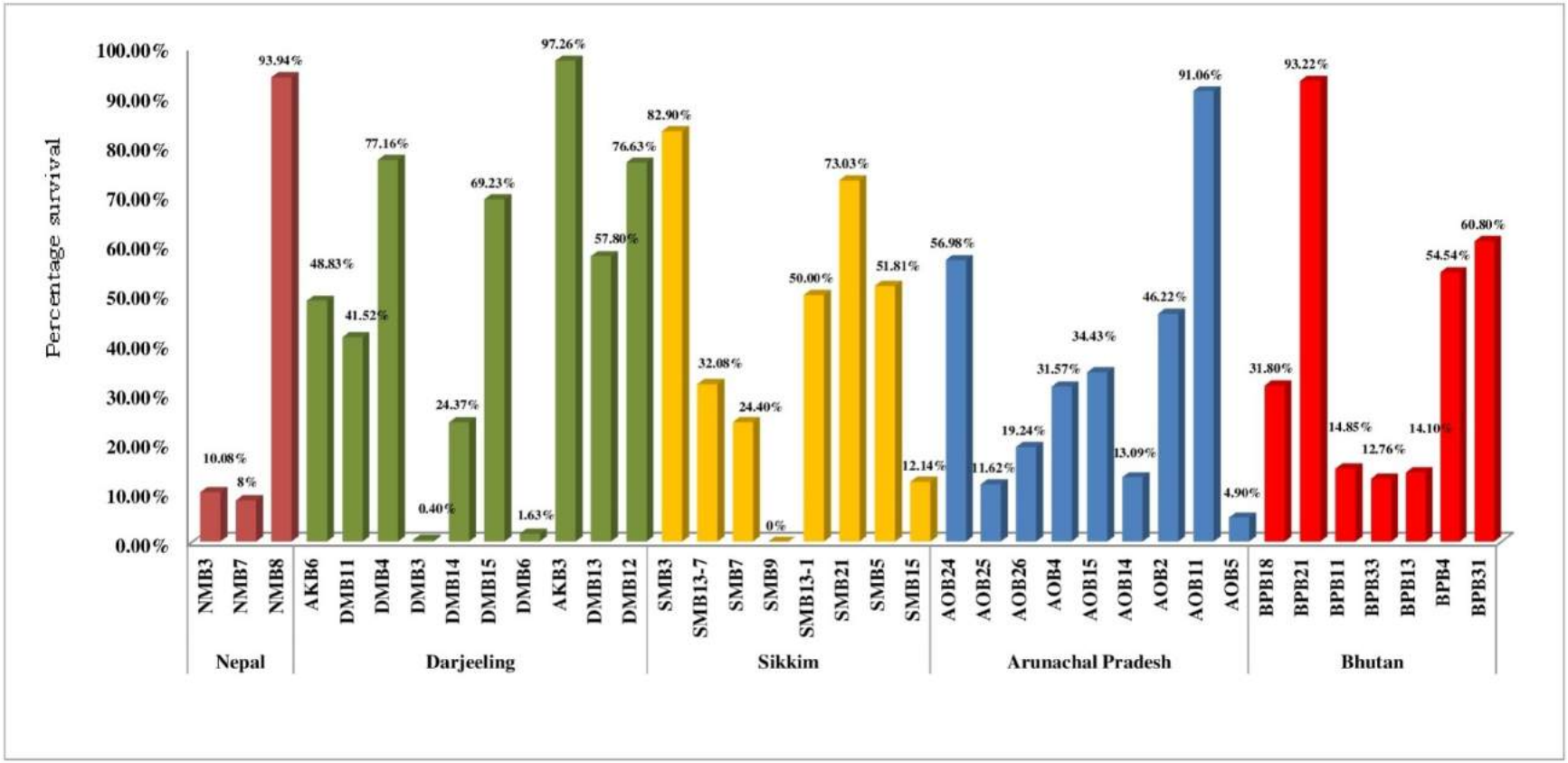


Figure 23: Percentage survival of bacterial isolates from different starters of the Eastern Himalayas in 0.3% bile.

Table 17: Probiotic characteristics and resistance to biological barriers of Lactic acid bacteria isolated from dry starter cultures of the Eastern Himalayas								
Starter	**Bacteria**	**pH 3 (% survival)**	**Bile (0.3%) (% survival)**	**Deconjugation of bile salts**		**H%**	**β Gal activity**	**Cholesterol Assimilation (%)**
				TDC	**TC**			
Marcha (Nepal)	*Lactobacillus pentosus* NMB3	41	10.08	-	+	47	-	29
Marcha(Nepal)	*Lactobacillus plantarum* subsp. *plantarum* NMB7	70	8.37	+	+	27	-	24
Marcha(Nepal)	*Lactobacillus plantarum* subsp. *plantarum* NMB8	36	93.94	-	-	57	+	37
Marcha(Darjeeling)	*Leuconostoc mesenteroides* AKB6	4.2	48.83	-	-	94	-	22
Marcha(Darjeeling)	*Enterococcus durans* DMB11	33	41.52	-	+ (w)	95	+	65
Marcha(Darjeeling)	*Enterococcus durans* DMB4	34	77.16	-	-	96	+	80
Marcha(Darjeeling)	*Enterococcus durans* DMB3	28	0.4	+	+	25	-	4
Marcha(Darjeeling)	*Pediococcus acidilactici* DMB14	21	24.37	+	+	92	+	43
Marcha(Darjeeling)	*Enterococcus durans* DMB15	0	69.23	-	-	90	+	63
Marcha(Darjeeling)	*Enterococcus durans* DMB6	37	1.63	-	-	92	-	9
Marcha(Darjeeling)	*Pediococcus acidilactici* AKB3	38	97.26	-	-	83	-	32
Marcha(Darjeeling)	*Enterococcus durans* DMB13	8.4	57.80	-	-	91	+	46
Marcha(Darjeeling)	*Pediococcus acidilactici* DMB12	92	76.63	-	-	77	+	53
Marcha(Sikkim)	*Enterococcus faecalis* SMB3	94	82.90	-	-	95	+	54
Marcha(Sikkim)	*Leuconostoc mesenteroides* SMB13-7	96	32.08	-	-	89	+	38
Marcha(Sikkim)	*Enterococcus durans* SMB7	7.6	24.40	-	+	98	+	78
Marcha(Sikkim)	*Weissella cibaria* SMB9	40	0	-	-	93	+	41

Marcha(Sikkim)	*Pediococcus pentosaceus* SMB13-1	49	50.00	+	+	96	-	72
Marcha(Sikkim)	*Enterococcus faecium* SMB21	23	73.03	-	-	96	+	38
Marcha(Sikkim)	*Enterococcus faecium* SMB5	39	51.81	+	+	77	+	51
Marcha(Sikkim)	*Enterococcus faecium* SMB15	42	12.14	+	+	86	+	25
Paa (Arunachal Pradesh)	*Enterococcus faecium* AOB4	75	31.57	-	-	57	+	20
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB2	53	46.22	-	-	86	-	57
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB11	72	91.06	+	+	92	+	5
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB5	23	4.90	-	-	84	-	11
Pee (Arunachal Pradesh)	*Enterococcus faecium* AOB15	02	34.43	-	+	97	+	4
Pee (Arunachal Pradesh)	*Enterococcus durans* AOB14	69	13.09	-	+	97	-	72
Phut(Arunachal Pradesh)	*Enterococcus hirae* AOB24	24	56.98	-	-	76	+	27
Phut(Arunachal Pradesh)	*Enterococcus faecium* AOB25	46	11.62	-	-	91	+	23
Phut(Arunachal Pradesh)	*Enterococcus lactis* AOB26	88	19.24	-	-	93	+	71
Marcha (Bhutan)	*Enterococcus durans* BPB18	55	31.80	-	-	91	+	43
Marcha (Bhutan)	*Enterococcus faecium* BPB11	51	14.85	+	+	96	+	23
Marcha (Bhutan)	*Pediococcus pentosaceus* BPB13	40	14.10	+	+	89	-	2
Marcha (Bhutan)	*Enterococcus durans* BPB4	87	54.54	-	-	86	+	63
Phab (Bhutan)	*Enterococcus durans* BPB21	86	93.22	-	-	98	-	31
Phab (Bhutan)	*Enterococcus faecium* BPB33	35	12.76	+	+	88	+	60
Phab (Bhutan)	*Enterococcus faecium* BPB31	65	60.80	-	-	83	+	26

All the tests were done in duplicates. The values given for pH 3 and 0.3% bile represents the percentage survival rate of bacterial isolates in these conditions; Values for cholesterol assimilation (%) was calculated by taking OD at 550nm; +: Positive; -: Negative; H% : Percentage hydrophobicity; (w): weak positive; TC: Taurocholic acid; TDC: Taurodeoxycholic acid.

Deconjugation of Bile Salts

The ability of bacterial isolates to deconjugate bile salts was done by plate assay method using two bile salts; sodium salts of taurocholic acid (TC) and taurodeoxycholic (TDC) acid. The appearance of zone of precipitation along the line of inoculation was taken as a positive result. About 24% of the total isolates showed precipitation on taurodeoxycholic acid plates and hence were tested positive for deconjugation of TDC. About 41% of the total bacterial isolates showed a positive result (precipitation) in taurocholic acid plates and hence has the ability to deconjugate TC (Table 17).

Hydrophobicity Test

The ability of lactic acid bacterial isolates to adhere onto cell surfaces was checked by measuring the percentage hydrophobicity in n- hexadecane (Table 17). About 86% of the bacterial isolates showed a percentage hydrophobicity of more than 70% and only 8% of the total isolates exhibited a poor adherence ability (<50 *H*%) (Figure 24). *Enterococcus durans* BPB21 (*phab*, Bhutan) and *Enterococcus durans* SMB7 (*marcha,* Sikkim) showed the highest hydrophobicity percentage of 98%. *Enterococcus durans* DMB3 (*marcha,* Darjeeling) showed the lowest percentage hydrophobicity of 25% only.

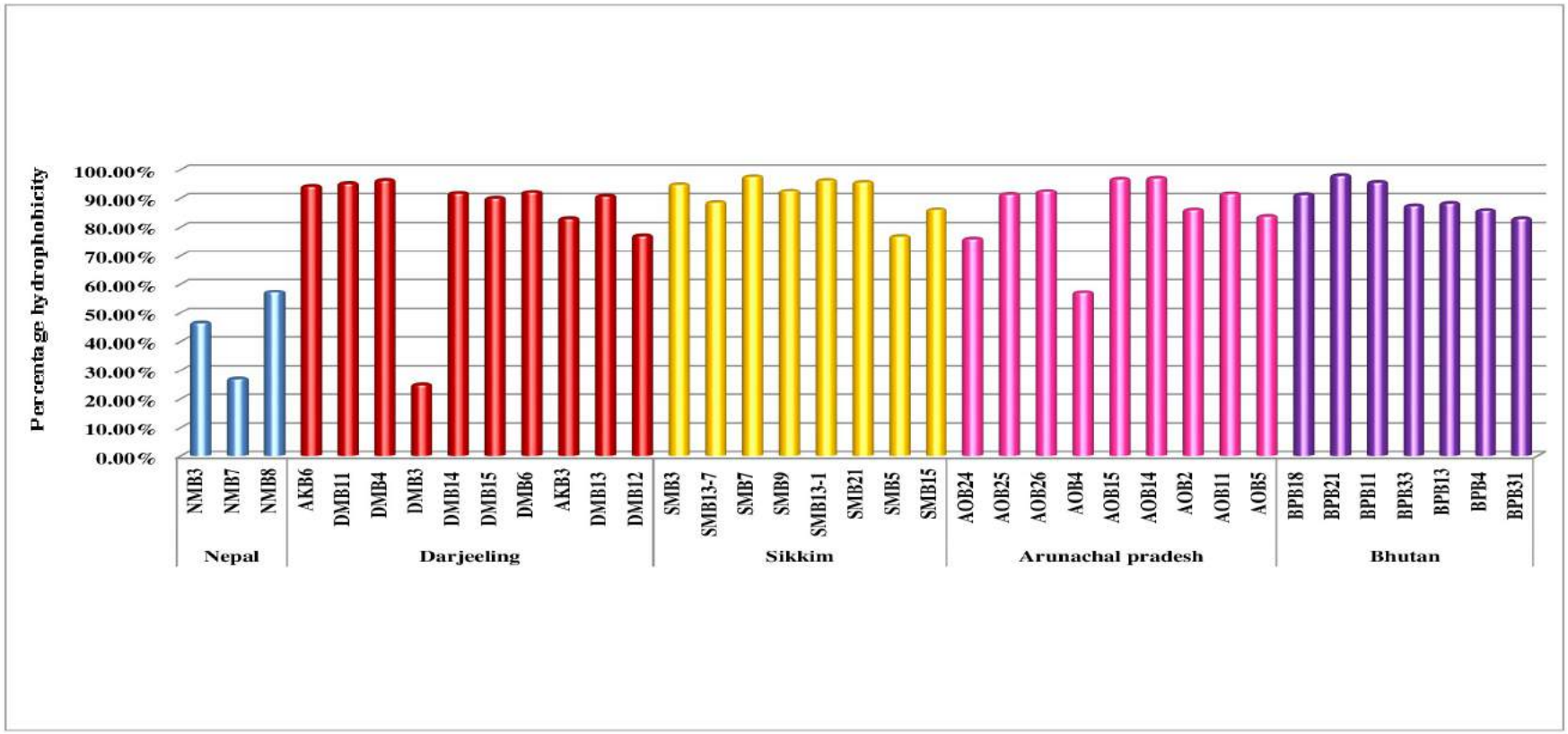


Figure 24: Percentage hydrophobicity of bacterial isolates from different starters of the Eastern Himalayas

β-Galactosidase Activity

β-galactosidase activity of the bacterial isolates was determined by plate assay technique using X gal and IPTG (isopropyl- thio β-D-galactopyranoside) as an inducer (Figure 25). About 65% of the total isolates showed positive result for beta galactosidase (Table 17).

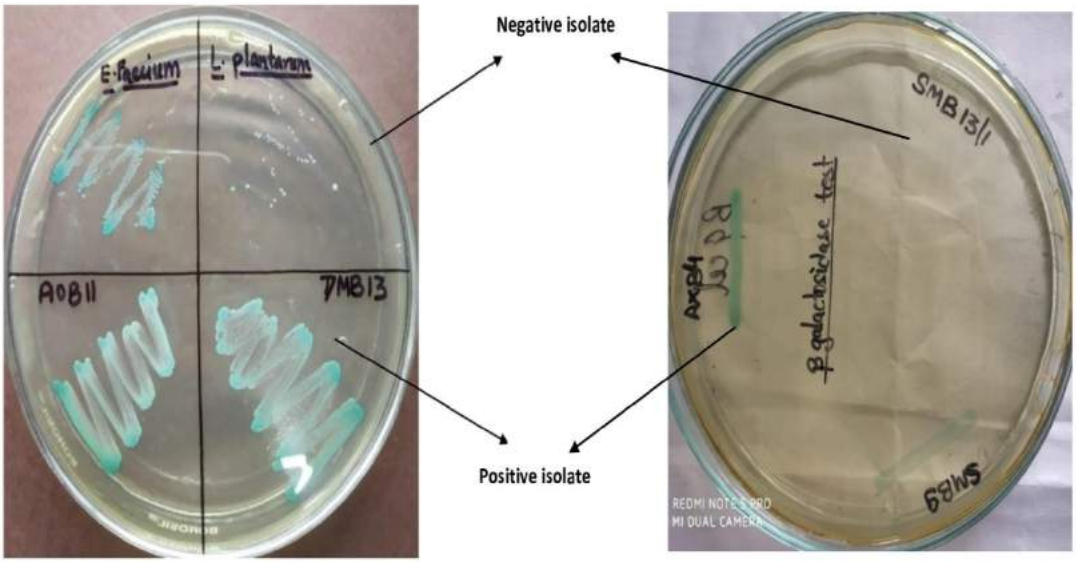


Figure 25: Plate screening assay showing β-galactosidase activity. Blue coloured colonies: positive isolates (producing β galactosidase); colourless/white coloured colonies: negative isolates (incapable of producing β galactosidase); positive control: *Lactobacillus plantarum* MTCC 1407(T) and *Enterococcus faceium* MCC 2763.

Cholesterol Assimilation

About 35% of the total isolates showed a cholesterol assimilation activity of more than 50% (Table 17). *Enterococcus durans* DMB4 (*marcha,* Darjeeling) showed the highest cholesterol assimilation of 80% (OD taken at 550nm). *Pediococcus pentosaceus* BPB13 (*marcha,* Bhutan) showed the lowest cholesterol assimilation activity of 2% only (Figure 26).

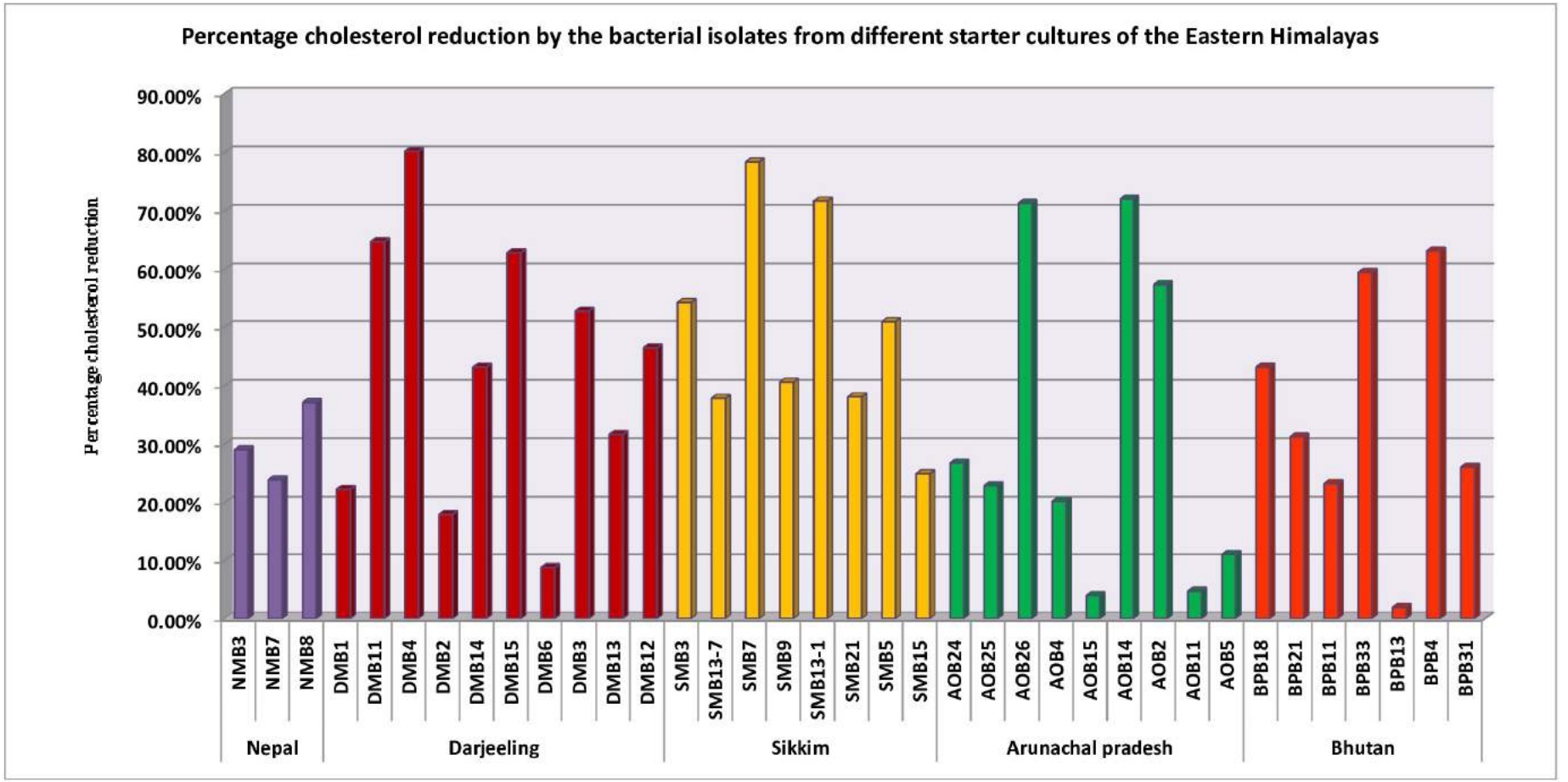


Figure 26: Percentage reduction of cholesterol by the bacterial isolates isolated from different starters of the Eastern Himalayas.

EVALUATION OF FUNCTIONAL ATTRIBUTES

Phytase Activity

Phytase activity of the bacterial isolates was checked by plate assay method using modified MRS agar and sodium phytate as a sole phosphate source. Appearance of clear zones around the line of inoculation was taken as a positive test (Figure 27). About 86% of the total test isolates showed the ability to produce the anti-nutritive degrading factor phytase (Table 18).

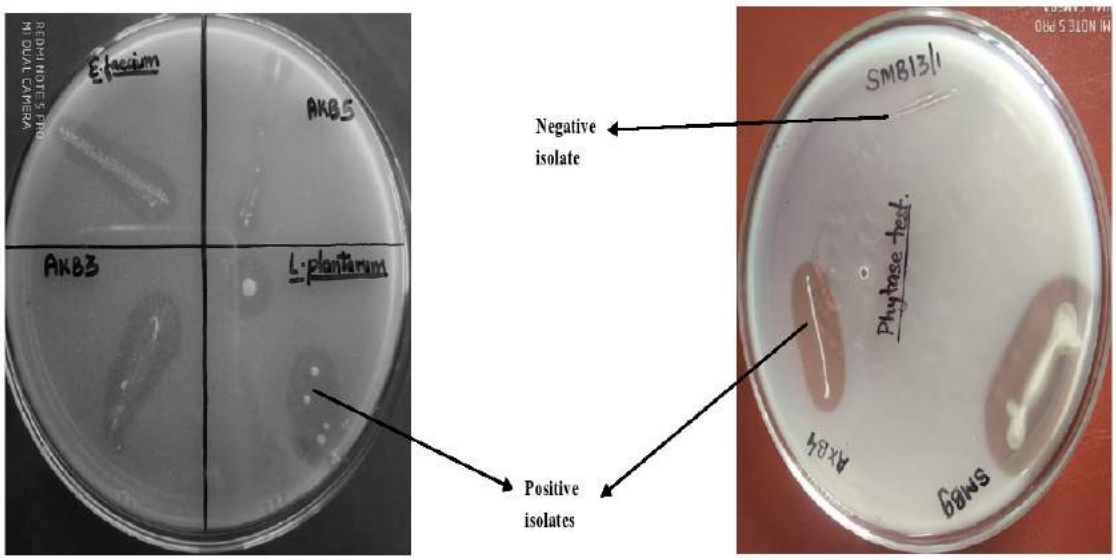


Figure 27: Plate screening assay showing phytase activity. Clear zone around the colonies: positive isolates (producing phytase); no zone of hydrolysis around the colonies: negative isolates (incapable of producing phytase); positive control: *Lactobacillus plantarum* MTCC 1407(T) and *Enterococcus faceium* MCC2763.

Table 18: Functional attributes and safety evaluation of lactic acid bacteria isolated from dry starter cultures of the Eastern Himalayas

Starter	Microorganisms/ isolates	Phytase activity	Amylase activity	Tolerance in 10% ethanol	Haemolysis
Marcha (Nepal)	*Lactobacillus pentosus* NMB3	-ve	-ve	-ve	-ve
Marcha (Nepal)	*Lactobacillus plantarum* subsp. *plantarum* NMB7	-ve	-ve	+ve	-ve
Marcha (Nepal)	*Lactobacillus plantarum* subsp. *plantarum* NMB8	-ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Leuconostoc mesenteroides* AKB6	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB11	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB4	+ve	-ve	-ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB3	-ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Pediococcus acidilactici* DMB14	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB15	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB6	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Pediococcus acidilactici* AKB3	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Enterococcus durans* DMB13	+ve	-ve	+ve	-ve
Marcha (Darjeeling)	*Pediococcus acidilactici* DMB12	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Enterococcus faecalis* SMB3	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Pediococcus pentosaceus* SMB13-7	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Enterococcus durans* SMB7	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Weissella cibaria* SMB9	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Leuconostoc mesenteroides* SMB13-1	-ve	-ve	+ve	-ve
Marcha (Sikkim)	*Enterococcus faecium* SMB21	+ve	-ve	+ve	-ve
Marcha (Sikkim)	*Enterococcus faecium* SMB5	+ve	-ve	+ve	-ve

Marcha (Sikkim)	*Enterococcus faecium* SMB15	+ve	-ve	+ve	-ve
Marcha (Bhutan)	*Enterococcus durans* BPB18	+ve	-ve	+ve	-ve
Marcha (Bhutan)	*Enterococcus durans* BPB21	+ve (w)	-ve	+ve	-ve
Marcha (Bhutan)	*Enterococcus faecium* BPB11	+ve	-ve	+ve	-ve
Marcha (Bhutan)	*Enterococcus faecium* BPB33	+ve (w)	-ve	+ve	-ve
Paa(Arunachal Pradesh)	*Enterococcus faecium* AOB4	+ve	-ve	+ve	-ve
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB2	+ve	-ve	+ve	-ve
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB11	+ve	-ve	+ve	-ve
Paa (Arunachal Pradesh)	*Enterococcus faecalis* AOB5	+ve	-ve	+ve	-ve
Pee (Arunachal Pradesh)	*Enterococcus faecium* AOB15	+ve	-ve	+ve	-ve
Pee (Arunachal Pradesh)	*Enterococcus durans* AOB14	+ve	-ve	+ve	-ve
Phut (Arunachal Pradesh)	*Enterococcus hirae* AOB24	+ve	-ve	+ve	-ve
Phut (Arunachal Pradesh)	*Enterococcus faecium* AOB25	+ve	-ve	+ve	-ve
Phut (Arunachal Pradesh)	*Enterococcus lactis* AOB26	+ve	-ve	+ve	-ve
Phab (Bhutan)	*Pediococcus pentosaceus* BPB13	+ve (w)	-ve	+ve	-ve
Phab (Bhutan)	*Enterococcus durans* BPB4	+ve	-ve	+ve	-ve
Phab (Bhutan)	*Enterococcus faecium* BPB31	+ve	-ve	+ve	-ve
+ve (w): weak positive; +ve: positive; -ve: negative					

Amylase Activity

The ability of the bacterial isolates to produce amylase was evaluated using plate screening method in starch agar. None of the isolates showed amylolytic activity indicating they have no role in saccharification and liquefaction of starchy substrates (Table 18).

Ethanol Tolerance Test

Tolerance of LAB cultures to ethanol was tested in MRS broth with varying concentration of ethanol (3%-10%) (Figure 28). About 86% of bacterial isolates showed optimum tolerance even at 10% ethanol (Table 19). *Enterococcus durans* DMB4 (*marcha,* Darjeeling) and *Lactobacillus pentosus* NMB3 (*marcha,* Nepal) showed tolerance to 7% ethanol after which no growth was observed in higher concentrations (Figure 29-33). 70% of the total isolates showed good tolerance ability at 3-9% ethanol (Figure 28).

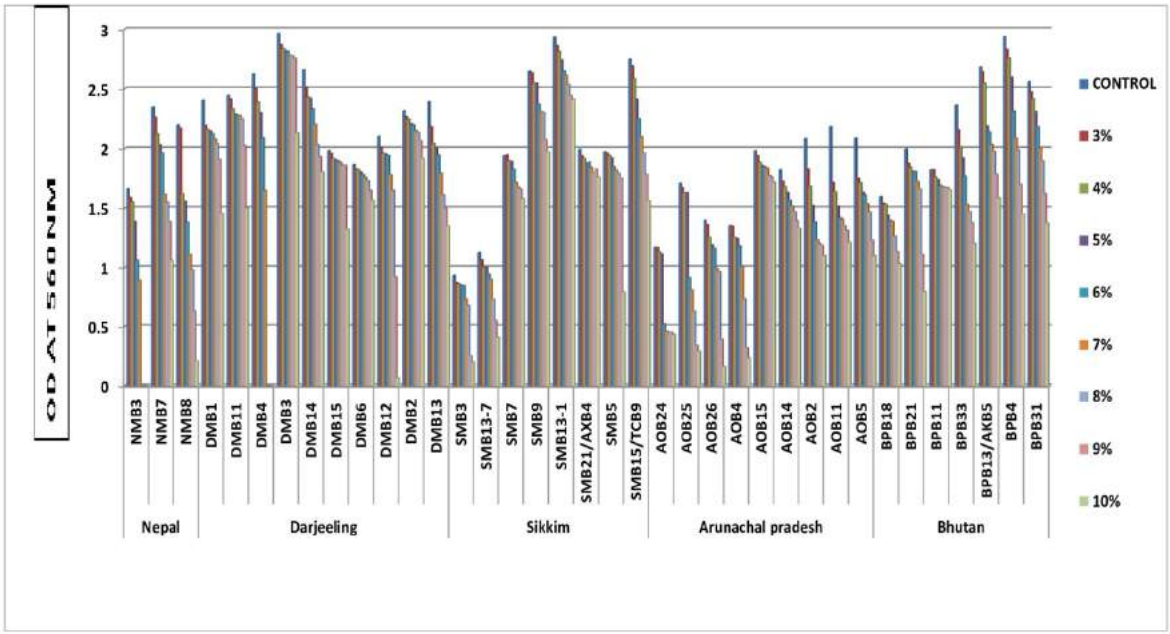


Figure 28: Growth of lactic acid bacteria isolated from starters of the Eastern Himalayas in different concentrations of ethanol.

Table 19: Ability of the bacterial isolates isolated from the dry starter cultures of the Eastern Himalayas to tolerate different concentration of ethanol										
Starter	**Bacteria**	**Control**	**Concentrations of Ethanol (%)**							
			3%	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**10%**
Marcha (Nepal)	*Lactobacillus pentosus* NMB3	1.66	1.59	1.55	1.39	1.06	0.89	0	0	0
	Lactobacillus plantarum subsp. *plantarum* NMB7	2.34	2.261	2.12	2.03	1.96	1.62	1.55	1.39	1.06
	Lactobacillus plantarum subsp. *plantarum* NMB8	2.19	2.17	1.62	1.55	1.38	1.11	0.98	0.63	0.21
Marcha (Darjeeling)	*Leuconostoc mesenteroides* AKB6	2.41	2.19	2.16	2.14	2.12	2.08	2.04	1.91	1.45
	Enterococcus durans DMB11	2.44	2.41	2.32	2.28	2.28	2.27	2.25	2.02	1.5
	Enterococcus durans DMB4	2.62	2.5	2.39	2.3	2.09	1.65	0	0	0
	Enterococcus durans DMB3	2.96	2.87	2.84	2.82	2.81	2.78	2.77	2.75	2.13
	Pediococcus acidilactici DMB14	2.66	2.51	2.43	2.41	2.33	2.20	2.03	1.93	1.80
	Enterococcus durans DMB15	1.98	1.95	1.91	1.91	1.89	1.88	1.86	1.85	1.31
	Enterococcus durans DMB6	1.86	1.82	1.82	1.81	1.78	1.75	1.73	1.65	1.57
	Pediococcus acidilactici AKB3	2.10	2.01	1.96	1.95	1.94	1.78	1.65	0.92	0.06
	Enterococcus durans DMB13	2.39	2.18	2.04	2.00	1.95	1.79	1.61	1.5	1.34
	Pediococcus acidilactici DMB12	2.31	2.26	2.24	2.21	2.19	2.15	2.13	2.07	1.92
Marcha (Sikkim)	*Enterococcus faecalis* SMB3	0.93	0.87	0.86	0.85	0.85	0.73	0.68	0.25	0.2
	Pediococcus pentosaceus SMB13-7	1.12	1.1	1.0	1.0	0.94	0.90	0.73	0.55	0.41
	Enterococcus durans SMB7	1.94	1.95	1.89	1.89	1.83	1.71	1.68	1.66	1.58
	Weissella cibaria SMB9	2.65	2.63	2.55	2.54	2.37	2.30	2.30	2.07	1.97
	Leuconostoc mesenteroides SMB13-1	2.93	2.86	2.81	2.74	2.65	2.61	2.53	2.44	2.41

Marcha (Sikkim)	*Enterococcus faecium* SMB21	1.99	1.94	1.91	1.88	1.89	1.84	1.8	1.83	1.76
	Enterococcus faecium SMB5	1.97	1.96	1.94	1.92	1.85	1.82	1.79	1.75	0.79
	Enterococcus faecium SMB15	2.75	2.69	2.58	2.41	2.24	2.09	1.96	1.79	1.56
Marcha (Bhutan)	*Enterococcus durans* BPB18	1.6	1.54	1.53	1.44	1.4	1.39	1.26	1.13	1.03
	Enterococcus faecium BPB11	1.82	1.82	1.76	1.74	1.69	1.68	1.67	1.67	1.65
	Pediococcus pentosaceus BPB13	2.68	2.64	2.55	2.19	2.14	2.03	1.98	1.79	1.59
	Enterococcus durans BPB4	2.94	2.83	2.76	2.60	2.31	2.09	1.99	1.70	1.45
Paa (Arunachal Pradesh)	*Enterococcus faecium* AOB4	1.35	1.34	1.25	1.24	1.18	1.00	0.73	0.32	0.23
	Enterococcus faecalis AOB2	2.08	1.83	1.68	1.52	1.39	1.23	1.2	1.19	1.09
	Enterococcus faecalis AOB11	2.19	1.71	1.64	1.52	1.42	1.41	1.35	1.31	1.21
	Enterococcus faecalis AOB5	2.08	1.75	1.71	1.63	1.61	1.54	1.47	1.22	1.2
Pee (Arunachal Pradesh)	*Enterococcus faecium* AOB15	1.98	1.94	1.88	1.86	1.85	1.84	1.77	1.76	1.72
	Enterococcus durans AOB14	1.82	1.73	1.68	1.63	1.56	1.52	1.47	1.39	1.33
Phut (Arunachal Pradesh)	*Enterococcus hirae* AOB24	1.17	1.17	1.13	1.11	0.52	0.46	0.45	0.45	0.43
	Enterococcus faecium AOB25	1.71	1.67	1.63	1.63	0.91	0.81	0.63	0.34	0.29
	Enterococcus lactis AOB26	1.4	1.36	1.25	1.19	1.16	0.99	0.96	0.39	0.16
Phab (Bhutan)	*Enterococcus durans* BPB21	1.99	1.88	1.84	1.81	1.81	1.72	1.66	1.1	0.80
	Enterococcus faecium BPB33	2.36	2.16	2.01	1.92	1.77	1.53	1.47	1.38	1.20
	Enterococcus faecium BPB31	2.56	2.47	2.41	2.31	2.18	2.00	1.90	1.62	1.38
All the values given above are OD taken at 560 nm after 24 h of incubation; Control: Cultures inoculated in MRS broth without ethanol										

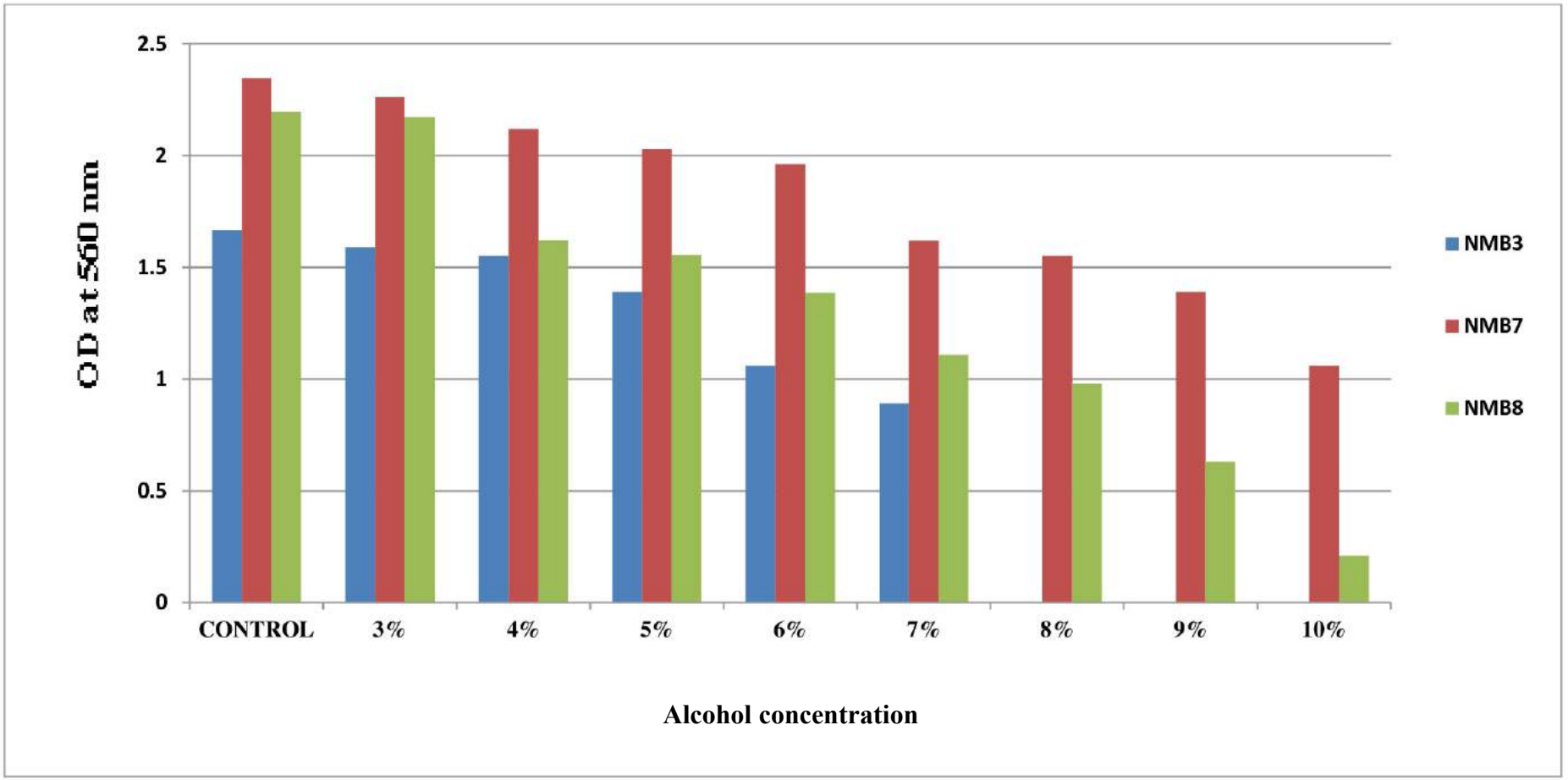


Figure 29: Growth of bacterial isolates from starters of Nepal at different concentration of ethanol.

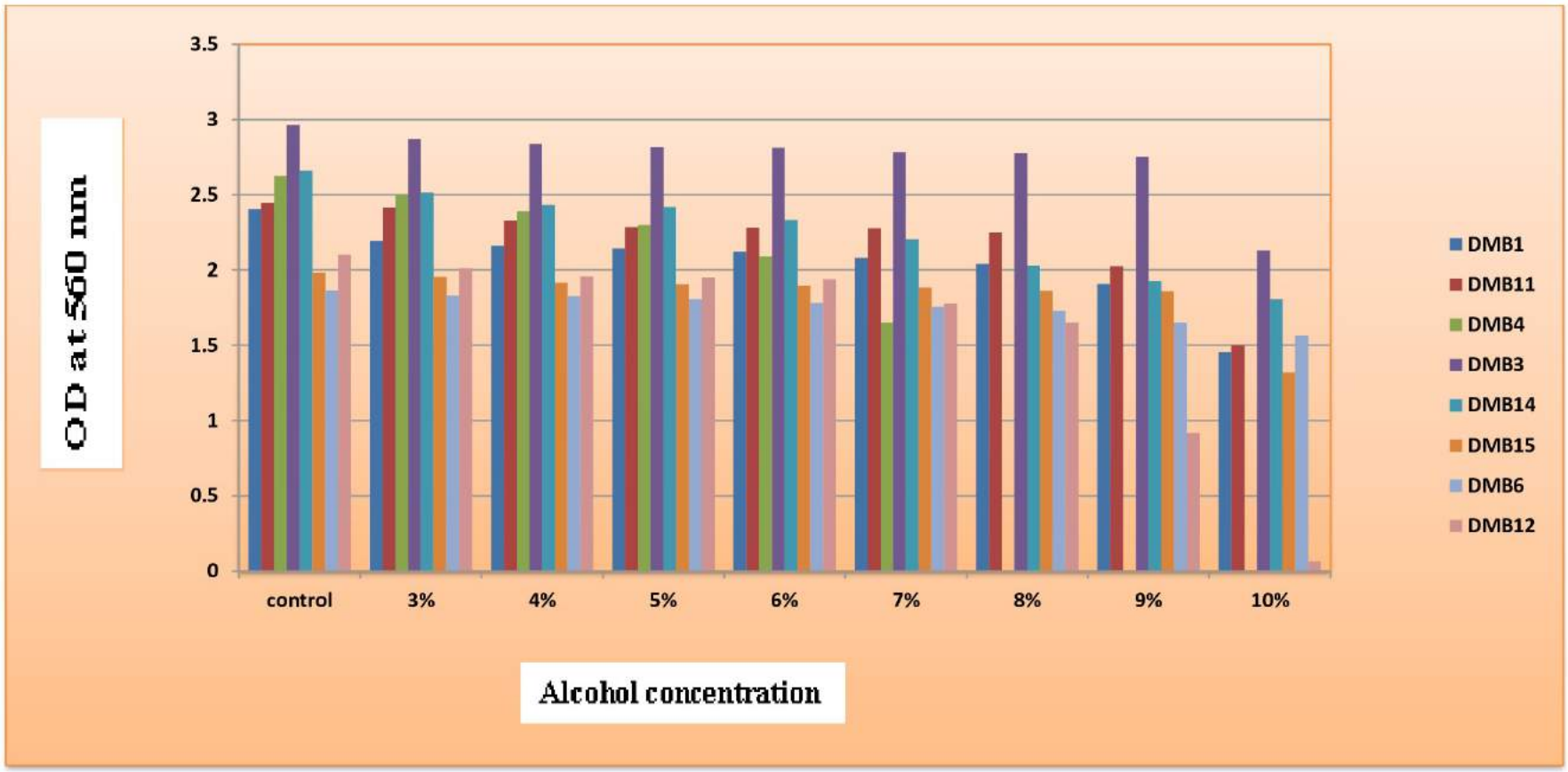


Figure 30: Growth of bacterial isolates from starters of Darjeeling at different concentration of ethanol.

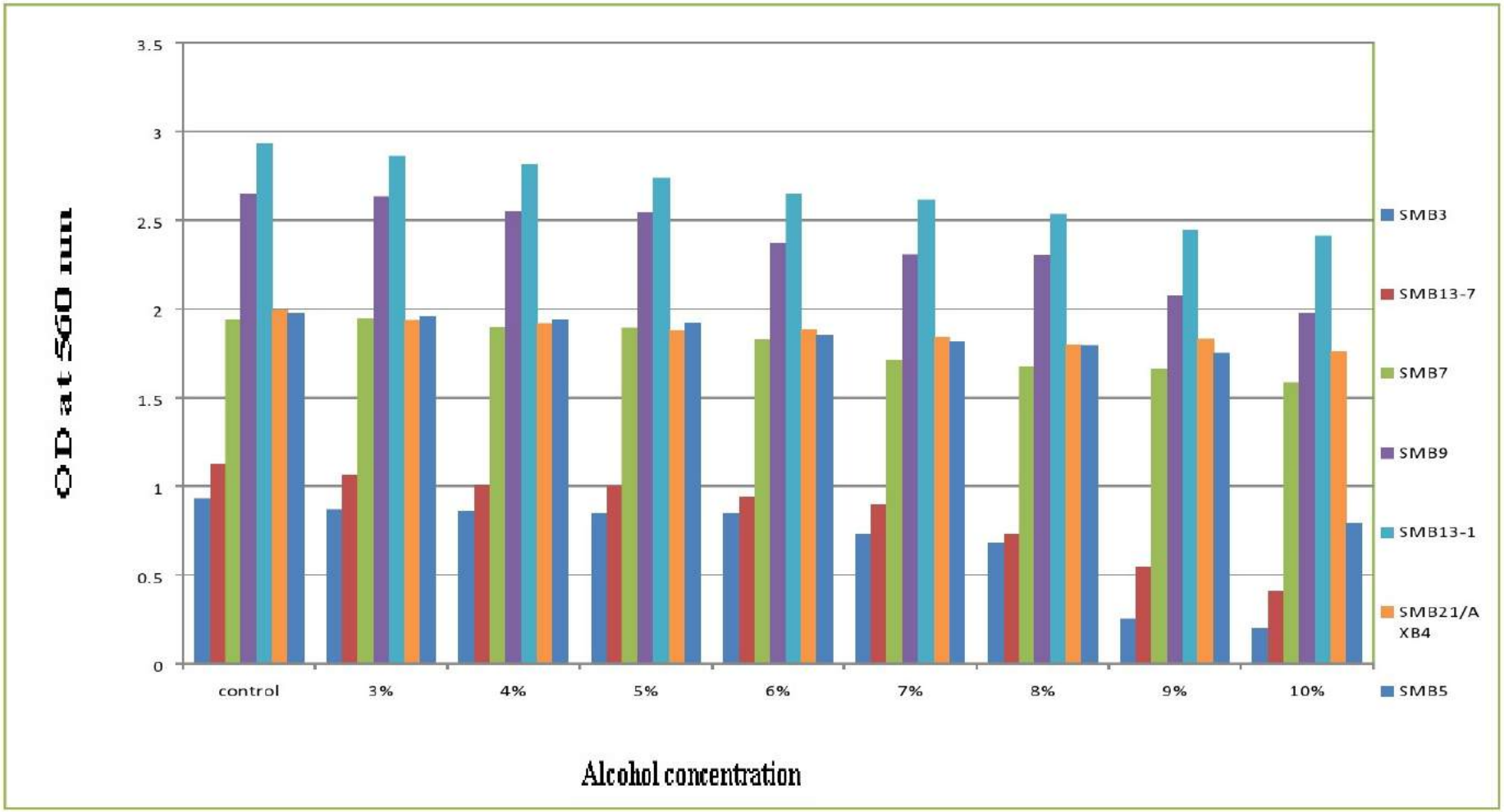


Figure 31: Growth of bacterial isolates from starters of Sikkim at different concentration of ethanol.

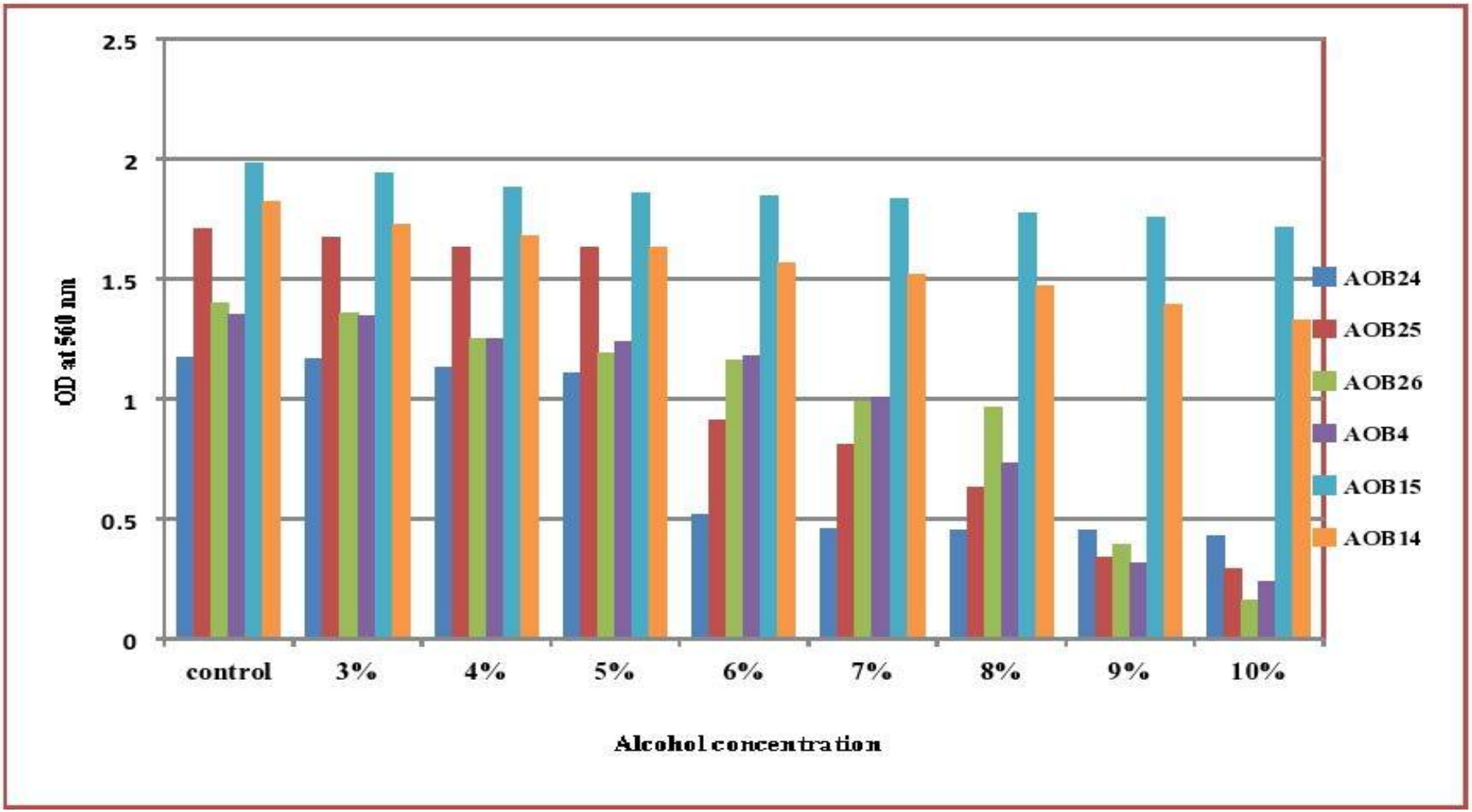


Figure 32: Growth of bacterial isolates from starters of Arunachal Pradesh at different concentration of ethanol.

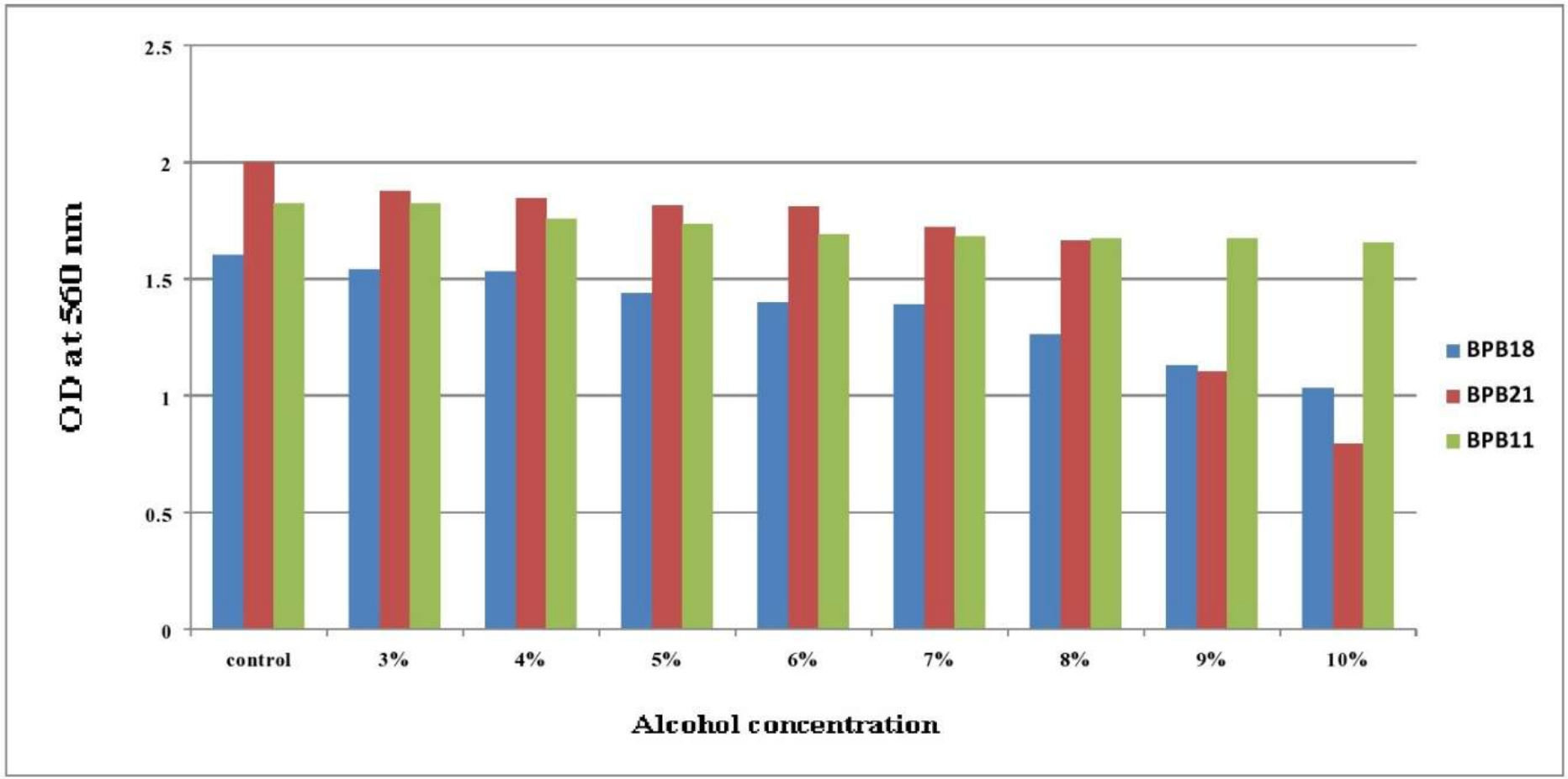


Figure 33: Growth of bacterial isolates from starters of Bhutan at different concentration of ethanol.

SCREENING FOR SAFETY EVALUATION

Haemolysis Activity

Plate screening assay in blood agar was performed to evaluate the safety of bacterial isolates. Occurrence of zone of α, β and γ haemolysis was observed in this test. No zone of haemolysis was observed in any of the inoculated plates hence the bacterial isolates were tested negative for haemolytic activity (Figure 34, Table 18).

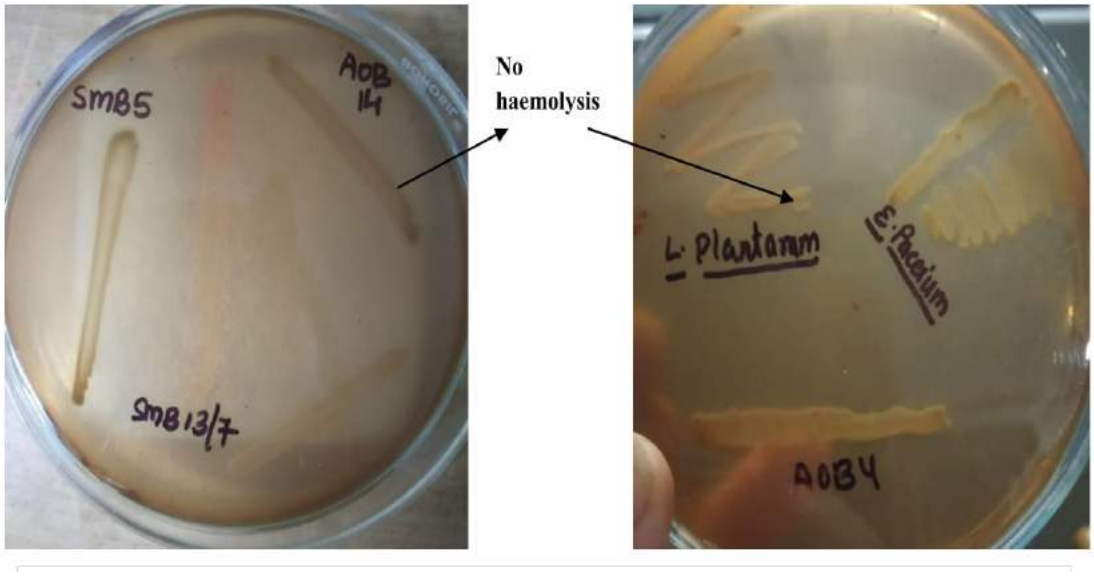


Figure 34: Plate screening assay on blood agar showing zone of γ haemolysis. Negative control: *Enterococcus faecium* MCC 2763 and *Lactobacillus plantarum* MTCC 1407(T).

SCREENING OF GENES CONTRIBUTING IN PROBIOTIC AND FUNCTIONAL ATTRIBUTES

Several genes that are involved in contributing essential attributes required for probiotic and functional characteristics were screened using primers in PCR based reactions. Specific primers with specific PCR conditions (PCR cycles, annealing temperature and product size in base pairs) (Figure 35) were followed for each of the genes that was screened (Table B). All 37 isolates of the lactic acid bacteria were screened for probiotic and functional genes.

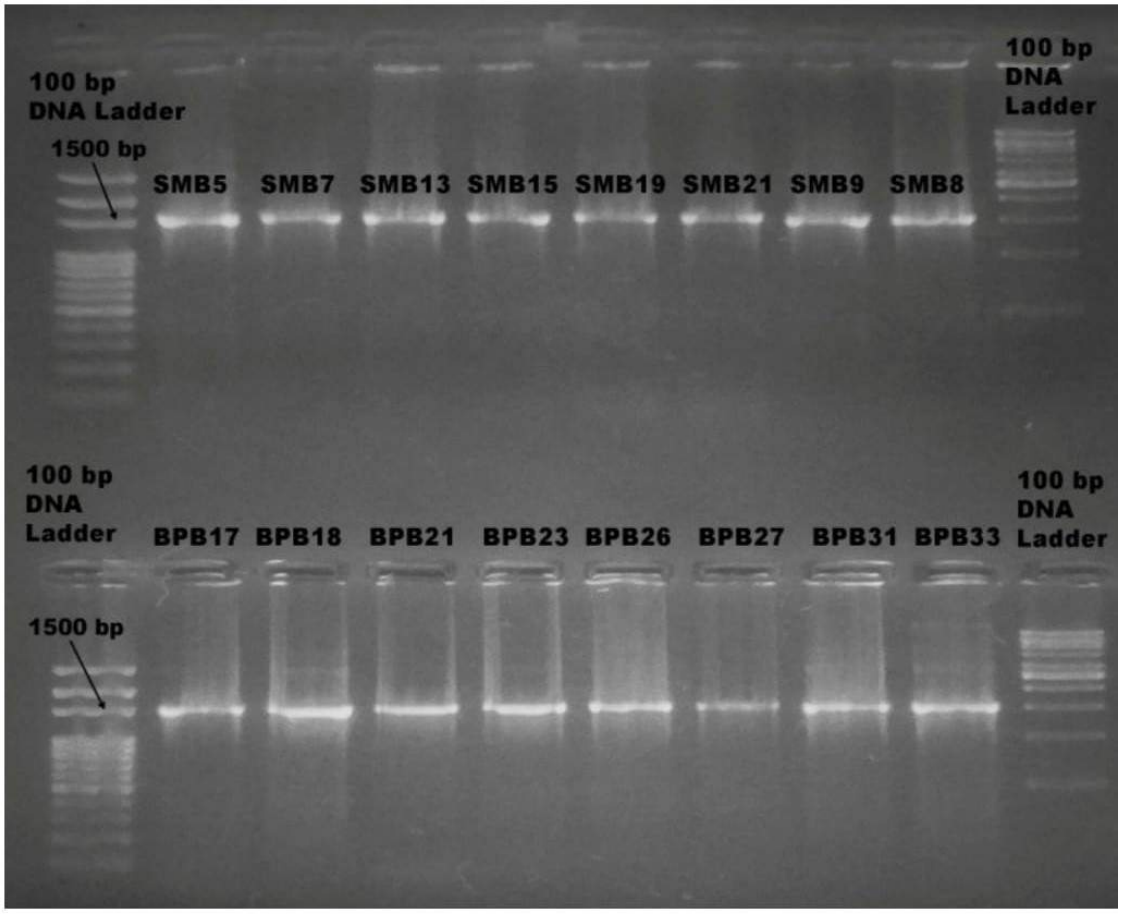


Figure 35: Amplified 16S rRNA gene product of size 1465 bp.

Detection of genes involved in biosynthesis of riboflavin

The ability of bacterial isolate to synthesize riboflavin was checked by screening the presence of *rib A* gene. Out of 37 bacterial isolates screened 22 LAB isolates were found positive for *rib A* gene. The product size of amplified *rib A* gene was 121 bp (Figure 36). *Enterococcus durans* BPB21 (*phab*, Bhutan); *Pediococcus acidilactici* DMB12 (*marcha,* Darjeeling); *Leuconostoc mesenteroides* SMB13-7 (*marcha,* Sikkim); *Enterococcus durans* SMB7 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Enterococcus durans* DMB6 (*marcha,* Darjeeling); *Enterococcus durans* DMB15 (*marcha,* Darjeeling); *Enterococcus faecalis* SMB3 (*marcha,* Sikkim); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh); *Enterococcus faecium* AOB15 (*pee*, Arunachal Pradesh); *Pediococcus acidilactici* DMB14 (*marcha,* Darjeeling); *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling); *Enterococcus faecalis* AOB2 (*paa*, Arunachal Pradesh); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) and *Enterococcus faecium* SMB21 (*marcha,* Sikkim) showed the presence of *rib A* gene for biosynthesis of riboflavin (Figure 36).

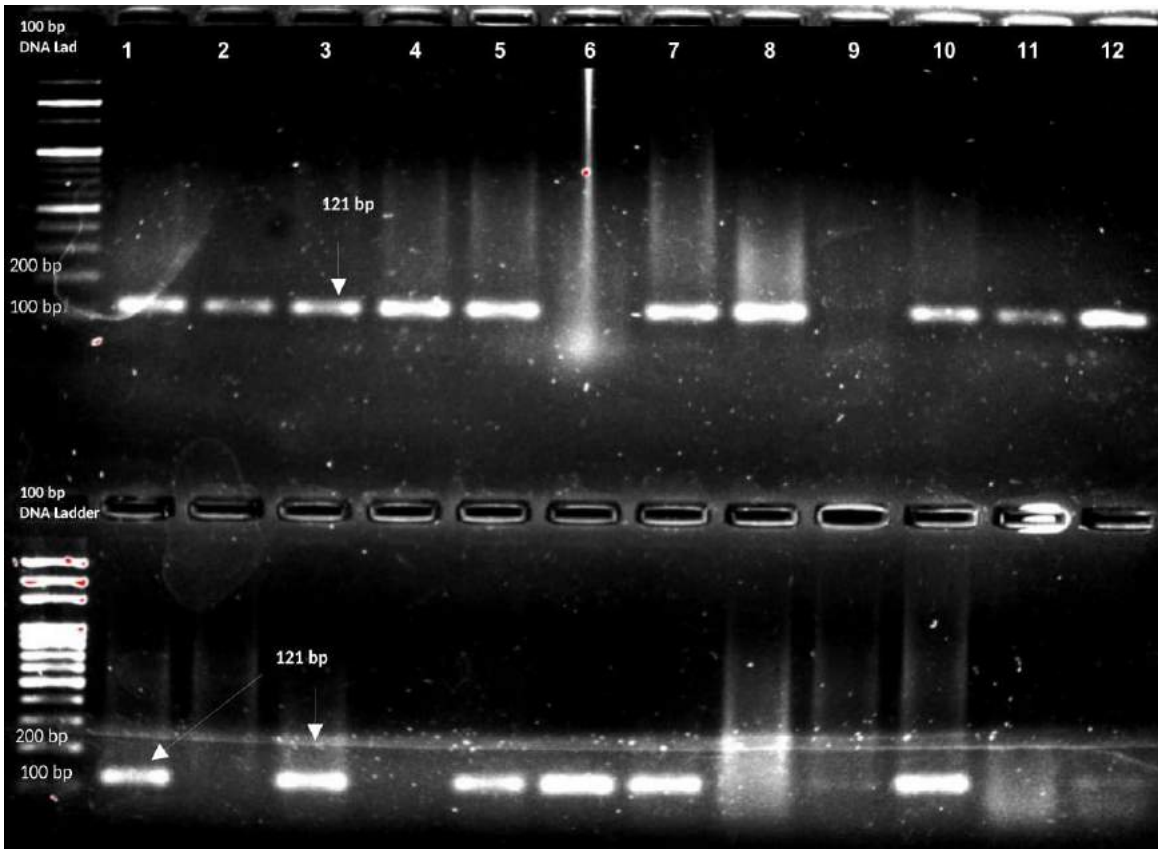


Figure 36: Amplified *rib A* gene of 121bp. Lane 1: *Enterococcus durans* AOB14; Lane 2: *Leuconostoc mesenteroides* AKB6; Lane 3: *Pediococcus acidilactici* AKB3; Lane 4: *Weissella cibaria* SMB9; Lane 5: *Enterococcus faecium* SMB21; Lane 7: *Enterococcus durans* SMB7; Lane 8: *Enterococcus faecalis* SMB3; Lane 10: *Enterococcus faecium* AOB4; Lane 11: *Lactobacillus plantarum* MTCC 2034 (Positive control); Lane 12: *Lactobacillus plantarum* MTCC 2794 (Positive control).

Detection of Gene Involved In Biosynthesis of Folate

Fol P gene primers were used to check the presence of *fol P* gene in the bacterial isolates to check their ability to biosynthesize folic acids. Out of 37 isolates screened only *Enterococcus faecium* AOB25 (*phut,* Arunachal Pradesh) possessed this gene for folate biosynthesis (Table 20). The amplified product size was 261 bp (Figure 37).

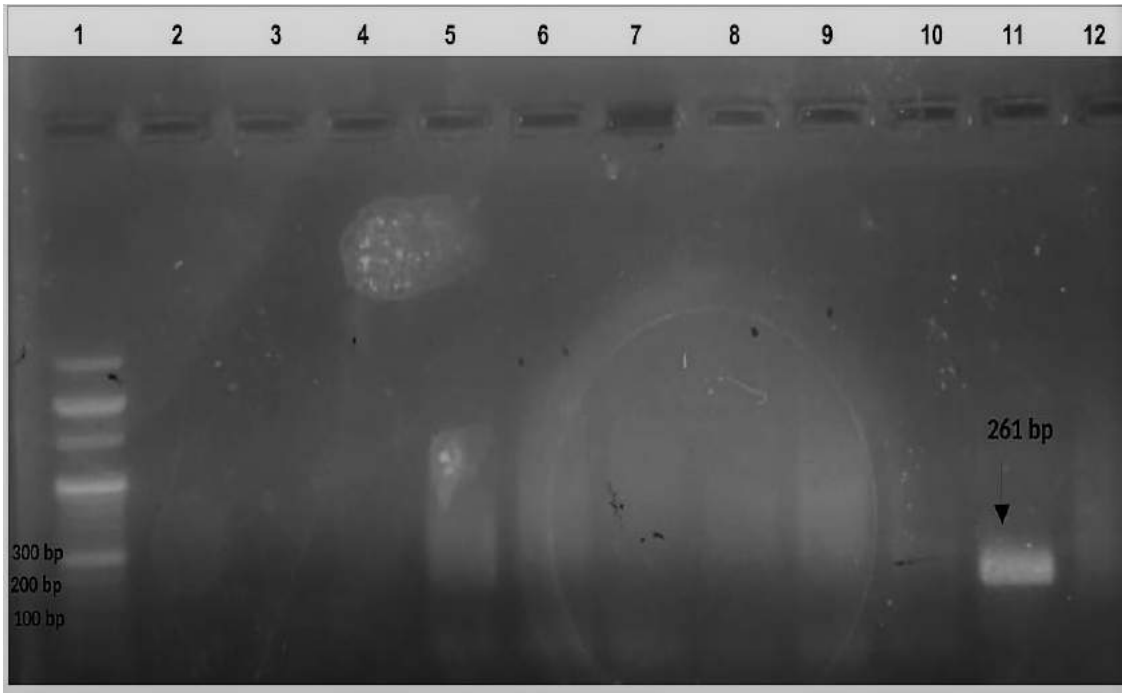


Figure 37: Amplified *fol p* gene of 261 bp. Lane 1:100 bp DNA ladder; Lane 11: *Enterococcus faecium* AOB 25.

Detection of Genes Involved In Bile Salt Survival

The bacterial isolates were screened for *clp L, bsh, Ir1584, Ir1516* and *LBA 1446* genes which were involved in imparting the ability to survive in bile concentrations.

The product size of *clp L* gene was 158 bp (Figure 38). Out of 37 isolates screened 24 isolates showed the presence of *clp L* gene. *Clp L* gene was detected in *Enterococcus durans* DMB4 (*marcha,* Darjeeling); *Enterococcus faecium* SMB5 (*marcha,* Sikkim); *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling); *Enterococcus durans* BPB21 (*phab,* Bhutan); *Enterococcus faecium* AOB25 (*phut,* Arunachal Pradesh); *Enterococcus faecalis* AOB11 (*paa*, Arunachal Pradesh); *Enterococcus faecium* BPB33 (*phab,* Bhutan); *Enterococcus durans* DMB13 (*marcha,* Darjeeling); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* BPB4 (*marcha*, Bhutan); *Enterococcus faecalis* AOB5 (*paa*, Arunachal Pradesh); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Enterococcus durans* SMB7 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Enterococcus faecalis* SMB3 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh); *Enterococcus faecium* BPB11 (*marcha*, Bhutan); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Enterococcus durans* DMB6 (*marcha,*Darjeeling); *Enterococcus durans* DMB15 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB8 (*marcha,* Nepal); and *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) (Figure 38).

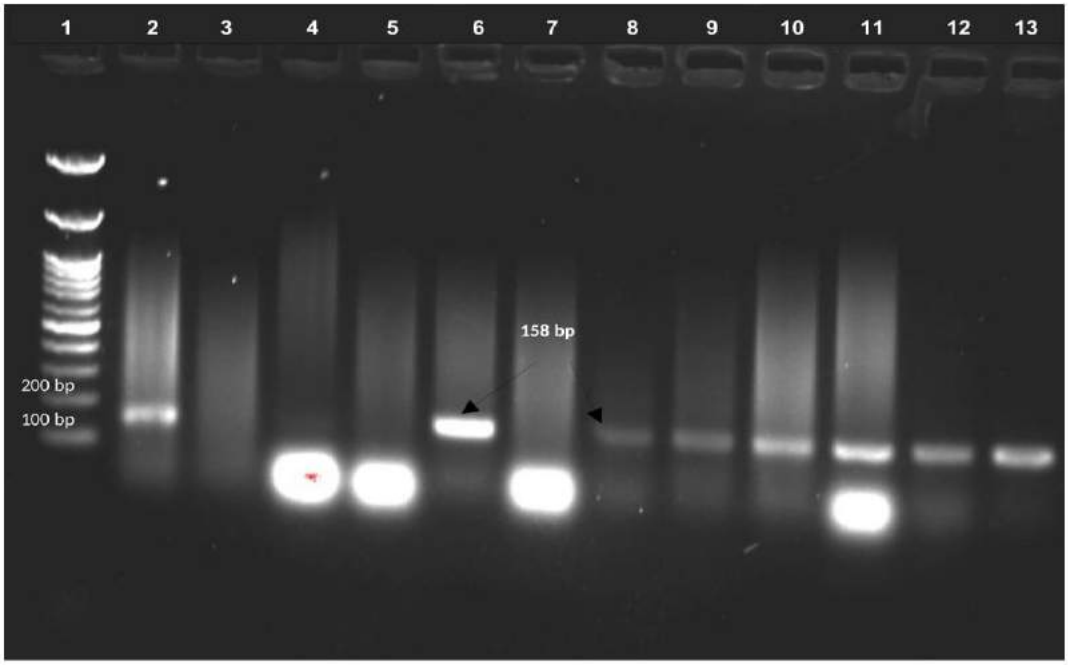


Figure 38: Amplified *clp L* gene of 158 bp. Lane 1: 100 bp DNA ladder; Lane 2: *Enterococcus durans* AOB14; Lane 6: *Enterococcus faecalis* SMB3; Lane 8: *Enterococcus lactis* AOB26; Lane 9: *Enterococcus faecium* BPB11; Lane 10: *Leuconostoc mesenteroides* AKB6; Lane 11: *Weissella cibaria* SMB9; Lane 12: *Lactobacillus plantarum* MTCC 2034 (positive control); Lane 13: *Lactobacillus plantarum* MTCC 2794 (positive control).

The amplified product size of *bsh* gene was 205 bp (Figure 39). Only two strains *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim) and *Enterococcus faecium* BPB11 (*marcha*, Bhutan) showed the presence of *bsh* gene (Table 20).

Size of *Ir1516* gene was 143 bp (Figure 40) which was detected in *Enterococcus faecium* SMB21 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus faecium* SMB15 (*marcha,* Sikkim); *Enterococcus faecium* BPB31 (*phab*, Bhutan); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) (Figure 40).

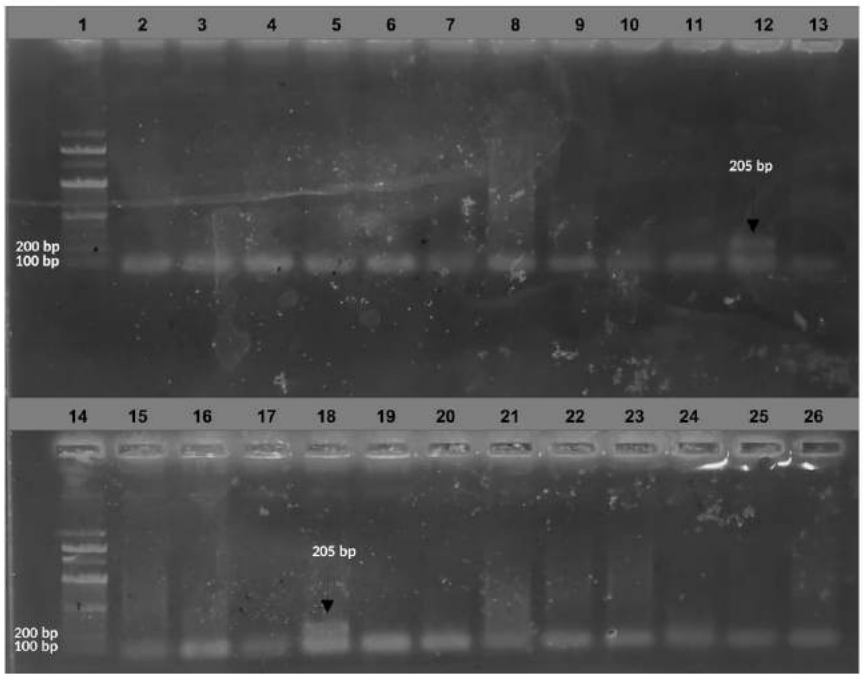


Figure 39: Amplified *bsh* gene of 205 bp. Lane 1: 100 bp DNA ladder; Lane 12: *Pediococcus pentosaceus* SMB13-1; Lane 14: 100 bp DNA ladder; Lane 18: *Enterococcus faecium* BPB11.

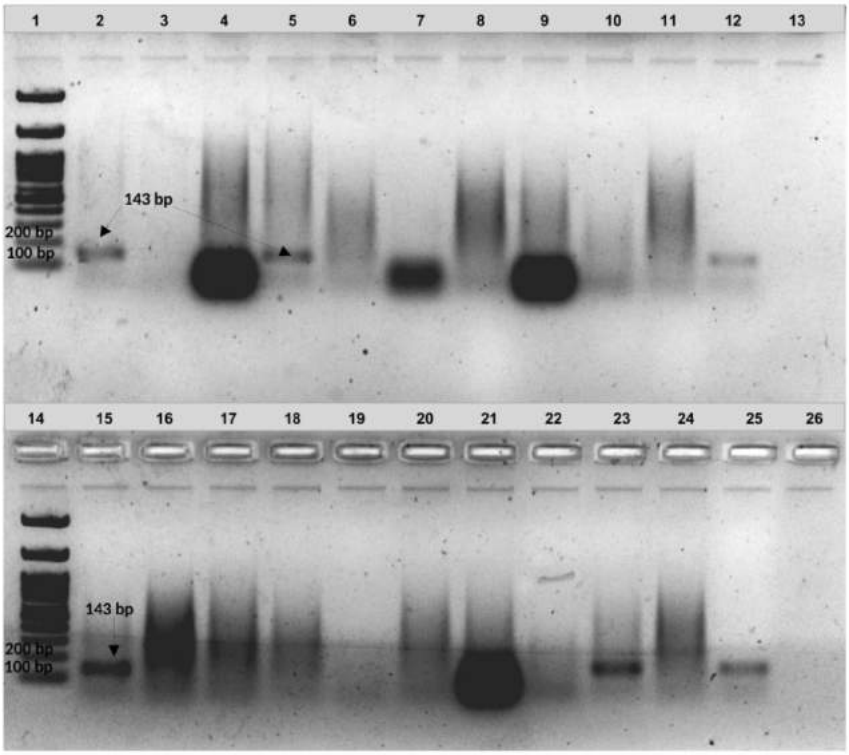


Figure 40: Amplified *Ir1516* gene of 143 bp. Lane 1: 100 bp DNA ladder; Lane 2: *Lactobacillus plantarum* MTCC 2034 (positive control), Lane 5: *Enterococcus durans* BPB18, Lane 12: *Pediococcus pentosaceus* SMB13-1, Lane 15: *Weissella cibaria* SMB9, Lane 23: *Pediococcus pentosaceus* BPB13, Lane 25: *Enterococcus faecium* SMB15.

LBA 1446 gene with size of 275 bp (Figure 41) was detected in 7 isolates; *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* BPB4 (*marcha*, Bhutan); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Enterococcus faecalis* SMB3 (*marcha,* Sikkim); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); and *Enterococcus hirae* AOB24 (*phut*, Arunachal Pradesh) (Figure 41). *Lactobacillus plantarum* MTCC 2794 was used a positive control for this test.

Ir1584 gene was not detected in any tested isolate.

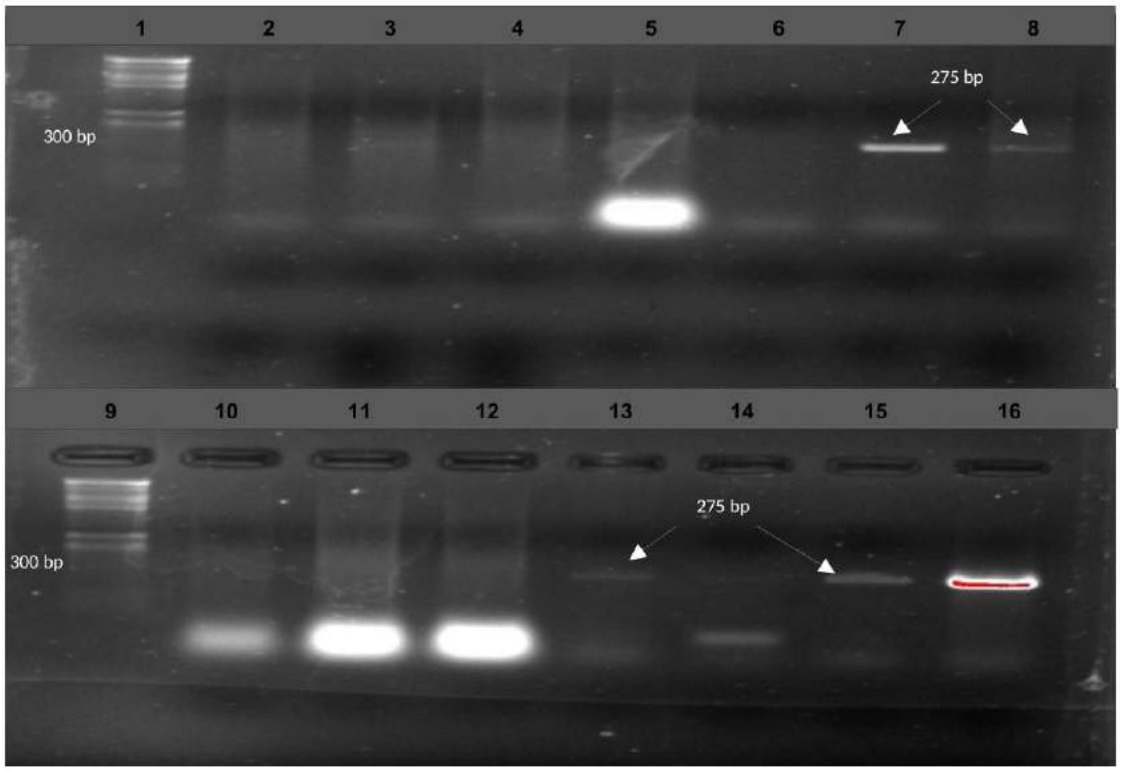


Figure 41: Amplified *LBA1446* gene of 275 bp. Lane 1: 100 bp DNA ladder; Lane 7: *Leuconostoc mesenteroides* AKB6; Lane 8: *Lactobacillus plantarum* MTCC 2034 (positive control); Lane 9: 100 bp DNA ladder; Lane 13: *Enterococcus faecalis* SMB3; Lane 15: *Pediococcus pentosaceus* SMB13-1; Lane 16: *Lactobacillus plantarum* MTCC 2794.

Detection of Genes Involved In Survival at Low pH

The ability of the bacterial isolates to survive and proliferate at low pH conditions were analyzed by screening the presence and absence of *hdc, odc, tdc, agu A, clp L* and *Ir1516* genes. *Clp L* and *Ir1516* gene were also involved in imparting tolerance in bile conditions. All 37 isolates screened were negative for *hdc, odc* and *tdc* gene.

Agu A gene product size was 542 bp (Figure 42) and 12 of the bacterial isolates tested were positive for this gene. *Enterococcus faecium* SMB21 (*marcha,* Sikkim); *Enterococcus durans* DMB6 (*marcha,* Darjeeling); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh), *Enterococcus hirae* AOB24 (*phut*, Arunachal Pradesh), *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) and *Lactobacillus plantarum* subsp. *plantarum* NMB8 (*marcha,* Nepal) were found to be positive for *agu A* gene (Figure 42).

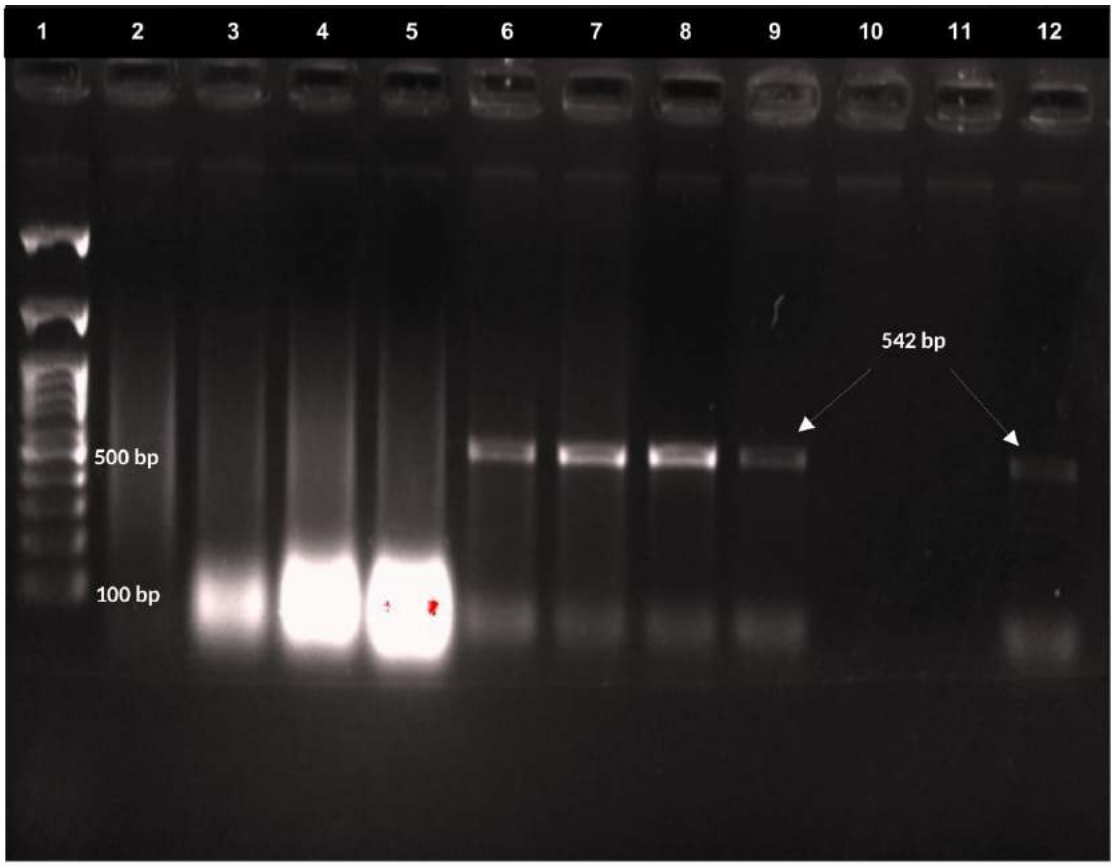


Figure 42: Amplified *agu A* gene of 542 bp. Lane 1: 100 bp DNA ladder; Lane 6: *Pediococcus pentosaceus* SMB13-1; Lane 7: *Enterococcus durans* DMB3; Lane 8: *Enterococcus lactis* AOB26; Lane 9: *Lactobacillus plantarum* subsp. *plantarum* NMB8; Lane 12: *Lactobacillus plantarum* MTCC 2794 (positive control).

Detection of Genes Involved In Adhesion

Mub, fbp, sor, sbp, msa, apf, map A and *mub1* genes were screened to check the ability of bacterial isolates to adhere to the mucoid membrane. All 37 isolates that were screened for the adhesion genes gave negative results for *mub, sor, sbp* and *msa* gene. Only one of the isolate *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling) was found to be positive for *fbp gene*. The amplicon product size of *fbp* gene was 835 bp (Figure 43).

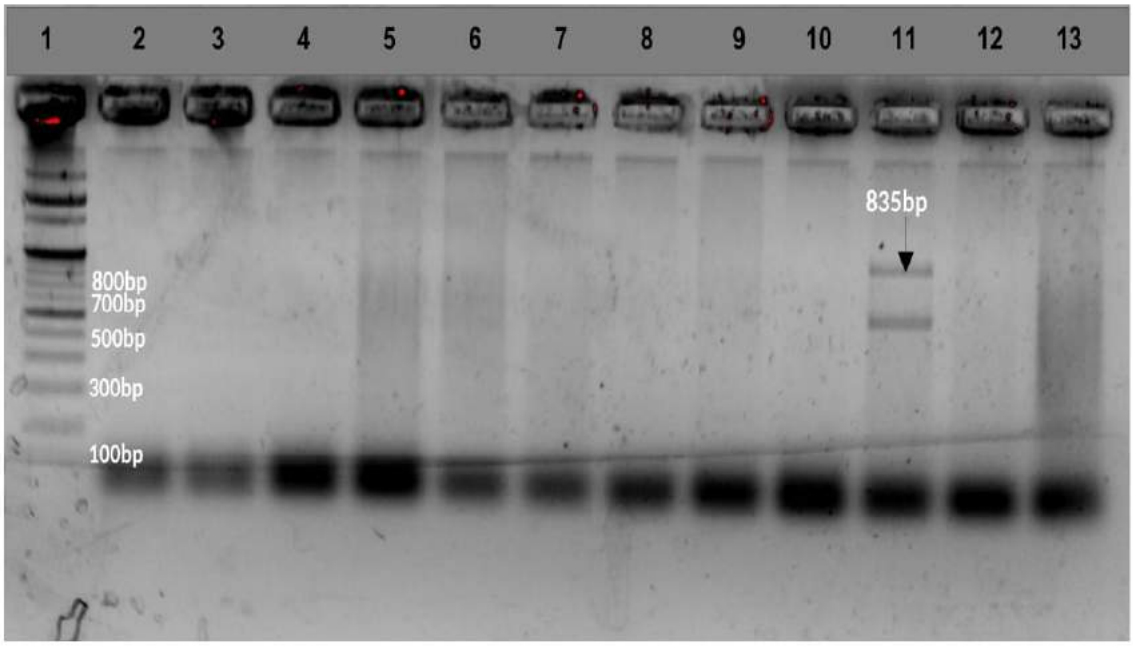


Figure 43: Amplified *fbp* gene of 835 bp. Lane 1: 100 bp DNA ladder; Lane 11: *Leuconostoc mesenteroides* AKB6.

Total 23 bacterial isolates screened for *apf* gene gave positive result. The product size of *apf* gene was 112 bp (Figure 44). *Enterococcus faecium* SMB21 (*marcha,* Sikkim); *Enterococcus durans* DMB6 (*marcha,* Darjeeling); *Leuconostoc mesenteroides* SMB13-7 (*marcha,* Sikkim); *Enterococcus durans* SMB7 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Enterococcus durans* DMB15 (*marcha,* Darjeeling); *Enterococcus faecalis* SMB3 (*marcha,* Sikkim); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh); *Enterococcus hirae* AOB24 (*phut*, Arunachal Pradesh); *Enterococcus faecium* BPB11 (*marcha*, Bhutan); *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); *Enterococcus durans* DMB4 (*marcha,* Darjeeling); *Enterococcus faecium* SMB5 (*marcha,* Sikkim); *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling); *Enterococcus faecium* AOB25 (*phut*,

Arunachal Pradesh); *Enterococcus faecalis* AOB2 (*paa*, Arunachal Pradesh); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* BPB4 (*marcha*, Bhutan); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) and *Lactobacillus plantarum* subsp. *plantarum* NMB8 (*marcha,* Nepal) were tested positive for *apf* gene (Figure 44). *Lactobacillus plantarum* MTCC 2794 and *Lactobacillus fermentum* MCC 2760 was used a positive control for this test.

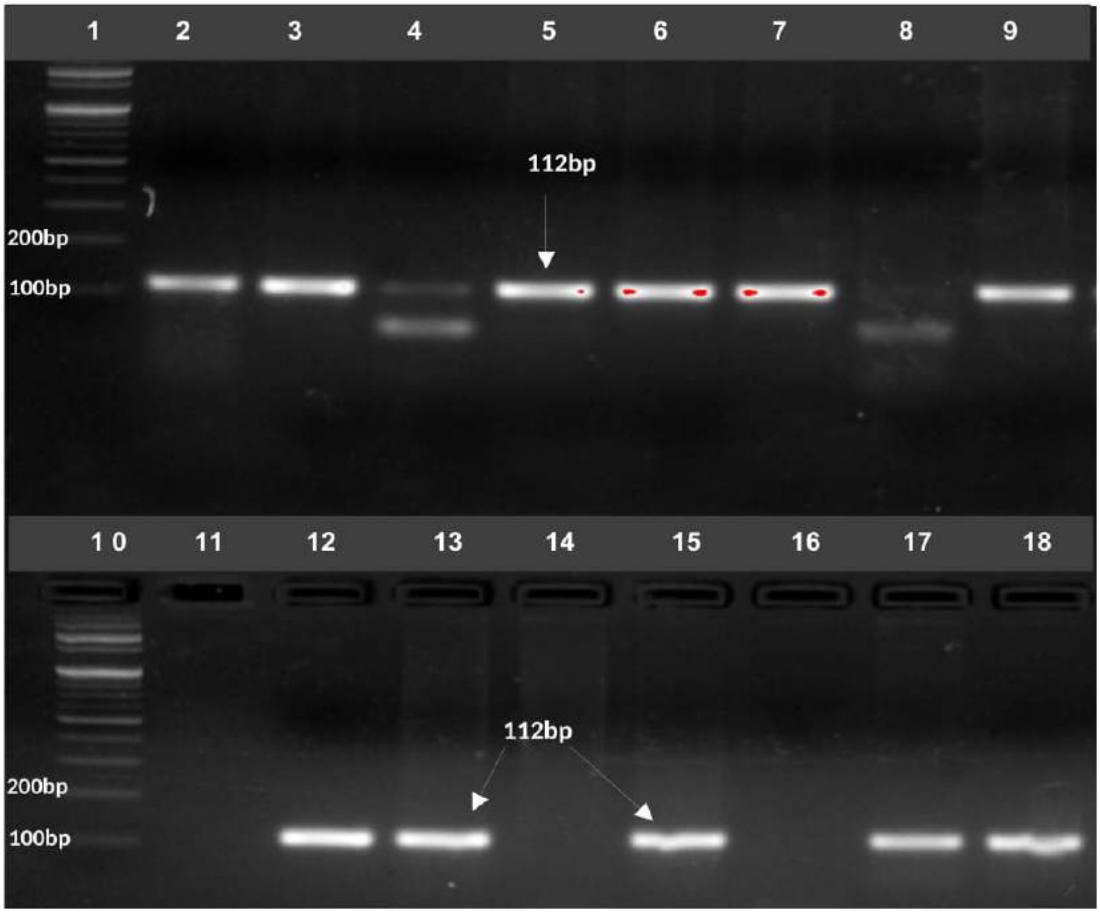


Figure 44: Amplified *apf* gene of 112 bp. Lane 1: 100 bp DNA ladder; Lane 2: *Enterococcus lactis* AOB26; Lane 3: *Pediococcus pentosaceus* SMB13-1; Lane 4: *Enterococcus durans* DMB6; Lane 5: *Lactobacillus plantarum* subsp. *plantarum* NMB8; Lane 6: *Enterococcus durans* DMB4; Lane 7: *Lactobacillus plantarum* subsp. *plantarum* NMB7; Lane 9: *Lactobacillus plantarum* MTCC 2794 (positive control); Lane 10: 100 bp DNA ladder; Lane 12: *Pediococcus pentosaceus* BPB13; Lane 13: *Enterococcus faecalis* AOB2; Lane 15: *Leuconostoc mesenteroides* AKB6; Lane 17: *Enterococcus hirae* AOB24; Lane 18: *Lactobacillus fermentum* MCC 2760 (positive control).

The amplicon size of *mapA* gene was 156 bp (Figure 45). *Lactobacillus plantarum* MTCC 2034 and *Lactobacillus plantarum* MTCC 2794 were used as a positive control in this test. *Enterococcus faecium* SMB21 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Enterococcus durans* DMB6 (*marcha,* Darjeeling); *Enterococcus durans* DMB15 (*marcha,* Darjeeling); *Pediococcus acidilactici* DMB12 (*marcha,* Darjeeling); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh); *Enterococcus faecium* BPB11 (*marcha*, Bhutan); *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling); *Enterococcus durans* BPB21 (*phab*, Bhutan); *Enterococcus faecalis* AOB2 (*paa*, Arunachal Pradesh); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus durans* BPB4 (*marcha*, Bhutan); *Enterococcus faecalis* AOB5 (*paa*, Arunachal Pradesh); *Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) and *Lactobacillus plantarum* subsp. *plantarum* NMB8 (*marcha,* Nepal) possessed *map A* gene (Figure 45).

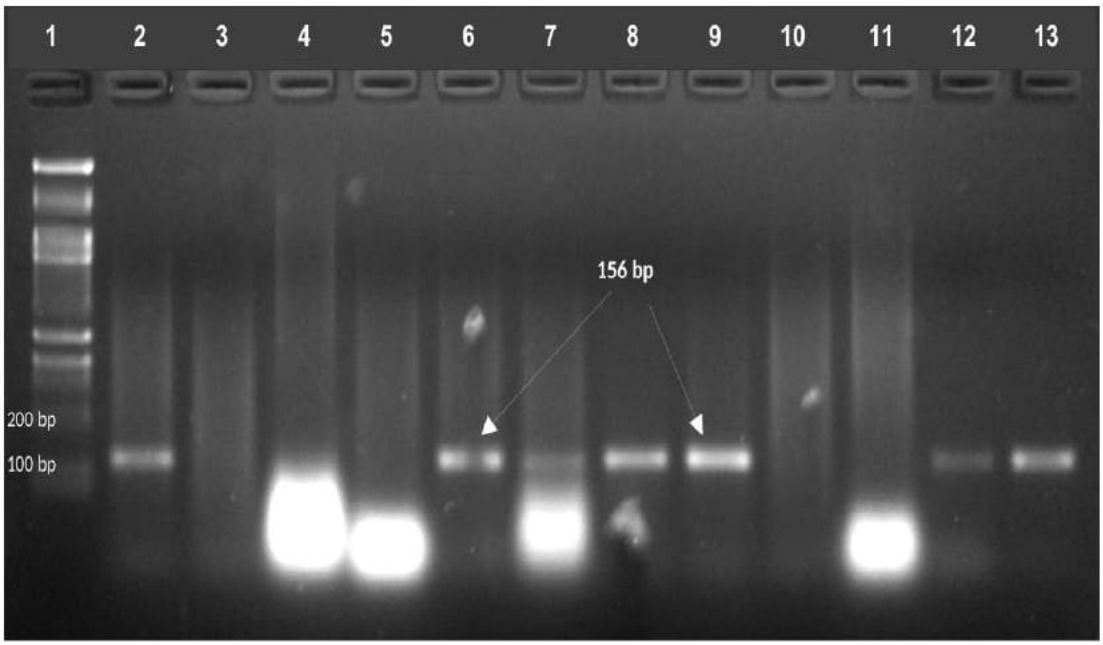


Figure 45: Amplified *map A* gene of 156 bp. Lane 1: 100 bp DNA ladder; Lane 2: *Enterococcus faecium* SMB21; Lane 6: *Enterococcus durans* DMB15; Lane 7: *Pediococcus acidilactici* DMB12; Lane 8: *Enterococcus durans* AOB14; Lane 9: *Pediococcus pentosaceus* SMB13-1; Lane 12: *Weissella cibaria* SMB9; Lane 13: *Lactobacillus plantarum* MTCC 2794 (positive control).

Mub 1 gene of 150 bp (Figure 46) was detected in *Enterococcus faecium* SMB21 (*marcha,* Sikkim); *Enterococcus durans* BPB18 (*marcha*, Bhutan); *Enterococcus durans* DMB6 (*marcha,* Darjeeling); *Enterococcus durans* DMB15 (*marcha,* Darjeeling); *Leuconostoc mesenteroides* SMB13-7 (*marcha,* Sikkim); *Enterococcus durans* DMB11 (*marcha,* Darjeeling); *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh); *Pediococcus pentosaceus* SMB13-1 (*marcha,* Sikkim); *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh); *Enterococcus faecium* BPB11 (*marcha*, Bhutan); *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh); *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling); *Weissella cibaria* SMB9 (*marcha,* Sikkim); *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling); *Enterococcus durans* BPB21 (*phab*, Bhutan); *Enterococcus hirae* AOB24 (*phut*, Arunachal Pradesh); *Enterococcus faecium* AOB25

(*phut*, Arunachal Pradesh); *Enterococcus faecalis* AOB2 (*paa*, Arunachal Pradesh); *Enterococcus faecium* BPB33 (*phab*, Bhutan); *Enterococcus durans* DMB13 (*marcha,* Darjeeling); *Pediococcus pentosaceus* BPB13 (*marcha*, Bhutan); *Enterococcus faecalis* AOB5 (*paa*, Arunachal Pradesh); *Enterococcus faecium* SMB15 (*marcha,* Sikkim); *Enterococcus faecium* BPB31 (*phab*, Bhutan);*Enterococcus durans* DMB3 (*marcha,* Darjeeling); *Lactobacillus plantarum* subsp. *plantarum* NMB7 (*marcha,* Nepal) and *Lactobacillus plantarum* subsp. *plantarum* NMB8 (*marcha,* Nepal) (Figure 46).

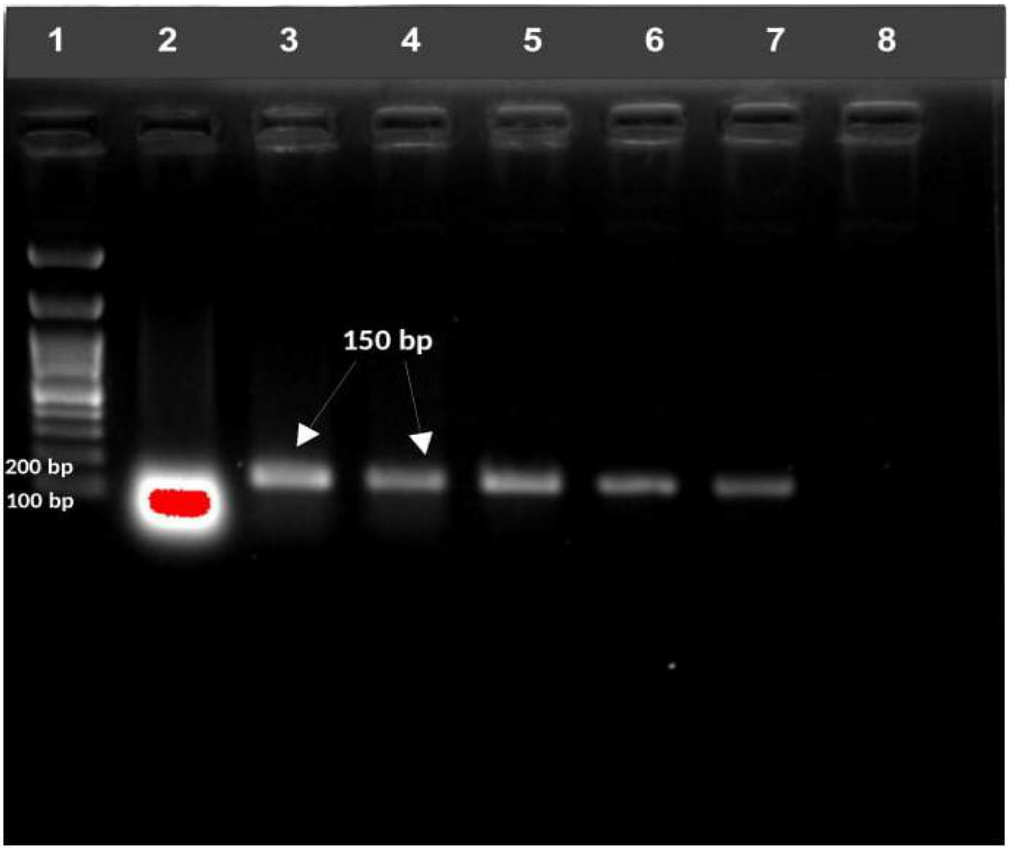


Figure 46: Amplified *mub 1* gene of 150 bp. Lane 1: 100 bp DNA ladder; Lane 3: *Enterococcus durans* BPB21; Lane 4: *Enterococcus hirae* AOB24; Lane 5: *Enterococcus faecium* AOB25; Lane 6: *Enterococcus faecalis* AOB2; Lane 7: *Enterococcus faecium* BPB33.

Detection of Bacteriocin Genes

Bacteriocin genes *ped A* (pediocin)*, ped B, ent A* (enterococin)*, ent B, ent P* and *cyl A* (cytolysin activator encoding gene) were screened to analyze the antimicrobial ability of the bacterial isolates. *cyl A* gene was also screened for the presence of virulence factors (haemolytic activity) as cytolysins act both as haemolytic toxins as well as bacteriocin. Only *ped B* gene with 375 bp size was detected in *Enterococcus durans* DMB13 (*marcha,* Darjeeling); and *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling) (Figure 47). None of the isolates tested were positive for virulence factor gene (*cyl A* gene).

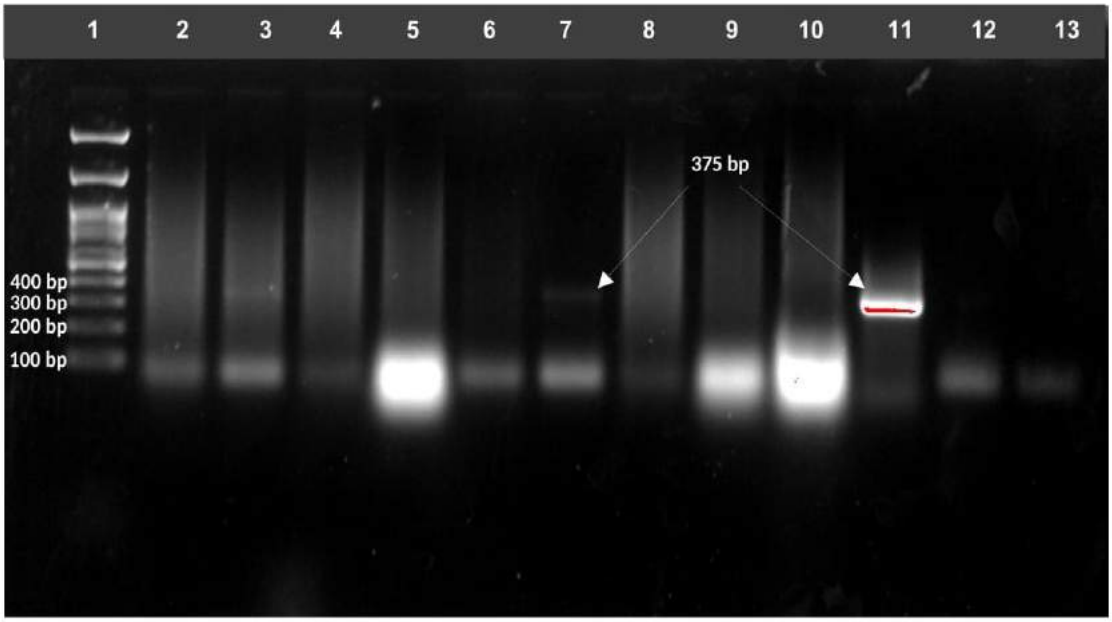


Figure 47: Amplified *ped B* gene of 375 bp. Lane 1: 100 bp DNA ladder; Lane 7: *Enterococcus durans* DMB13; Lane 11: *Pediococcus acidilactici* AKB3.

Detection of β-Glucosidase Gene

Bgl gene was screened to test the ability of bacterial isolates to produce β glucosidase enzyme. *Bgl* gene with a size of 1392 bp (Figure 48) was detected only in *Enterococcus faecium* SMB5 (*marcha,* Sikkim).

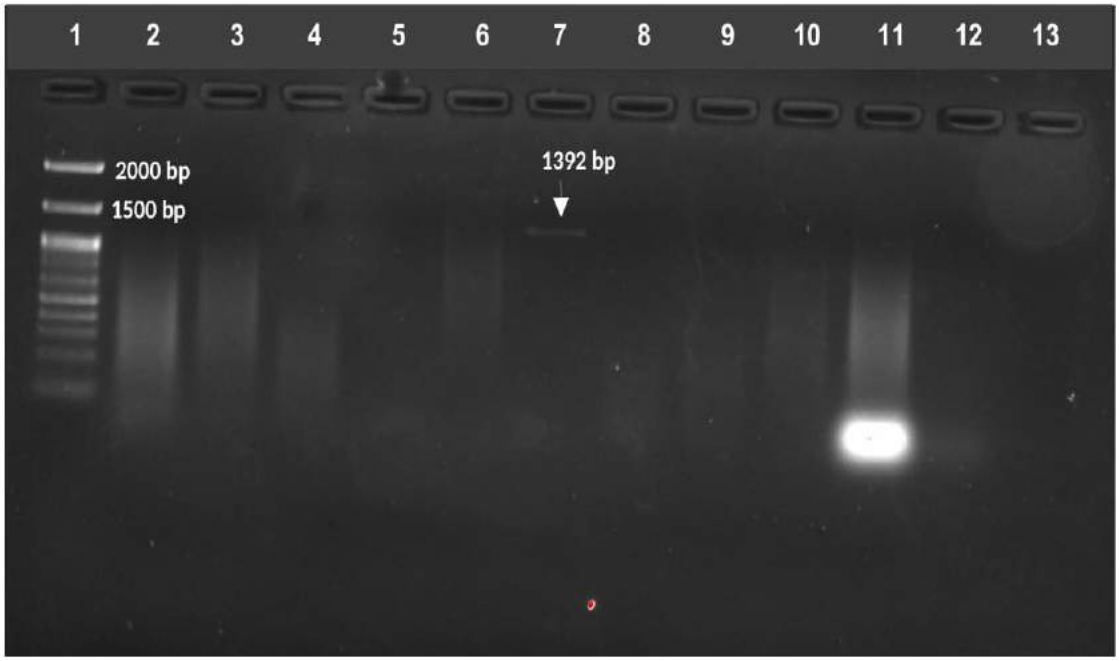


Figure 48: Amplified *bgl* gene of 1392 bp. Lane 1: 100 bp DNA ladder; Lane 7: *Enterococcus faecium* SMB5.

Selection of Best Probiotic Candidates

Based on the results of test for probiotic attributes and probiotic gene detection 18 bacterial strains from the dry samples of the Eastern Himalayas were selected (Table 20). These bacterial strains exhibited the best probiotic characteristics and were selected for further analysis. These probiotic strains were *Lactobacillus plantarum subsp. plantarum* NMB7 (*marcha,* Nepal), *Leuconostoc mesenteroides* AKB6 (*marcha,* Darjeeling), *Enterococcus durans* DMB3 (*marcha,* Darjeeling), *Enterococcus durans* DMB6 (*marcha,* Darjeeling), *Pediococcus acidilactici* AKB3 (*marcha,* Darjeeling), *Weissella cibaria* SMB9 (*marcha,* Sikkim), *Leuconostoc mesenteroides* SMB13-1 (*marcha,* Sikkim), *Pediococcus pentosaceus* BPB13 (*marcha,* Bhutan), *Enterococcus durans* AOB14 (*pee*, Arunachal Pradesh), *Enterococcus lactis* AOB26 (*phut*, Arunachal Pradesh), *Leuconostoc mesenteroides* SMB13-7 (*marcha,* Sikkim), *Enterococcus durans* SMB7 (*marcha,* Sikkim), *Enterococcus durans* DMB4 (*marcha,* Darjeeling), *Enterococcus faecalis* AOB11 (*paa*, Arunachal Pradesh), *Enterococcus durans* BPB21 (*paa,* Bhutan), *Enterococcus faecalis* SMB3 (*marcha,* Sikkim), *Pediococcus acidilactici* DMB12 (*marcha,* Darjeeling) and *Enterococcus durans* BPB4 (*marcha,* Bhutan) (Table 20).

The bacterial isolates from the dry starters of the Eastern Himalayas also exhibited certain functional properties along with probiotic activities. Functional attributes such as phytase test, amylase test, high-alcohol tolerance ability, riboflavin and folic acid producing ability were tested (Table 18). Based on the results obtained from these tests 8 isolates showing excellent functional properties were selected: *Pediococcus acidilactici* DMB14 (*marcha,* Darjeeling), *Enterococcus durans* DMB15 (*marcha,* Darjeeling), *Enterococcus faecium* SMB21 (*marcha,* Sikkim), *Enterococcus durans* BPB18 (*marcha,* Bhutan), *Enterococcus faecium* AOB4 (*paa*, Arunachal Pradesh),

Enterococcus faecalis AOB2 (*paa*, Arunachal Pradesh), *Enterococcus faecium* AOB15 (*pee*, Arunachal Pradesh) and *Enterococcus faecium* AOB25 (*phut*, Arunachal Pradesh) (Table 20).

Table 20: Selection of the best bacterial strain isolated from dry starters of the Eastern Himalayas showing genes for probiotic and functional properties

Starter	Strain	Probiotic Attributes										Functionality		
		Bile Tolerance			Low pH			Adhesion				Bacteriocin	β-glucosidase	Riboflavin	Folate
		Genes													
		Clp L	*LBA 1446*	*Bsh*	*Clp L*	*Agu A*	*Ir 1516*	*Apf*	*Mub 1*	*Map A*	*Fbp*	*Ped B*	*bgl*	*Rib A*	*Fol P*
Marcha (Nepal)	*Lactobacillus pentosus* NMB3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lactobacillus plantarum subsp. *plantarum* NMB7	+	-	-	+	+	+	+	+	+	-	-	-	+	-
	Lactobacillus plantarum subsp. *plantarum* NMB8	+	-	-	+	+	-	+	+	+	-	-	-	-	-
Marcha (Darjeeling)	*Leuconostoc mesenteroides* AKB6	+	+	-	+	-	-	+	+	+	+	-	-	+	-
	Enterococcus durans DMB11	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Enterococcus durans DMB4	+	-	-	+	-	-	+	-	-	-	-	-	-	-
	Enterococcus durans DMB3	+	+	-	+	+	+	-	+	+	-	-	-	+	-
	*Pediococcus acidilactici*DMB14	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	Enterococcus durans DMB15	+	-	-	+	-	-	+	+	+	-	-	-	+	-
	Enterococcus durans DMB6	+	-	-	+	+	-	+	+	+	-	-	-	+	-
	Pediococcus acidilactici AKB3	+	-	-	+	-	-	+	+	+	-	+	-	+	-
	Enterococcus durans DMB13	+	-	-	+	-	-	-	+	-	-	+	-	-	-
	Pediococcus acidilactici DMB12	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Marcha (Sikkim)	*Enterococcus faecalis* SMB3	+	+	-	+	-	-	+	-	-	-	-	-	+	-
	*Pediococcus pentosaceus*SMB13-7	-	-	-	-	-	-	+	+	-	-	-	-	+	-
	Enterococcus durans SMB7	+	-	-	+	-	-	+	-	-	-	-	-	+	-
	Weissella cibaria SMB9	+	-	-	+	+	+	-	+	+	-	-	-	+	-
	Leuconostoc mesenteroides SMB13-1	-	+	+	-	+	+	+	+	+	-	-	-	+	-

	Enterococcus faecium SMB21	-	-	-	-	+	+	+	+	+	-	-	-	+	-
	Enterococcus faecium SMB5	+	-	-	+	-	-	+	-	-	-	-	+	-	-
	Enterococcus faecium SMB15	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Marcha (Bhutan)	*Enterococcus durans* BPB18	+	-	-	+	-	+	+	+	+	-	-	-	+	-
	Enterococcus faecium BPB11	+	-	+	+	-	-	+	+	+	-	-	-	-	-
	Pediococcus pentosaceus BPB13	+	+	-	+	+	+	+	+	+	-	-	-	+	-
	Enterococcus durans BPB4	+	+	-	+	-	-	+	-	+	-	-	-	-	-
Paa (Arunachal Pradesh)	*Enterococcus faecium* AOB4	-	-	-	-	+	-	+	+	+	-	-	-	+	-
	Enterococcus faecalis AOB2	-	-	-	-	-	-	+	+	+	-	-	-	+	-
	Enterococcus faecalis AOB11	+	-	-	+	-	-	-	-	-	-	-	-	-	-
	Enterococcus faecalis AOB5	+	-	-	+	-	-	-	+	+	-	-	-	-	-
Pee (Arunachal Pradesh)	*Enterococcus faecium* AOB15	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	Enterococcus durans AOB14	+	-	-	+	+	-	+	+	+	-	-	-	+	-
Phut (Arunachal Pradesh)	*Enterococcus hirae* AOB24	-	+	-	-	+	-	+	+	-	-	-	-	-	-
	Enterococcus faecium AOB25	+	-	-	+	-	-	+	+	-	-	-	-	-	+
	Enterococcus lactis AOB26	+	-	-	+	+	-	+	+	+	-	-	-	+	-
Phab (Bhutan)	*Enterococcus durans* BPB21	+	-	-	+	-	-	-	+	+	-	-	-	+	-
	Enterococcus faecium BPB33	+	-	-	+	-	-	-	+	-	-	-	-	-	-
	Enterococcus faecium BPB31	-	-	-	-	-	+	-	+	-	-	-	-	-	-
+ve: positive; -ve: negative															

DISCUSSION

Documentation

This study was focused on the documentation of traditional practices of sub-culturing of essential bacterial flora in the form of dry starters by the ethnic people of the Eastern Himalayan regions of India, Nepal and Bhutan, bacterial diversity and their probiotic and functional properties present in dry starters. Five different types of dry starters viz. *marcha, paa, pee*, *phut* and *phab* were documented and collected from different regions of the Eastern Himalayan regions of India, Nepal and Bhutan and were analysed. During the documentation, we found that the preparation of *marcha* in Sikkim and Bhutan was similar and it differed from the preparation process of *marcha* in Nepal especially in the herbs that were used. Preparation practices of *marcha* of North East India have been documented earlier by Tamang and Sarkar (1995) and Tamang et al. (2007) and their report matches the information we have obtained in this documentation. The preparation process of *paa, pee* and *phut* of Arunachal Pradesh was also documented. The preparation process of *paa* and *phut* differed in the herbs that were used and no herbs were used for the preparation of *pee*. The preparation process of *paa, pee* and *phut* has also been reported earlier by Shrivastava et al. (2012) and the information given is similar to our documented report. Documentation of traditional method of preparation of *phab*, dry starter made by *Drukpa* community of Bhutan has been reported for the first time in this study.

Culture dependent analysis

Average bacterial population of all samples was 10^8 cfu/g which was not reported earlier except for *marcha* of Darjeeling hills and Sikkim (Tamang and Sarkar 1995; Tsuyoshi et al. 2005; Tamang et al. 2007). Bacterial load of *marcha* of Sikkim was 10^6 to 10^8 cfu/g which was almost same as that of populations of yeasts and filamentous

moulds in *marcha* of Sikkim (Tsuyoshi et al. 2005). This shows that bacterial populations in traditionally prepared starters of the Eastern Himalayas may have equally co-existed with filamentous moulds and yeasts (Heseltine et al. 1988; Zheng et al. 2015). The moisture content of all starters was low due to sun-drying process immediately after the fermentation, the step which is necessary to maintain the potency of traditionally prepared starters to store in dry place at room temperature for future use. *Phab*, dry starters made in Ladhak was also reported to have a low moisture content ranging 2.9%-3.4 % (Angmo and Bhalla 2014). The pH of all samples was mild acidic which may be due to the dominance of lactic acid bacteria (~10^8 cfu/g) in dry starters (Tamang and Sarkar 1995).

All 201 bacterial strains isolated from samples of *marcha, paa, pee, phut* and *phab* were morphologically and phenotypically characterized and tentatively identified into four major genera of lactic acid bacteria (LAB) viz. *Enterococcus, Pediococcus, Leuconostoc* and *Lactobacillus* two genera of non-LAB viz. *Bacillus* and *Staphylococcus*; and two Gram-negative bacterial genera viz. *Enterobacter* and *Citrobacter.* Based on similar phenotypic characterizations and biochemical tests, 201 bacterial isolates were grouped into 68 representative bacterial strains for confirmation of their taxonomical identification by 16S rRNA gene sequencing method (Sanger et al. 1977; Heather and Chain 2016). Firmicutes (85%) was the dominant phylum, followed by Proteobacteria (9%) and Actinobacteria (6%) in dry starters of the Eastern Himalayas. Firmicutes was also reported as the abundant phylum in *daqu,* a starter for Chinese strong flavour liquor (Zou et al. 2018; He et al. 2019) and also in *nuruk*, a dry starter of Korean used to make to mild-alcoholic beverage *makgeolli* (Jung et al. 2012). The sequence data based on constructed phylogenetic tree revealed dominance of lactic acid bacteria (59%) with 5 different genera and 11 species represented by

Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilacti, P. pentosaceus, Lactobacillus plantarum subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*. The abundance of lactic acid bacteria (LAB) could be related to the known fact that these bacteria can tolerate and proliferate in extreme conditions such as high alcohol concentration (14% v/v); low pH (>3.5) and low temperature (18-20˚C) (Versari et al. 1999; Zhang et al. 2018). Only two genera of LAB represented by *Pediococcus pentosaceus* and *Lactobacillus brevis* were reported earlier from *marcha* samples of Sikkim and Darjeeling hills (Tamang and Sarkar 1995; Tamang et al. 2007). However, in this study we found a wide diversity of LAB in samples of *marcha* collected from Darjeeling hills and Sikkim in India which included *Pediococcus pentosaceus, P. acidilactici, Enterococcus faceium, E. durans, E. faecalis, Leuconostoc mesenteroides* and *Weissella cibaria*, whereas, *Lactobacillus pentosus,* and *Lb. plantarum* subsp. *plantarum* were found only in *marcha* samples of Nepal. Geographical and altitudinal variations may affect the composition of microorganisms in dry starters (Jeyaram et al. 2011; Lv et al. 2012). Traditional methods of preparation of *marcha, phab, paa, pee* and *phut* are more or less similar except some variations were observed in use of substrates such as rice for *marcha, phut, paa* and *pee*, and maize-rice husk for *phab* of Bhutan, and also wrapping materials for fermenting substrates such as fern leaves (*Glaphylopteriolopsis erubeseens*) for *marcha* preparation, dry paddy straws for *phab*, and locally available plant leaves for preparation of *paa, pee* and *phut*, respectively. Bacterial diversity in dry starters of the Eastern Himalayas may be influenced by hygienic conditions, quality of cereal substrates, wrapping materials and source of natural or tap water during traditional methods of preparation (Peter-Ikechukwu et al. 2016; Gonelimali et al. 2018; Sha et al. 2019).

Bacterial profile in *marcha* of Nepal and Bhutan, *paa, pee* and *phut* of Arunachal Pradesh and *phab* of Bhutan has been reported for the first time in our study. Similar types of dry starter of Assam in North East India called *xaj-pitha* also contained several species of LAB such as *Lactobacillus plantarum, Lb. brevis, Weissella cibaria, W. paramesenteroides, W. confusa, Lactococcus lactis, Lactobacillus casei group, Leuconostoc lactis, Leuconostoc pseudomesenteroides, Pediococcus pentosus, Lactococcus garvie,* and *Enterococcus* sp. (Bora et al. 2016). Thanh et al. (2008) reported many species of lactic acid bacteria in Vietnamese *banh men* which included *Pediococcus pentosaceus, Lactobacillus plantarum, Lb. brevis, Lb. fermentum, Lb. agilis, W. confusa, W. paramesenteroides,* and *Lactococcus lactis*. *Enterococcus faecium, Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weissella paramesenteroides, and W. cibaria* were reported from *nuruk* of Korea (Hoon et al. 2013). Several species of LAB in Cambodian *dombea* were also reported *Weissella cibaria, Lactobacillus plantarum, Lactococcus lactis, Pediococcus pentosaceus* and *Enterococcus durans* (Ly et al. 2018). This justifies that species of LAB predominate the microbial composition of traditionally prepared dry starters in Asia including the Eastern Himalayas. LAB may be favourable bacteria in cereal based beverages due to their ability to improve protein digestibility, enhance organoleptic quality and increase nutritional bioavailability (Luana et al. 2014). Species of *Weissella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Enterococcus* are known for flavour enrichment, production of organic acids and antimicrobial activities in Chinese *daqu* used for liquor production (Gou et al. 2015). *Enterococcus* sp. has been reported to produce enterocins which play a major role in preventing the growth of pathogens (Javed et al. 2011). *Pediococcus* has fermentative role in winemaking (Wade et al. 2018).

Bacillus spp. (32%) as non-LAB species was also detected in dry starters of the Eastern Himalayas. Prevalence of *Bacillus* sp. may be due to its ability to survive in low moisture and high temperature environment (Nuding et al. 2017). *Bacillus* species are also important sources of amylase and protease enzymes involved in saccharification and flavour production (Beaumont 2002). Dominance of *Bacillus* sp. was also reported in *daqu* of China (Wang et al. 2008; Zheng et al. 2012) and *banh men* of Vietnam (Thanh and Tuan 2008). The next abundant bacterium was *Staphylococcus* spp. in the Himalayan starters, which secrete amylase (Li et al. 2014) and protease in Chinese *daqu* (Yang et al. 2017), and also produce lipases for production of esters for flavour (Talon et al. 1996) and thus this group of bacteria probably play a major role in flavour enhancement in the final product. The prevalence of phylum Actinobacteria in some starters of the Eastern Himalayas was only 6% which were represented by *Kocuria rosea*, *Micrococcus yunnanensis* and *Brevibacterium frigotolerans*. Presence of *Actinobacteria* has been reported in Chinese *daqu* (Zou et al. 2018) and Indian *marcha* and *thiat* (Sha et al. 2017).

Few species of opportunist pathogens and environmental contaminants such as *Micrococcus*, *Stenotrophomonas*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* were detected only in samples of *paa, pee,* and *phut* of Arunachal Pradesh and *phab* of Bhutan. However, the prevalence and populations of these contaminants were low and probably that these organisms might have contaminated in the samples during traditional method of preparation from substrates and ingredients such as herbs, water, utensils, wrapping materials, etc. Gram-negative bacteria were not detected in any sample of *marcha* collected from Nepal, India and Bhutan. In our previous study on *marcha* no pathogenic Gram-negative bacteria were found at genus level analysed by

high-throughput sequencing method (Sha et al. 2017). Although most of these bacteria are opportunists and probable food borne pathogens, however, some of them such as *Enterobacter* sp. are involved in production of amylases and lipases and also in formation of flavour in *daqu,* starter of China (Li et al. 2015). LAB prevents the growth of pathogenic and spoilage microorganisms in foods (Cizeikiene et al. 2013; Castellano et al. 2017) and also produce flavour compound (Mukisa et al. 2017).

A diversity index or phylogenetic metrics is a quantitative measure to show phylogenetic relations within different species in a community (Birtel et al. 2015). Hence we characterized diversity indexes of bacterial community present in starters of the Eastern Himalayas by Shannon diversity index *H*, Simpson's index, Dominance and Chao1 index. Shannon diversity index *H* for evaluating bacterial diversity was recorded highest in *marcha* of Sikkim (*H*:2.305) and lowest in *marcha* of Darjeeling (*H*:1.121) indicating higher bacterial diversity in *marcha* of Sikkim as compared to other starters. Simpson's diversity index (1-*D*) index which considers both number of species as well as relative abundance of each species for evaluating diversity showed highest value for *marcha* of Sikkim. The dominance D values were recorded highest for *marcha* samples of Darjeeling and lowest for *marcha* samples of Sikkim, which supports the above inference on bacterial diversity. The dominance D value ranges between 0-1, where the value 0 indicates that all taxa are equally present where as a value 1 indicates dominance of one taxon over the whole community (Wagner et al. 2018). Thus the values near to zero indicates a highly diverse eco-system and values near to 1 indicates less or homogenous eco-system (Lv et al. 2012). Hence phylogenetic matrix of bacterial community present in dry starters of the Eastern Himalayas showed high diversity within the community. The Eastern Himalayas was known for their rich flora and fauna diversity within a wide eco-system (Chettri et al.

2010), our findings also supplement the richness of microbial diversity in food eco-system of the Eastern Himalayas.

Bacterial community and their interactions in starters are extremely important for fermentation which may enhance the productivity and develop flavour quality to end product (Cai et al. 2018). There has been an increasing concern on the safety of fermented beverages, due to presence of ethyl carbamate which is considered as carcinogenic (Ryu et al. 2015), biogenic amines (Liu et al. 2016), mycotoxin (Sivamaruthi et al. 2019), and contamination by opportunistic microbial pathogens (Hong et al. 2016). All these considerations mandate a deep understanding of the microbial community in starters. Also, profile of native microorganism in these starters opens a possibility of finding novel strain(s) with functional properties for industrial purposes. This study also records the bacterial diversity of *phab* of Bhutan which is found to be produced rarely by few ethnic people of Bhutan, probably due to their more preference to commercial *marcha,* similar to *phab*, sold in local markets. Bacteria present in traditionally prepared dry starters have no amylolytic activities (Thapa and Tamang 2004), however, they may contribute in acidification of fermenting substrates and impart flavour with mild acidic and sour taste to traditional alcoholic beverages (*kodo ko jaanr, opo, apong* and *themsing*) preferred by the Himalayan people (Tamang and Thapa 2006; Tamang et al. 2007).

Culture Independent Analysis

The alpha diversity analysis based on Shannon, Chao1 and Simpson's diversity index revealed a very rich bacterial diversity in *marcha* of Sikkim. The results obtained in Metagenomic analysis using high-throughput sequencing (HTS) tool of *marcha* of Sikkim showed significant variations from those obtained in 16S rRNA analysis. The

amplicon sequencing yielded Proteobacteria (53%), Firmicutes (45.4%) and other minor phyla (0.8%) showing the abundance of phylum Proteobacteria. At the family level, Acetobacteraceae (50.6%) was found to be the dominant family in *marcha* samples. The predominant bacterial genera found in *marcha* were *Acetobacter* (52.6%), *Fructobacillus* (21.1%), *Lactococcus* (10.3%), *Lactobacillus* (8.4%), *Leuconostoc* (4.0%), *Burkholderia* (2.1%) and *Gluconacetobacter* (1.4%). *Acteobacter,* a genus of acetic acid bacteria though found to be dominant in HTS result was not found in culture dependent analysis which could be due to their viable but not culturable (VBNC) nature (De Roos and De Vuyst 2018). The abundance of *Acetobacter* could be related to the aerobic nature, ability to tolerate high ethanol concentration (10-14%) (Du Toit and Pretorius 2002), along with its enrichment during fermentation. The percentage occurrence of *Lactobacillus* was higher than that of *Lactococcus* and *Streptococcus* which could be due to the higher tolerance at acidic/low pH conditions as compared to the later (Rogosa et al. 1951; Alegría et al. 2012). Gram negative bacterial genera such as *Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea* were also found in HTS results but the percentage occurrence was quite low (>3.9%). *Pediococcus* species which was prominently found in 16S rRNA analysis and also reported in earlier culture dependent studies on *marcha* (Tamang and Sarkar 1995; Tamang et al. 2007) was however not observed in the bacterial diversity profile of HTS.

Probiotic Properties

Human body and microbes have existed in a close symbiotic association where the bacteria have been well studied to play a role in maintaining host homeostasis (Lebeer et al. 2008). Bacteria such as LAB form a major and beneficial microbiota of

gastrointestinal tract (GIT) and thus have gained importance as being a "green therapeutic alternative" (Hemarajata and Versalovic 2012; Archer and Halami 2015). Hence in this study we also focused on screening the probiotic attributes of lactic acid bacteria such as tolerance to low pH, bile concentration, deconjugation of bile salts, hydrophobicity, β galactosidase activity, cholesterol assimilation ability and screening of probiotic marker genes.

The foremost and the most important property that a probiotic strain should possess is the ability to adhere and colonize the host intestinal epithelial cells (Garcia-Gonzalez et al. 2018; Monteagudo-Mera et al. 2019). The adherence ability of a probiotic strain is greatly contributed by cell surface molecules such as proteins, teichoic acids and polysaccharides as well as different environmental factors (Polak-Berecka et al. 2014). Thus the cell surface hydrophobicity is correlated to adhesion and the bacterial cell exhibiting high surface hydrophobicity is considered to have good adherence ability (Krasowska et al. 2014). In this study, nine 9 isolates have shown more than 95% hydrophobicity in n-hexadecane indicating good adherence property, since hydrophobicity index of more than 70% is considered to be hydrophobic (Nostro et al. 2004). *Enterococcus durans* BPB21 and *Enterococcus durans* SMB7 showed the highest hydrophobicity of 98%. In earlier reports *Enterococcus durans* strain VJ119 isolated from GI of broiler chicken was shown to have 41% cell surface hydrophobicity which is quite low compared to our findings (Jeevaratnam and Nallala 2017). Further the genus *Enterococci* is also known for its ability to form biofilms which is a desirable trait as it prevents the colonization of intestinal epithelium by the enteric pathogens (Martin et al. 2008). Thus our isolates showing excellent cell surface hydrophobicity (98%) along with well known biofilm forming trait could be an appropriate probiotic candidate (Schiffer et al. 2019).

The ability to tolerate acid and bile is another mandatory requirement for any strain to be probiotic as after colonization the primary barrier is to survive the adverse environment of GIT (Terpou et al. 2019). Due to the production of gastric juice (2.5 L) in stomach a pH of 3-5 is created post feeding (Cotter and Hill 2003) and thus we have checked the ability of LAB strains to tolerate a pH of 3. Majority of the isolates showed a high tolerance (between 50% - 96%) at pH 3 in 3 h of exposure, exhibiting a good survival capacity in acidic conditions. *Leuconostoc mesenteroides* SMB13-7 isolated from *marcha* of Sikkim showed an excellent viable rate of 96% at pH 3. Diana et al. (2015) reported that strains of *Leuconostoc mesenteroides* isolated from aguamiel sap could survive at a pH 2 after 3 h of incubation however; there was a significant decrease in viability percentage which ranged between 40% - 49%. *Leuconostoc mesenteroides* strain 41Lac isolated from curd showed a survival rate of 84% in pH 2.5 (Haghshenas et al. 2017). *Enterococcus durans* DMB15 from *marcha* of Darjeeling showed no growth (0% viability) at pH 3 which contradicts the earlier findings where different strains of *Enterococcus durans* have been reported to exhibit high survival rate at acidic conditions (pH 2 and pH3) (Jeevaratnam and Nallala 2017; Aspri et al. 2017; Yerlikaya and Akbulut 2020).

The human liver secretes around 1 L of bile ranging between 0.3%-0.5% in small intestine per day (Begley et al. 2005) with the retention time of 4 h (Kiela and Ghishan 2016). A probiotic strain should be able to tolerate the effects of such bile concentration (Ruiz et al. 2013), thus we have checked the ability of LAB to tolerate 0.3% of bile in this study. *Pediococcus acidilactici* AKB3, *Enterococcus faecalis* AOB11, *Lactobacillus plantarum* subsp. *plantarum* NMB8 and *Enterococcus durans* BPB21 showed a good survival rate of more than 90% in 0.3% bile salts. De Oliveira

Vieira et al. (2020) also reported a good tolerance ability of *Pediococcus acidilactici* CE51 strain isolated from meat sausage to 0.3%, 0.6% and 0.9% bile.

Bile salt hydrolase (BSH) activity is an important probiotic attribute as it is involved in deconjugation of bile salts by catalyzing the hydrolysis of amide bond between the steroid moiety and amino acid side chain of the bile salts, thus enabling the bacteria to endure bile stress in the intestine (Lebeer et al. 2008; Horackova et al. 2020). BSH is also known to reduce serum cholesterol level (Corzo and Gilliland 1999; Adebola et al. 2020). The deconjugated bile salts co-precipitates with cholesterol at a pH below 5 which then gets bound to the bacterial cells and is excreted through faeces, thus reducing the serum cholesterol level (Mathara et al. 2008). About 24% of the isolates showed deconjugation of taurodeoxycholic acid (TDC) and 41% of the test isolates could deconjugate taurocholic acid (TC). *Pediococcus pentosaceus* SMB13-1, *Enterococcus faecium* SMB5, *Enterococcus faecium* SMB15, *Enterococcus faecalis* AOB11, *Enterococcus faecium* BPB11, *Pediococcus pentosaceus* BPB13, *Enterococcus faecium* BPB33, *Enterococcus durans* DMB3, *Pediococcus acidilactici* DMB14 and *Lactobacillus plantarum subsp. plantarum* NMB7 showed positive results with both TDC and TC bile salts.

Lactose intolerance is a common problem observed in children and adults worldwide (Harvey et al. 2018). In this condition the intake of unfermented dairy products leads to bloating, abdominal pain, diarrhoea and flatulence (Lomer et al. 2008). This condition is marked by the deficiency of the enzyme lactase which breaks down lactose into glucose and galactose in the small intestine (Tamang et al. 2016b). The ability of microorganisms to produce β-galactosidase enzyme which can breakdown lactose and thus alleviate lactose intolerance is a desirable probiotic attribute. In our study 65 % of the total test isolates showed a positive result for β-galactosidase and

majority of the positive isolates belonged to the genus *Enterococcus*. Similar findings of *Enterococci* isolates isolated from dairy products showing high β-galactosidase activity was reported by Nami et al. (2019).

Hypercholesterolemia, a condition of elevated serum cholesterol which is one of the major risk factor of coronary and cardiovascular diseases has become a reason for increasing mortality rate every year (Shehata et al. 2016). The ability of microorganism to lower the serum cholesterol level is another attribute that is desired in a probiotic candidate (Albano et al. 2018). We have screened the cholesterol assimilation ability of our test isolates in this study and we found five isolates showing >70% cholesterol assimilation. *Enterococcus durans* DMB4, *E. durans* SMB7, *E. durans* AOB14, *E. lactis* AOB26 and *Pediococcus pentosaceus* SMB13-1 showed a cholesterol assimilation ability of 80%, 78%, 72%, 71% and 72%, respectively. Previous studies have also reported *Enterococcus durans* strains to have good cholesterol assimilation ability (Guo et al. 2016; Nami et al. 2019).

Functional Properties

Phytic acid or phytate are a major storage of myoinositol and phosphate in plant seeds, legumes, cereals, nuts and oil seeds (Priyodip et al. 2017). The phytic acids are considered an antinutritive factor as it binds to the divalent cations such as Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and amino group derivatives in protein decreasing its bioavailability for absorption by the human body (Waters et al. 2015). Further the phytic acid also decreases the digestibility of starch by binding to it directly or by inhibiting the α-amylase enzyme (Oatway et al. 2001; Lee et al. 2015). Phytases are the enzymes that breakdown phytic acid and are produced by several plants, microorganism and animals (Gupta et al. 2015). However, human body is incapable of producing phytase and thus

the ability to produce phytase is an additional and desirable attribute in a probiotic candidate (Priyodip et al. 2017). In the present study we also screened the phytase producing ability of lactic acid bacterial isolates and we found 86% of the test isolates to be positive. An interesting observation in this study was that all *Lactobacillus* isolates were negative for phytase production. However, *Lactobacillus* species such as *L. plantarum* and *L. amylovorus* have been reported to be eminent phytase producers (Sreeramulu et al. 1996). Scheers et al. (2016) in their study conducted on human volunteers have reported that ingestion of vegetables fermented with *Lactobacillus plantarum* increased the bioavailability of iron (Sharma et al. 2020).

Amylases are the enzymes that break down starch by the hydrolysis of α-D 1, 4 glycosidic bonds (De Souza and De Oliveira Magalhaes 2010). The insufficient production of amylases by the pancreas often causes incomplete digestion of starch in the body (Peyrot des Gachons et al. 2016). Probiotic bacteria with functional properties such as ability to produce amylases are often a desirable alternative for such problems (Guo et al. 2010). We screened the LAB isolates for amylase activity in the present study and found that none of the test isolates produced amylase. Similar results were also obtained in studies conducted by Tamang et al. (2007). In contradiction to our findings, many studies have reported amylase activity in different *Lactobacillus* species (Padmavathi et al. 2018). Vakevainen et al. (2018) have reported the amylolytic activity of *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides* and *Pediococcus pentosaceus* strains isolated from *atoleagrio,* a fermented Mexican maize-based beverage.

Since alcoholic beverages are the final product produced using the dry starter cultures we have also screened the ethanol tolerance ability of LAB isolates. The alcohol content of *kodo ko jaanr* (fermented finger millet beverage) which is prepared using

marcha is 4.8% (1.8% – 8.7%) (Thapa and Tamang 2004). Taking this information as a standard we have used the ethanol concentration range of 3% - 10% in this test. 86% of the test isolates showed optimum tolerance even at 10% ethanol. *Enterococcus durans* DMB4 and *Lactobacillus pentosus* NMB3 showed tolerance only upto 7% ethanol after which no growth was observed. Earlier studies have reported the tolerance of *Lactobacillus* strain up to 16% ethanol (Gold et al. 1992). The ability of LAB to tolerate ethanol is substrate as well as strain dependent and is mainly associated with the change in the fatty acid composition (from saturated to mono unsaturated fatty acids) of cell membrane upon exposure to ethanol (Dombek and Ingram 1984).

Safety Evaluation

One of the major steps before considering any strain to be used as a probiotic candidate is its safety assessment. Screening for haemolytic activity and virulence genes was done for the safety evaluation of all LAB isolates. High frequency of β haemolytic activity by *Enterococci* strains have been reported in earlier studies (Hallgren et al. 2009; Semedo et al. 2003). However, in this study haemolytic activity was not found in any of the test isolates. Another mandatory and desirable attribute of probiotic strain is the absence of virulence genes. In this study we have screened the presence of virulence gene *cyl A* that codes for cytolysin which causes haemolysis. Cytolysin is a haemolytic toxin produced by some strains of *Enterococcus fecalis* and is considered a major risk factor especially in food industry (Nami et al. 2019). High prevalence of *cyl A* genes have been reported in *Enterococcus* strains by Semedo et al. (2003). In this study majority of the LAB isolates belonged to the genera *Enterococcus* because of which we have specifically screened *cyl A* gene for virulence factor. None

of the isolates tested showed the presence of *cyl A* gene and thus can be considered safe in this regard.

Probiotic marker gene detection

Genetic screening of the probiotic marker gene in the test isolates were also done to characterize a potential probiotic candidate. 26 probiotic and functional marker genes: *rib A, fol P, clp L, bsh, Ir1584, Ir1516*, *LBA 1446, hdc, odc, tdc, agu A, Mub, fbp, sor, sbp, msa, apf, map A, mub1, ped A, ped B, ent A, ent B, ent P,cyl A* and *bgl* gene were targeted in this study. Two nutritional marker gene, *rib A* and *fol P* which are involved in synthesis of B vitamins, riboflavin and folate respectively were also screened. Maximum number of the test isolates was positive for *rib A* gene however only one isolate, *Enterococcus faecium* AOB25 was positive for *fol P* gene. Probiotic strains capable of synthesizing B vitamins is a desirable trait and can be used in food fortification as these vitamins are not synthesized by human body. Previous studies have also reported the presence of complete pathway for folate synthesis in *Lactobacillus reuteri* strains (Saulnier et al. 2011) and riboflavin biosynthetic operon in *Lactococcus lactis* subsp. *cremoris* strain NZ9000 (Burgess et al. 2004). The detection of these nutrition marker genes in our test isolates suggest the potential of these isolates to produce B vitamins, the deficiency of which can cause severe health problems.

Clp L, bsh, Ir1584, Ir1516 and *LBA 1446* marker genes for survival in bile salts were screened for all the test isolates. The occurrence frequency of *bsh* gene was quite low compared to other genes and was found in only two strains *Pediococcus pentosaceus* SMB13-1 and *Enterococcus faecium* BPB11. These two strains have also shown positive result for deconjugation of TDC as well as TC bile salts in in-vitro analysis

suggesting a probable role of *bsh* gene in hydrolysis of bile salts by these isolates. *Ir1584* gene was not found in any of the test isolates. From the results obtained we can say that *clp L, bsh, Ir1516* and *LBA 1446* are the genes that are majorly involved in tolerance of these isolates to bile salts.

Genes *hdc, odc, tdc, agu A, clp L* and *Ir1516* were screened for the ability of the test isolates to survive low pH. *Hdc, odc* and *tdc* genes were not detected in any of the test isolates however; the frequency of *agu A, clp L* and *Ir1516* genes was comparatively high in all the test isolates. Only four of the isolates; *Lactobacillus plantarum subsp. plantarum* NMB7, *Enterococcus durans* DMB3, *Weissella cibaria* SMB9 and *Pediococcus pentosaceus* BPB13 showed the presence of all the 3 genes for low pH (*agu A, clp L* and *Ir1516*) however, except for *Lactobacillus plantarum subsp. plantarum* NMB7 (70%) all the other isolates showed a very low viability rate at pH 3 (<40%). Further, *Leuconostoc mesenteroides* SMB13-7 which showed the highest survival rate of 96% at pH 3 did not harbour any of the 6 six genes tested for survival at low pH. From this observation we can conclude that some other genes may be involved in the survival of these isolates at low pH. The absence of *hdc, odc* and *tdc* genes could also be considered as a positive characteristic as these genes are also associated with the formation of biogenic amines such as putrescine, histamine, and tyrosine (Halász et al. 1994, Lu et al. 2007; Turpin et al. 2011; Zhang et al. 2018). Among the different biogenic amines histamine is found to have highest toxicity (Dadáková et al. 2009) and a concentration level of not more than 2-10 mg/L has been recommended to reduce its toxicological effects in alcoholic drinks (Soufleros et al. 2007).

Genetic screening of 8 genes (*Mub, fbp, sor, sbp, msa, apf, map A* and *mub1*) known to confer adherence ability to bacteria to GI tract were done for all the isolates. All the

test isolates were negative for *mub, sor, sbp* and *msa* gene. *Mub 1* and *map A* gene were detected in 73% and 57% of the test isolates. Similar high occurrence of *mub 1* and *map A* gene in LAB strains isolated from fermented pearl millet slurries were reported by Turpin et al. (2012) in their study. *Fbp* gene which codes for fibronectin binding protein was detected in *Leuconostoc mesenteroides* AKB6 only. Archer and Halami (2015) have reported the presence of *fbp* in *Lactobacillus fermentum* strains isolated from fermented dairy products and infant faecal samples. 62% of the total test isolates were positive for *apf* gene. The *apf* gene codes for *apf* proteins which aids the bacterial cell in auto-aggregation, co-aggregation and maintenance of cell shape. The high occurrence of *apf* gene in all the bacteria could be related to the small conserved sequence of the C terminal region of the *apf* protein (Goh and Klaenhammer 2010). Similar observations were also reported in the findings of Turpin et al. (2012). Only one of the isolate *Leuconostoc mesenteroides* AKB6 showed the presence of all four adhesion genes (*apf, fbp, map A* and *mub1*). *Leuconostoc mesenteroides* AKB6 also exhibited a high hydrophobicity percentage of 94% suggesting a good adherence ability of this isolate.

The test isolates were also screened for the presence of bacteriocin genes and six bacteriocin marker genes were targeted; *ped A* (pediocin)*, ped B, ent A* (enterococin)*, entB, ent P* and *cyl A*. Bacteriocins are proteins or peptide complexes with antimicrobial activity and synthesized by several lactic acid bacteria. Bacteriocin producing LAB strains are a demand in pharmaceutical and food industry due to its antagonistic activity against spoilage and pathogenic bacteria (Porto et al. 2017). Enterococins are bacteriocin produced by *Enterococcus* genus and *ent A, entB* and *ent P* genes are mostly found in *E. fecalis* and *E, faecium* strains (Moreno et al. 2003). Though most of the isolates screened in this study belonged to the genus *Enterococcus,*

ent A, ent B and *ent P* genes were not detected in any of the test isolates. *Cyl A* gene code for cytolysins which are enterococcal lantibiotics effective against broad range of Gram positive bacteria (Ness et al. 2014). *Cyl A* was not detected in any of the test isolates. *Ped B* was detected in two test isolates; *Enterococcus durans* DMB13 and *Pediococcus acidilactici* AKB3. *Ped B* genes are involved in the production of pediocin A, a bacteriocin majorly produced by *Pediococcus* genus. Previous studies have shown the occurrence of *ped B* gene in *P. pentoceseus* AH1 strain (El-Arabi et al. 2018).

Apart from imparting health benefits LAB also play an important role in improving the organoleptic properties of fermented foods and beverages. LAB are well known producers of β glucosidase enzyme which cause hydrolysis of plant β-D-glucosylated precursors and releases a wide range of plant secondary metabolites (Michlmayr et al. 2014). Such secondary modifications were shown to enhance the flavour and fragrance of fermented products and also increase the bioavailability of antioxidative plant metabolites (Xiang et al. 2019). The functional marker gene *bgl* which codes for β glucosidase enzyme was detected only in *Enterococcus faecium* SMB5 suggesting the probable role of this isolate in flavour enhancement of final alcoholic beverage.

Based on the results of probiotic test, functional test, probiotic and functional marker gene detection 26 LAB strains showing the best results were selected for further analysis. A significant variation in the expression of different probiotic attributes was observed amongst the test isolates in this study. The probable reason for such variation could be the species and strain specificity in the mechanisms involved in the expression of such probiotic attributes.

CONCLUSION

CONCLUSION

The preparation of dry starter cultures is an ancient traditional technology which prevails amongst the ethnic communities residing in the Eastern Himalayan regions of eastern Nepal, India (Darjeeling hills, Sikkim and Arunachal Pradesh) and Bhutan. The consumption of fermented alcoholic beverages prepared using these dry starters is a major part of socio-cultural activities of indigenous communities of the Eastern Himalayas. Traditionally prepared dry starter cultures consist of consortia of bacteria, filamentous fungi and yeast which has been conserved, sub-cultured and passed on for centuries. The diminishing practise of making traditional dry starters for fermented beverages demands and necessitates a comprehensive study on these dry starter cultures. Information on microbial composition of traditionally prepared dry starters viz. *phab, paa, pee* and *phut* of the Eastern Himalayan regions of India, Nepal and Bhutan was unknown except *marcha* of Sikkim in India. This study aims to generate a complete profile on bacterial diversity of traditional starters of the Eastern Himalayas based on 16S rRNA sequencing. Metagenomic study of *marcha* of Sikkim was also done and a significant variation was found in the comparative analysis of bacterial diversity profile obtained from culture dependent and independent technique. Interestingly a very high diversity of bacterial community was found in the traditional dry starters which open a new area of research on importance and mode of coexistent of different bacterial genera and different microbial communities within these traditional dry starters. An attempt has been made to screen out few potent probiotic candidate strain based on probiotic and functionality tests of the LAB isolates. Detection of probiotic and functional marker genes of the test isolates has also been done. Eighteen LAB isolates with the best probiotic and functionality attribute and distinct marker genes have been chosen for further studies. This study holds its

uniqueness in being the first report on bacterial diversity of dry starters of the Eastern Himalayas along with extensive information on probiotic, functional characteristics and genetic screening of probiotic marker gene of the LAB isolated from these dry starters.

SUMMARY

This Thesis was aimed to study the bacterial diversity in traditionally prepared dry starters of the Eastern Himalayan regions of India, Nepal and Bhutan. The probiotic and functional attributes of the LAB isolates from the dry starters were also evaluated which were further aided by the genetic screening of probiotic and functional marker genes. A complete documentation on the traditional methods of preparation of dry starters found in the Eastern Himalayan region was also done during this research. During this study, I personally visited and documented by interviewing the ethnic communities involved in preparations of these starters, the different places in the Eastern Himalayas viz. Dharan, Dhankutta, Hattikharka and Hiley districts in Nepal, Darjeeling and Kalimpong districts of West Bengal in India, Pakyong, Gangtok, Recab and Basilakha village of Sikkim in India, lower Subansiri, upper Subansiri and Ziro valley of Arunachal Pradesh in India and Gedumari, Thimpu and Dhonakha in Bhutan were visited for this study. Fresh samples of dry starters along with plant samples used for the starter preparation were collected for microbial analysis and study of ethno-microbiology, respectively. This study also documented the preparation process of *phab* (dry starter prepared only by *Drupka* community of Bhutan), a rare and unique dry starter which is being constantly replaced by the use of *marcha* in Bhutan and hence is at the verge of extinction. During this study, 35 different traditionally prepared dry starters viz 8 samples of *marcha* from Nepal, 5 samples of *marcha* from Darjeeling hills, 8 samples of *marcha* from Sikkim, 5 samples of *marcha* from Bhutan, 2 samples of *paa*, 3 samples of *pee* and 2 samples of *phut* from Arunachal Pradesh and 2 samples of *phab* from Bhutan were collected. The moisture contents of all dry starter samples ranged between 6%-15%. The moisture content of *marcha* of Bhutan was recorded to be the highest amongst the starter samples collected with an average moisture content of 13.76%. Average moisture content of *phab* samples of Bhutan was

found to be lowest with the moisture content of 6.17%. The pH of all dry starter samples was acidic and ranged within 5.0-5.9. Culture-dependent as well as culture-independent techniques were used in this study for bacterial community analysis and for comparative evaluation of the results obtained from both the techniques. The bacterial count of all the 35 traditionally prepared dry starter samples ranged from $1.0x10^5$ cfu/g to $2.7x10^8$ cfu/g. The highest microbial load was recorded in *marcha* samples of Sikkim with an average bacterial load of $1.9\ x10^8$ cfu/g, whereas the lowest bacterial count was recorded in *marcha* samples of Bhutan with the average bacterial load of $1.0x10^5$cfu/g. A total of 201 bacterial isolates were isolated from 35 traditionally prepared dry starter viz. 49 isolates from *marcha* of Sikkim, 38 isolates from *marcha* of Darjeeling hills, 34 isolates from *marcha* of Nepal, 18 isolates from *marcha* of Bhutan, 12 isolates from *paa*, 17 isolates from *pee,* 11 isolates from *phut* all from Arunachal Pradesh, and 22 isolates from *phab* of Bhutan. Out of 201 isolates, 183 bacterial isolates were Gram-positive and 18 isolates were Gram-negative. All the isolates were subjected to phenotypic and biochemical tests and based on the results obtained from these tests the isolates were tentatively identified into 6 genera of Gram-positive bacteria: *Pediococcus, Enterococcus, Leuconostoc, Bacillus, Lactobacillus* and *Staphylococcus* and 2 genera of Gram-negative bacteria: *Enterobacter* and *Citrobacter* (Bergey's manual of bacteriological classification). Sixty-eight representative strains of bacteria were randomly selected from 201 isolates based on phenotypic characterizations and biochemical tests for genotypic characterization. The PCR amplification of 16S rRNA gene of all the 68 isolates were done and sequenced using Sanger sequencing method. The sequences retrieved from 16S rRNA gene sequencing were assigned the nucleotide accession numbers (MK748250-MK748278, MK202997-MK203032 and MK752675-MK752677) and deposited in GeneBank-

NCBI. Identification of the isolates by 16S rRNA gene sequence and phylogenetic analysis was performed by using Basic Local Alignment Search Tool (BLAST) and MEGA-7, respectively. According to the results obtained from 16S rRNA sequencing data, three phyla of bacteria were observed in the starter samples: *Firmicutes* (85%) was dominant phylum, followed by *Proteobacteria* (9%) and *Actinobacteria* (6%). In *marcha* samples of Nepal, dominance of phylum *Firmicutes* represented by 80% was observed. Phylum *Firmicutes* (100%) was observed in *marcha* samples of Darjeeling and Bhutan. Dominance of phylum *Firmicutes* (92%) followed by *Actinobacteria* (8%) was found in *marcha* sample of Sikkim. Dominance of phylum *Firmicutes* (80%) followed by phylum *Proteobacteria* (20%) was observed in *paa* samples. *Pee* samples had 67% of phylum *Firmicutes,* 16% of phylum *Proteobacteria* and 17% of phylum *Actinobacteria.* In *phut* samples dominance of phylum *Firmicutes* (75%) was observed followed by phylum *Proteobacteria* (25%). Similarly, *Firmicutes* (57%) was the dominant phylum in *phab* of Bhutan, followed by phylum *Proteobacteria* (43%). Based on 16S rRNA sequencing data, 15 genera with 32 species of bacteria were identified in dry starters of the Eastern Himalayas, which included *Leuconostoc* (3%), *Enterococcus* (41%), *Bacillus* (13%), *Staphylococcus* (12%), *Lactobacillus* (5%), *Enterobacter* (4%), *Klebsiella* (2%), *Pseudomonas* (2%), *Pediococcus* (9%), *Strenotrophomonas* (2%), *Kocuria* (2%), *Brevibacterium* (3%), *Lysinibacillus* (2%), *Weisella* (2%) and *Micrococcus* (2%). In *marcha* samples of Nepal, *Brevibacterium frigoritolerans* (20%) and *Lactobacillus plantarum* (20%) were found dominant bacterial species. In *marcha* samples of Darjeeling and Sikkim *Enterococcus durans* (55%) and *Enterococcus faecium* (22%) were found dominant species. In samples of Arunachal Pradesh, *Enterococcus faecium* (20%) was the dominant species. *Staphylococcus warneri* represented by 24% of the total isolates was the dominant

bacterium in starter sample *marcha* of Bhutan. The lactic acid bacteria (LAB) were found dominant group represented 59% of total bacterial isolates followed by non-LAB (31%) and Gram-negative bacteria (9%). *Enterococcus durans* was found the dominant LAB species represented by 18%, followed by *E. faecium* (13%) of the total bacterial isolates. Other LAB species found in the starter samples were *Enterococcus faecalis, E. hirae, E. lactis, Pediococcus acidilacti, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides* and *Weissella cibaria.* Species of LAB were found in all samples with the highest occurrence in *marcha* samples of Darjeeling (91%) and lowest in *marcha* of Nepal (30%). The non-LAB species found in samples were *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans* and *Micrococcus yunnanensis.* The highest occurrence of non-LAB species of bacteria (70%) were found in *marcha* samples of Nepal and lowest in *marcha* samples of Darjeeling (9%). Gram negative bacteria: *Stenotrophomonas maltophilia, Klebsiella pneumonia, Pseudomonas putida* and *Enterobacter hormaechei* subsp. *xiangfangensis* and *E. hormaechei* subsp. *steigerwaltii* were found in dry starter samples of Arunachal Pradesh and Bhutan however, this group of bacteria were not found in any of the *marcha* samples in this study.

We also profiled the bacterial community in samples of *marcha* of Sikkim by high-throughput sequencing (HTS). Based on operational taxonomic units (OTUs), 15 bacterial phyla were recovered in *marcha* samples, out of which phylum *Proteobacteria* and *Firmicutes* were observed. *Acetobacteraceae* (50.6%) was found dominant family followed by *Leuconostocaceae* (25.5%), *Streptococcaceae* (10.5%),

Lactobacillaceae (8.38%), *Burkholderiaceae* (2.13%) and *Staphylococcaceae* (0.54%). In genus level *Acetobacter* (52.6%) was dominant genus, followed by *Fructobacillus* (21.1%), *Lactococcus* (10.3%), *Lactobacillus* (8.4%), *Leuconostoc* (4.0%), *Burkholderia* (2.1%) and *Gluconacetobacter* (1.4%). High occurrence of Gram-negative bacteria such as *Acetobacter, Burkholderia, Gluconacetobacter, Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea* was detected in *marcha* of Sikkim by HTS data, whereas, this group of bacteria was not observed in culture-dependent study. Our study concludes a very high and rich diversity within the bacterial community in traditionally prepared starters of the Eastern Himalayas.

Probiotic characteristics such as resistance to acidic condition (pH 3), 0.3% Bile concentration, deconjugation of bile salts, hydrophobicity, β-galactosidase and cholesterol assimilation ability of 37 LAB isolates were also screened. *Leuconostoc mesenteroides* SMB13-7 isolated from *marcha* of Sikkim showed the highest survival rate of 96% in pH 3. *Pediococcus acidilactici* AKB3 from *marcha* of Darjeeling showed a highest survival rate of 97.26% in 0.3% bile salts. About 24% and 41% of the total isolates showed the ability to deconjugate taurodeoxycholic acid and taurocholic acid respectively. About 86% of the bacterial isolates showed high adherence ability of more than 70%, among which, *Enterococcus durans* BPB21 of *phab* (Bhutan) and *Enterococcus durans* SMB7 isolated from *marcha* (Sikkim) showed the highest hydrophobicity percentage of 98%. Hydrophobicity is directly related to the adherence ability of bacteria to host cell surfaces hence, from the results obtained we can conclude that the LAB isolates have good adherence ability indicating their projection as probiotic candidates. Similarly, 65% and 35% of the total isolates showed positive results for β galactosidase activity and cholesterol assimilation activity, respectively. *Enterococcus durans* DMB4 showed the highest cholesterol

assimilation of 80%. The β galactosidase and cholesterol assimilation activity is a desirable probiotic characteristic especially in patients suffering from lactose intolerance and increased serum cholesterol level and coronary heart disease respectively. The results obtained in *in-vitro* probiotic screening tests were further validated with genetic screening of probiotic marker genes such as *clp L, bsh, Ir1584, Ir1516* and *LBA 1446* genes for survival in bile; *hdc, odc, tdc, agu A, clp L* and *Ir1516* genes for survival in low pH; *Mub, fbp, sor, sbp, msa, apf, map A* and *mub1* genes for adherence and *ped A, ped B, ent A, entB, ent P* and *cyl A* bacteriocin genes for antimicrobial activity.

Apart from probiotic attributes, the LAB isolates in this study were also screened for functional properties such as production of phytase, amylase and β-glucosidase, ability to tolerate various concentrations of ethanol and ability to synthesize vitamins such as riboflavin and folate. About 86% of the total isolates showed the ability to produce phytase, the anti-nutritive degrading factor indicating significant role in enhancing the nutritional quality of food. None of the tested isolates were able to produce amylase enzyme hence, we can conclude that they have no role in saccharification and liquefaction of starchy substrates. About 86% of bacterial isolates showed optimum tolerance at 10% ethanol. *Enterococcus durans* DMB4 and *Lactobacillus pentosus* NMB3 could not survive in ethanol concentration of >7% ethanol suggesting a poor alcohol tolerance ability. Twenty two LAB isolates were found positive for *rib A* gene involved in riboflavin biosynthesis and only one strain *Enterococcus faecium* AOB25 showed the presence of *fol P* gene for folate biosynthesis. The ability to synthesize vitamins is a desired attribute in a probiotic candidate which can thus be used for food fortification. The ability of the tested isolates to produce β-glucosidase was evaluated by genetic screening of *bgl* gene which codes for the enzyme β-glucosidase. *Bgl* gene

was detected only in *Enterococcus faecium* SMB5 suggesting the probable role of this isolate in flavour enhancement of final alcoholic beverage.

For any strain to be considered a potent probiotic candidate it should mandatorily qualify the safety evaluation tests. In this study we have screened the *in-vitro* haemolytic activity and presence of virulence gene *cyl A* for the safety evaluation of the LAB isolates. None of the isolates showed hemolysis in *in-vitro* test. Virulence gene *cyl A* was not found in any of the tested isolates hence, the bacterial isolates from dry starters of the Eastern Himalayas may be considered safe. Based on the probiotic and functional test results, a total of 26 LAB isolates have been selected as a potential probiotic candidate strains which may be further evaluated and tested in future study.

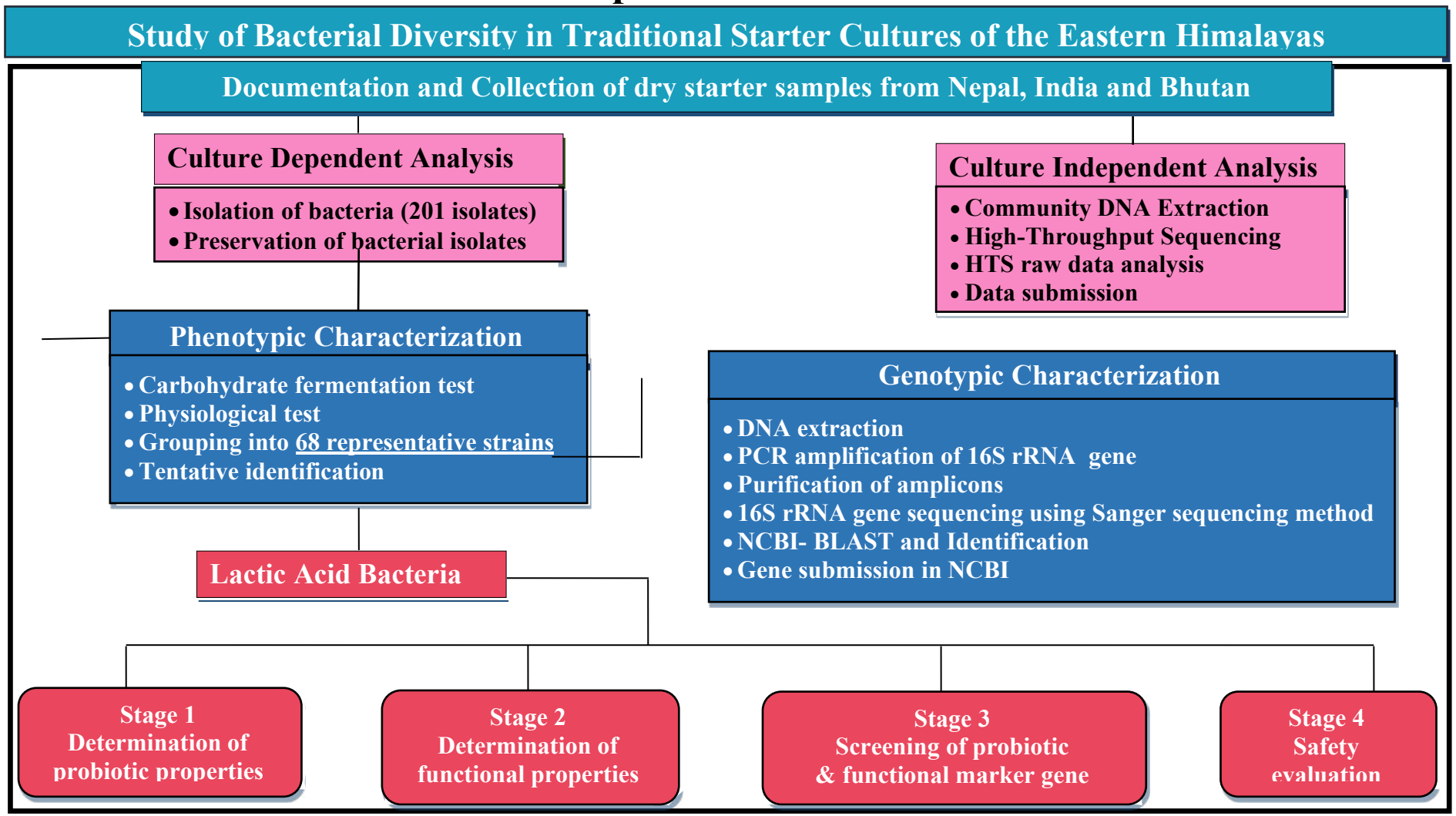
This study holds its uniqueness in presenting the first report on the complete information on bacterial diversity, probiotic and functional characterization of the bacterial isolates, and genetic screening of probiotic and functional marker genes in the bacteria of traditionally prepared dry starters of the Eastern Himalayas.

Highlights of Findings

- Traditional prepared dry starters of the Eastern Himalayan regions of India, Nepal and Bhutan were documented and studied: *marcha, phab, paa, pee* and *phut*.
- Based on 16S rRNA gene sequencing: LAB- *Enterococcus durans, E. faecium, E. faecalis, E. hirae, E. lactis, Pediococcus acidilacti, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*; Non-LAB- *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans* and *Micrococcus yunnanensis*; Gram-negative bacteria- *Stenotrophomonas maltophilia, Klebsiella pneumonia, Pseudomonas putida* and *Enterobacter hormaechei* subsp. *xiangfangensis* and *E. hormaechei* subsp. *Steigerwaltii.*
- Very rich and diverse ecosystem in *marcha* of Sikkim
- High-throughput sequence data of *marcha* of Sikkim showed the bacterial community: *Acetobacter, Fructobacillus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Burkholderia, Gluconacetobacter, Citrobacter, Cronobacter, Enterobacter, Escherichia, Shigella* and *Pantoea.*
- Based on the results of test for probiotic attributes and gene detection, 18 bacterial strains were selected as probiotics candidates. *Lactobacillus plantarum subsp. plantarum* NMB7; *Leuconostoc mesenteroides* AKB6, SMB13-1, SMB13-7; *Enterococcus durans* DMB3, DMB6, AOB14, SMB7, DMB4, BPB21, BPB4; *Pediococcus acidilactici* AKB3, DMB12; *Weissella cibaria* SMB9; *Pediococcus pentosaceus* BPB13; *Enterococcus lactis* AOB26; *Enterococcus faecalis* AOB11, SMB3.

- Strains showing excellent functional properties were selected: *Pediococcus acidilactici* DMB14, *Enterococcus durans* DMB15, BPB18; *Enterococcus faecium* SMB21, AOB4, AOB15, AOB25; *Enterococcus faecalis* AOB2.

Schematic Representation of PhD Work



BIBLOGRAPHY

Abriouel, H., Pérez Montoro, B., Casimiro-Soriguer, C.S., Pérez Pulido, A.J., Knapp, C.W., Caballero Gómez, N., Castillo-Gutiérrez, S., Estudillo-Martínez, M.D., Gálvez, A. and Benomar, N. (2017). Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence. *Frontiers in Microbiology* 8: 891. doi: 10.3389/fmicb.2017.00891.

Achaya, K.T. (1991). Alcoholic fermentation and its products in ancient India. *Indian Journal of History of Science* 26(2): 123-129.

Adebola, O.O., Corcoran, O. and Morgan, W.A. (2020). Prebiotics may alter bile salt hydrolase activity: Possible implications for cholesterol metabolism. *Pharma Nutrition* 12: 100182. doi: 10.1016/j.phanu.2020.100182.

Adhikari R.R. and Ghimirey, H. (2000). *Nepalese Society and Culture*. Vidharthi Pushtak Bhandar, Kathmandu (Nepali).

Aidoo, K.E. and Nout, M.R. (2010). Functional yeasts and molds in fermented foods and beverages. In: *Fermented foods and beverages of the world* (Ed: Tamang, J.P. and Kailasapathy, K.), pp. 127-148. CRC Press, Taylor & Francis Group, New York.

Albano, C., Morandi, S., Silvetti, T., Casiraghi, M.C., Manini, F. and Brasca, M. (2018). Lactic acid bacteria with cholesterol-lowering properties for dairy applications: In vitro and in situ activity**.** *Journal of Dairy Science* 101(12): 10807-10818**.**

Albuquerque, M.A.C., Bedani, R., Vieira, A.D.S., LeBlanc, J.G. and Saad, S.M.I. (2016). Supplementation with fruit and okara soybean by-products and amaranth flour increases the folate production by starter and probiotic cultures. *International Journal of Food Microbiology* 236: 26-32.

Albuquerque, M.A.C., del Milagro, M., Saad, S.M.I. and de Melo Franco, B.D.G. (2020). B-Group Vitamin-Producing Lactic Acid Bacteria. In: *Lactic Acid Bacteria: A Functional Approach*. CRC press, Tylor and Francis, New York.

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

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215(3): 403-410.

Álvarez-Ríos, D.G., Figueredo-Urbina, C.J. and Casas, A. (2020). Physical, chemical, and microbiological characteristics of pulque: management of a fermented beverage in Michoacán, Mexico. *Foods* 9*:* 361. doi: 10.3390/foods9030361.

Angmo, K. and Bhalla, T.C. (2014). Preparation of Phabs-an indigenous starter culture for production of traditional alcoholic beverage, Chhang, in Ladakh. *Indian Journal of Traditional Knowledge* 13(2): 347-351.

Angmo, K., Kumari, A. and Bhalla, T.C. (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT-Food Science and Technology* 66: 428-435.

Anupma, A. and Tamang, J.P. (2020). Diversity of filamentous fungi isolated from some amylase and alcohol-producing starters of India. *Frontiers in Microbiology* 11: 905. doi: 10.3389/fmicb.2020.00905.

Anupma, A., Pradhan, P., Sha, S.P. and Tamang, J.P. (2018). Traditional skill of ethnic people of the Eastern Himalayas and North East India in preserving microbiota as dry amylolytic starters. *Indian Journal of Traditional Knowledge* 17: 184-190.

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

Ardhana, M.M. and Fleet, G.H. (1989). The microbial ecology of tape *ketan* fermentation. *International Journal of Food Microbiology* 9(3): 157-165.

Aspri, M., Bozoudi, D., Tsaltas, D., Hill, C. and Papademas, P. (2017). Raw donkey milk as a source of Enterococcus diversity: Assessment of their technological properties and safety characteristics. *Food Control* 73: 81-90.

Bae, K.H, Kee, S.S., Ryu, H.Y., Kwon, C. and Sohn, H.Y. (2007). Identification and fermentation characteristics of lactic acid bacteria isolated from the fermentation broth of Korean traditional liquor, Andong-Soju. *Korean Journal of Microbiology and Biotechnology* 35: 310-315.

Bal, J., Yun, S.H., Chun, J., Kim, B.T. and Kim, D.H. (2016). Taxonomic characterization, evaluation of toxigenicity, and saccharification capability of *Aspergillus* section Flavi isolates from Korean traditional wheat-based fermentation starter *nuruk*. *Mycobiology* 44(3): 155-161.

Bartram, A.K., Lynch, M.D., Stearns, J.C., Moreno-Hagelsieb, G. and Neufeld, J.D. (2011). Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Applied and Environmental Microbiology* 77(11): 3846-3852.

Bassi, D., Orrù, L., Cabanillas Vasquez, J., Cocconcelli, P.S. and Fontana, C. (2020). Peruvian *chicha*: a focus on the microbial populations of this ancient maize-based fermented beverage. *Microorganisms* 8: 93. doi: 10.3390/microorganisms8010093

Beaumont, M. (2002). Flavouring composition prepared by fermentation with *Bacillus* spp. *International Journal of Food Microbiology* 75(3): 189-196.

Begley, M., Gahan, C.G. and Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews* 29(4): 625-651.

Berbegal, C., Spano, G., Tristezza, M., Grieco, F. and Capozzi, V. (2017). Microbial resources and innovation in the wine production sector. *South African Journal of Enology and Viticulture* 38(2): 156-166.

Bhalla, T.C. (2007). Traditional foods and beverages of Himachal Pradesh. *Indian Journal of Traditional Knowledge* 6(1): 17-24.

Bhasin, V. (2013). Pastoralists of Himalayas. *Journal of Biodiversity* 4(2): 83-113.

Birtel, J., Walser, J.C., Pichon, S., Bürgmann, H. and Matthews, B. (2015). Estimating bacterial diversity for ecological studies: methods, metrics, and assumptions. *PLoS ONE* 10(4): e0125356. doi: 10.1371/journal.pone.0125356.

Blandino, A., Al-Aseeri, M.E., Pandiella, S.S., Cantero, D. and Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International* 36(6): 527-543.

Bora, S.S., Keot, J., Das, S., Sarma, K. and Barooah, M. (2016). Metagenomics analysis of microbial communities associated with a traditional rice wine starter culture (Xaj-pitha) of Assam, India. *3 Biotechnology* 6: 153. doi: 10.1007/s13205-016-0471-1.

Burgess, C., O'Connell-Motherway, M., Sybesma, W., Hugenholtz, J. and Van Sinderen, D. (2004). Riboflavin production in Lactococcus lactis: potential for in situ production of vitamin-enriched foods. *Applied Microbiology and Biotechnology* 70(10): 5769-5777.

Cai, H., Zhang, T., Zhang, Q., Luo, J., Cai, C. and Mao, J. (2018). Microbial diversity and chemical analysis of the starters used in traditional Chinese sweet rice wine. *Food Microbiology* 73: 319-326.

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

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7(5): 335-336.

Cappuccino, J.C. and Sherman, N. (1992). In: *Microbiology: A Laboratory Manual* (third ed), pp. 125-179. Benjamin-cummings Publishing Co., New York.

Castellano, P., Pérez Ibarreche, M., Blanco Massani, M., Fontana, C. and Vignolo, G.M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: a focus on meat ecosystems and industrial environments. *Microorganisms* 5(3): 38. doi: 10.3390/microorganisms5030038.

Chagnaud, P., Machinis, K., Coutte, L.A., Marecat, A. and Mercenier, A. (2001). Rapid PCR –based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. *Journal of Microbiological Methods* 44(2): 139-148.

Chao, A. and Chiu, C. (2016). Nonparametric estimation and comparison of species richness. In eLS, John Wiley & Sons, Ltd (Ed.). doi:10.1002/9780470015902.a0026329.

Chay, C., Elegado, F.B., Dizon, E.I., Hurtada, W.A., Norng, C. and Raymundo, L.C. (2017). Isolation and identification of mold and yeast in *medombae,* a rice wine starter culture from Kompong Cham Province, Cambodia. *International Food Research Journal* 1(6): 708-717.

Chen, B., Wu, Q. and Xu, Y. (2014). Filamentous fungal diversity and community structure associated with the solid state fermentation of Chinese Maotai-flavor liquor. *International Journal of Food Microbiology* 179: 80-84.

Cheng, H.R. and Jiang, N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letters* 28(1): 55-59.

Chettri, N., Sharma, E., Shakya, B., Thapa, R., Bajracharya, B., Uddin, K., Oli, K.P. and Choudhury, D. (2010). Biodiversity in the Eastern Himalayas: status, trends and vulnerability to climate change. Technical Report 2, ICIMOD, Kathmandu.

Chim, C., Erlinda, I.D., Elegado, F.B., Hurtada, A.W., Chakrya, N. and Raymundo, C.L. (2015). Traditional dried starter culture (Medombae) for rice liquor production in Cambodia. *International Food Research Journal* 22(4): 1642-1650.

Cizeikiene, D., Gražina, J., Paškevičius, A. and Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control* 31(2): 539-545.

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

Coburn, P.S. and Gilmore, M.S. (2003). The Enterococcus faecalis cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cellular microbiology* 5(10): 661-669.

Corzo, G. and Gilliland, S.E. (1999). Measurements of bile salt hydrolase activity from Lactobacillus acidophilus based on disappearance of conjugated bile salts. *Journal of Dairy Sciences* 82(3): 466-471.

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

Cotter, P.D. and Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* 67(3): 429-453.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R. and Baldassarri, L. (2004). Survey for virulence determinants among Enterococcus faecalis isolated from different sources. *Journal of Medical Microbiology* 53(1): 13-20.

Cronk, T.C., Steinkraus, K.H., Hackler, L.R. and Mattick, L.R. (1977). Indonesian tape ketan fermentation. *Applied and Environmental Microbiology* 33(5): 1067-1073.

Dadáková, E., Krížek, P. and Pelikánová, T. (2009). Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). *Food Chemistry* 116(1): 365-370.

Damerow, P. (2012). Sumerian beer: the origins of brewing technology in ancient Mesopotamia. *Cuneiform Digital Library Journal* 2(1): 1-20.

Das, C.P. and Pandey, A. (2007). Fermentation of traditional beverages prepared by Bhotiya community of Uttaranchal Himalaya. *Indian Journal of Traditional Knowledge* 6(1):136-140.

Das, D. and Goyal, A. (2015). Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim. *LWT-food Science and Technology* 61(1): 263-268.

De Crécy-Lagard, V., El Yacoubi, B., de la Garza, R.D., Noiriel, A. and Hanson, A.D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics* 8(1): 245. doi: 10.1186/1471-2164-8-245.

De Oliveira Vieira, K.C., Ferreira, C.D.S., Bueno, E.B.T., De Moraes, Y.A., Toledo, A.C.C.G., Nakagaki, W.R., Pereira, V.C. and Winkelstroter, L.K. (2020). Development and viability of probiotic orange juice supplemented by *Pediococcus acidilactici* CE51. *LWT* 109637. doi: 10.1016/j.lwt.2020.109637.

De Roos, J. and De Vuyst, L. (2018). Acetic acid bacteria in fermented foods and beverages. *Current Opinion in Biotechnology* 49: 115-119.

De Souza, P.M. and de Oliveira Magalhães, P. (2010). Application of microbial α-amylase in industry - A review. *Brazilian Journal of Microbiology* 41(4): 850-861.

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

Denou, E., Pridmore, R.D., Berger, B., Panoff, J.M., Arigoni, F. and Brüssow, H. (2008). Identification of genes associated with the long-gut-persistence phenotype of the probiotic Lactobacillus johnsonii strain NCC533 using a combination of genomics and transcriptome analysis. *Journal of Bacteriology* 190(9): 3161-3168.

Devi, S.M., Archer, A.C. and Halami, P.M. (2015). Screening, characterization and in Vitro evaluation of probiotic properties among lactic acid bacteria through comparative analysis. *Probiotics and Antimicrobial Proteins* 7(3): 181-192.

Dhamcharee, B. (1982). Traditional fermented food in Thailand. *In: Traditional Food Fermentation as Industrial Resources in ASCA Countries* (Ed: Saono, S., Winarno, W.J. and Karjarki, D.), pp. 85-90. Jakarta, Indonesia: The Indonesian Institute of Science (LIPI).

Diana, C., Humberto, H. and Jorge, Y.F. (2015). Probiotic Properties of *Leuconostoc mesenteroides* Isolated from Aguamiel of *Agave salmiana*. *Probiotics and Antimicrobial Proteins* 7(2): 107-117.

Diaz-Ruiz, G., Guyot, J.P., Ruiz-Teran, F., Morlon-Guyot, J. and Wacher, C. (2003). Microbial and physiological characterization of weakly amylolytic but fast-growing lactic acid bacteria: a functional role in supporting microbial diversity in pozol, a Mexican fermented maize beverage. *Applied Environmental Microbiology* 69(8): 4367-4374.

Dolci, P., Alessandria, V., Rantsiou, K. and Cocolin, L. (2015). “Advanced methods for

the identification, enumeration, and characterization of microorganisms in fermented foods," In: *Advances in Fermented Foods and Beverages* (Ed: Holzapfel, W.H.), pp. 157-176. London: Elsevier.

Dombek, K.M. and Ingram, L.O. (1984). Effects of ethanol on the Escherichia coli plasma membrane. *Journal of Bacteriology* 157(1): 233-239.

Du Toit, W.J. and Pretorius, I.S. (2002). The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. *Annals of Microbiology* 52(2): 155-179.

Du, H., Wang, X., Zhang, Y. and Yan Xu, Y. (2019). Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter. *International Journal of Food Microbiology* 297: 32-40.

Dung, N.T.P. (2004). Defined fungal starter granules for purple glutinous rice wine. PhD Thesis, Wageningen University, Wageningen, the Netherlands.

Dung, N.T.P., Rombouts, F.M. and Nout, M.J.R. (2006). Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. *Food Microbiology* 23(4): 331-340.

Dung, N.T.P., Rombouts, F.M. and Nout, M.J.R. (2007). Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). *LWT-Food Science and Technology* 40(1): 130-135.

Dwidjoseputro, D. and Wolf, F.T. (1970). Microbiological studies of Indonesian fermented foodstuffs. *Mycopathologia et Mycologia Applicata* 41(3-4): 211-222.

Edgar, R.C. (2010). Search and clustering orde of magnitude faster than BLAST. *Bioinformatics* 26(19): 2460-2461.

El-Arabi, N.I., Salim, R.G., Abosereh, N.A. and Abdelhadi, A.A. (2018). Molecular Characterization of Some Antilisterial Bacteriocin Genes from *Enterococcus faecium*

and *Pediococcus pentosaceus*. *Microbiology and Biotechnology Letters* 46(3): 288-299.

Elshaghabee, F., Rokana, N., Gulhane, R.D., Sharma, C. and Panwar, H. (2017). *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Frontiers in Microbiology* 8: 1490. doi.org/10.3389/fmicb.2017.01490.

Eom, H.J., Moon, J.S., Seo, E.Y. and Han, N.S. (2009). Heterologous expression and secretion of *Lactobacillus amylovorus* alpha-amylase in *Leuconostoc citreum*. *Biotechnology Letters* 31: 1783-1788.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods* 56(3): 297-314.

Erdo grul, O. and Azirak, S. (2004). Review of the studies on the red yeast rice (Monascus purpureus). *Turkish Electronic Journal of Biotechnology* 2(5): 37-49.

Escalante, A., López Soto, D.R., Velázquez Gutiérrez, J.E., Giles-Gómez, M., Bolívar, F. and López-Munguía, A. (2016). *Pulque*, a traditional Mexican alcoholic fermented beverage: historical, microbiological, and technical aspects. *Frontiers in Microbiology* 7: 1026.doi: 10.3389/fmicb.2016.01026.

Fang, F., Li, Y., Bumann, M., Raftis, E.J., Casey, P.G., Cooney, J.C., Walsh, M.A. and O'Toole, P.W. (2009). Allelic variation of bile salt hydrolase genes in Lactobacillus salivarius does not determine bile resistance levels. *Journal of Bacteriology* 191(18): 5743-5757.

Faria-Oliveira, F., Diniz, R.H., Godoy-Santos, F., Pilo, F.B., Mezadri, H., Castro, I.M. and Brandao, R.L. (2015). The role of yeast and lactic acid bacteria in the production of fermented beverages in South America. *Food Production and Industry* 107-135.

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

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

Fischer, M.M., Egli, I.M., Aeberli, I., Hurrell, R.F. and Meile, L. (2014). *International Journal of Food Microbiology* 190: 54-60.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology* 66(5): 365-378.

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

Garcia-Ruiz, A., Esteban-Fernandez, D.G.A., Requena, T., Bartolome, B. and Moreno-Arribas, M.V. (2014). Assessment of probiotic properties in lactic acid bacteria isolated from wine. *Food Microbiology* 44: 220-225.

Gascuel, O. and Steel, M. (2006). Neighbor-Joining revealed. *Molecular Biology and Evolution* 23(11): 1997-2000.

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

Giri, S.S., Sen, S.S., Saha, S., Sukumaran, V. and Park, S.C. (2018). Use of a potential probiotic, *Lactobacillus plantarum* L7, for the preparation of a rice-based fermented beverage. *Frontiers in Microbiology* 9: 473. doi.org/10.3389/fmicb.2018.00473.

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

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

Gold, R.S., Meagher, M.M., Hutkins, R. and Conway, T. (1992). Ethanol tolerance and carbohydrate metabolism in lactobacilli. *Journal of Industrial Microbiology* 10(1): 45-54.

Gomi, K. and Abe, K. (2007). Food products fermented by Aspergillus oryzae. In: *The Aspergilli: Genomics, Medical Applications, Biotechnology and Research Methods (Mycology)*, pp. 427-437. CRC Press (Boca Raton, FL).

Gonelimali, F.D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M. and Hatab, S.R. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. *Frontiers in Microbiology* 9: 1639. doi: 10.3389/fmicb.2018.01639.

Gonzalez, C.J., Encinas, J.P., Garcıa-López, M.L. and Otero, A. (2000). Characterization and identification of lactic acid bacteria from freshwater fishes. *Food Microbiology* 17(4): 383-391.

Goodman, S. (2018). Unpalatable truths: food and drink as medicine in colonial British India. *Journal of the History of Medicine and Allied Sciences* 73(2): 205-222.

Gorer, G. (1938). *The Lepchas of Sikkim*. Gian Publishing House, Delhi.

Gou, M., Wang, H., Yuan, H., Zhang, W., Tang, Y. and Kida, K. (2015). Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production. *Journal of the Institute of Brewing* 121(4): 620-627.

Grosu-Tudor, S.S. and Zamfir, M. (2012). Probiotic potential of some lactic acid bacteria isolated from romanian fermented vegetables. *Annals of the Romanian Society for Cell Biology* 17(1): 234-239.

Grumezescu, A.M. and Holban, A.M. (2019). *Alcoholic Beverages*, The Science of Beverages. Vol. 7, Woodhead Publishing, Cambridge.

Guan, X., Xu, Q., Zheng, Y., Qian, L. and Lin, B. (2017). Screening and characterization of lactic acid bacterial strains that produce fermented milk and reduce cholesterol levels. *Brazilian Journal of Microbiology* 48(4): 730-739.

Guo, L., Li, T., Tang, Y., Yang, L. and Huo, G. (2016). Cholesterol-lowering effects of Enterococcus. *Microbial Biotechnology* 9(6): 737-745.

Guo, X.H., Kim, J.M., Nam, H.M., Park, S.Y. and Kim, J.M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. *Anaerobe* 16(4): 321-326.

Gupta, N. (2018). Ethnicity in north-east India: A challenge to identity. *International Journal of Advanced Educational Research* 3(2): 292-294.

Gupta, R.K., Gangoliya, S.S. and Singh, N.K. (2015). Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *Journal of Food Science and Technology* 52(2): 676-684.

Haard, N.F. (1999). In: *Fermented cereals: a global perspective* (No. 138). Haard, N.F., Odunfa, S.A., Lee, C.H., Quintero-Ramićrez, R., Lorence-Quiñones, A., Wacher-Radarte, C. Food and Agriculture Organization.

Haghshenas, B., Nami, Y., Almasi, A., Abdullah, N., Radiah, D., Rosli, R., Barzegari, A. and Khosroushahi, A.Y. (2017). Isolation and characterization of probiotics from dairies. *Iranian Journal of Microbiology* 9(4): 234-243.

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

Hällgren, A., Claesson, C., Saeedi, B., Monstein, H.J., Hanberger, H. and Nilsson, L.E. (2009). Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes and in vitro adhesion to urinary catheters of Enterococcus faecalis and E. faecium of clinical origin. *International Journal of Medical Microbiology* 299(5): 323-332.

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

Hammes, W.P. and Hertel, C. (2003). The genus *Lactobacillus*. In: *The Prokaryotes an Electronic Resource for the Microbiological Community.* (Ed: Dworkin, M., Flakow, S., Rosenberg, E., Schleifer, K.H. and Stackbrandt, E.). New York, Springer-Verlag

Harrigan, W.F. and McCance, M.E. (1976). *Laboratory methods in food and dairy microbiology*. Academic Press Inc. (London) Ltd.

Harvey, L., Ludwig, T., Hou, A.Q., Hock, Q.S., Tan, M.L.N., Osatakul, S., Bindels. J. and Muhardi, L. (2018). Prevalence, cause and diagnosis of lactose intolerance in children aged 1–5 years: a systematic review of 1995–2015 literature. *Asia Pacific Journal of Clinical Nutrition* 27(1): 29-46.

He, G., Huang, J., Zhou, R., Wu, C. and Jin, Y. (2019). Effect of fortified daqu on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Frontiers of Microbiology* 10: 56. doi: 10.3389/fmicb.2019.00056.

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

Heber, D., Yip, I., Ashley, J.M., Elashoff, D.A., Elashoff, R.M. and Vay Liang, W.G. (1999). Cholesterol-lowering effects of a proprietary Chinese red-yeast rice dietary supplement. *The American Journal of Clinical Nutrition* 69(2): 231-236.

Hemarajata, P. and Versalovic, J. (2012). Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic Advances in Gastroenterology* 6(1): 39-51.

Hesseltine, C.W. (1991). Zygomycetes in food fermentations. *Mycologist* 5(4): 162-169.

Hesseltine, C.W. and Kurtzman, C.P. (1990). Yeasts in amylolytic food starters. *Anales del Instituto de Biología: serie Botánica* 60(1): 1-7.

Hesseltine, C.W. and Ray, M.L. (1988). Lactic acid bacteria in murcha and ragi. *Journal of Applied Microbiology* 64(5): 395-401.

Hesseltine, C.W. and Wang, H.L. (1980). The importance of traditional fermented foods. *BioScience* 30(6): 402-404.

Hesseltine, C.W., Rogers, R. and Winarno, F.G. (1988). Microbiological studies on amylolytic Oriental fermentation starters. *Mycopathologia* 101: 141-155.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., *et al*. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Review in Gastroenterology and Hepatology* 11: 506-514.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). In: *Bergey's Manual of Determinative Bacteriology.* ninth ed. Williams & Wilkins, Baltimore.

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

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

Hong, X.T., Chen, J., Liu, L., Wu, H., Tan, H.Q., Xie, G.F., Xu, Q., Zou, H.J., Yu, W.J., Wang, L. and Qin, N. (2016). Metagenomic sequencing reveals the relationship between microbiota composition and quality of Chinese rice wine. *Science Reporters* 6(1): 1-11.

Hooker, J.D. (1854). *Himalayan Journals: Notes of a Naturalist in Bengal, the Sikkim and Nepal Himalayas, the Khasia Mountains* John Murray, London.

Hoon, S.S., Lee, C., Lee, S., Park, J.M., Lee, H.J., Bai, D.H., Yoon, S.S., Choi, J.B. and Park, Y.S. (2013). Analysis of microflora profile in Korean traditional nuruk. *Journal of Microbiology and Biotechnology* 23: 40-46.

Horackova, S., Vesela, K., Klojdova, I., Bercikova, M. and Plockova, M. (2020). Bile salt hydrolase activity, growth characteristics and surface properties in Lactobacillus acidophilus. *European Food Research and Technology* 246: 1627-1636.

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

Huang, Y., Yi, Z., Jin, Y., Zhao, Y., He, K., Liu, D., Zhao, D., He, H., Luo, H., Zhang, W., Fang, Y. and Zhao, H. (2017). New microbial resource: microbial diversity, function and dynamics in Chinese liquor starter. *Scientific Reports* 7(1): 1-14.

Hutkins, R. (2019). Microbiology and Technology of Fermented Foods. Wiley Press, London.

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

Ishimaru, Y. and Nakano, M. (1960). Studies on the microorganisms in the ragi. *Report of Food Research Institute* 18: 158-163.

Ives, J.D. (2006). *Himalayan perception: Environmental change and the well-being of mountain peoples.* Routledge, London.

Javed, A., Masud, T., Ain, Q.U., Imran, M. and Maqsood. S. (2011). Enterocins of *Enterococcus faecium*, emerging natural food preservatives. *Annals of Microbiology* 61(4): 699-708.

Jeevaratnam, K. and Nallala, V. (2017). Probiotic evaluation of Enterococcus duransVJI19 isolated from gastrointestinal tract of broiler chicken. *International Journal of Advanced Life Sciences* 10(1): 139-155.

Jeyaram, K., Singh, W.M., Capece, A. and Romano, P. (2008). Molecular identification of yeast species associated with '*Hamei*'-a traditional starter used for rice wine production in Manipur, India. *International Journal of Food Microbiology* 124(2): 115-125.

Jeyaram, K., Tamang, J.P., Capece, A. and Romano, P. (2011). Geographical markers for *Saccharomyces cerevisiae* strains with similar technological origins domesticated for

rice-based ethnic fermented beverages production in North East India. *Antonie Van Leeuwenhoek* 100(4): 569-578.

Ji, Z., Jin, J., Yu, G., Mou, R., Mao, J., Liu, S., Zhou, Z. and Peng, L. (2018). Characteristic of filamentous fungal diversity and dynamics associated with wheat Qu and the traditional fermentation of Chinese rice wine. *International Journal of Food Science and Technology* 53(7): 1611-1621.

Jung, M.J., Nam, Y.D., Roh, S.W. and Bae, J.W. (2012). Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food Microbiology* 30(1): 112-123.

Kam, N. and Fiona, M. (2018). Development of natural flavor enhancer using shio koji fermentation from menir rice (Oryza sativa L.). In: *AIP Conference Proceedings* (Vol. 2024, No. 1, p. 020057). AIP Publishing LLC.

Kapse, N.G., Engineer, A.S., Gowdaman, V., Wagh, S. and Dhakephalkar, P.K. (2019). Functional annotation of the genome unravels probiotic potential of *Bacillus coagulans* HS243. *Genomics* 111 (4): 921-929.

Khare, A. and Gaur, S. (2020). Cholesterol-lowering effects of *Lactobacillus* species. *Current Microbiology* 77: 1-7.

Khatri, P.K. (1987). *Nepali Samajra Sanskriti (Prachin-Madhyakal*). Shaja Prakashan, Kathmandu (Nepali).

Kiela, P.R. and Ghishan, F.K. (2016). Physiology of intestinal absorption and secretion. *Best Practice & Research Clinical Gastroenterology* 30(2): 145-159.

Kim, N.H., Jun, S.H., Lee, S.H., Hwang, I.G and Rhee, M.S. (2018). Microbial diversities and potential hazards of Korean turbid rice wines (makgeolli): multivariate analyses. *Food Microbiology* 76: 466-472.

Kimoto, H., Ohmomo, S. and Okamoto, T. (2002). Cholesterol removal from media by *lactococci. Journal of Dairy Science* 85(12): 3182-3188.

Kishida, M., Seike, Y. and Kawasaki, H. (2009). Identification and characterization of a psychrophilic yeast strain newly isolated from the fermentative starter (Loog-pang) of a traditional drink in Thailand. *Biocontrol Science* 14(3):119-122.

Kitamura, Y., Kusumoto, K.I., Oguma, T., Nagai, T., Furukawa, S., Suzuki, C., Satomi, M., Magariyama, Y., Takamine, K. and Tamaki, H. (2016). Ethnic fermented foods and alcoholic beverages of Japan. In: *Ethnic fermented foods and alcoholic beverages of Asia*, pp. 193-236. Springer, New Delhi.

Koyanagi, T., Nakagawa, A., Kiyohara, M., Matsui, H., Tsuji, A., Barla, F., Take, H., Katsuyama, Y., Tokuda, K., Nakamura, S., Minami, H., Enomoto, T., Katayama, T. and Kumagai, H. (2016). Tracing microbiota changes in *yamahai-moto*, the traditional Japanese sake starter. *Bioscience, Biotechnology and Biochemistry* 80(2): 399-406.

Kozaki, M. and Uchimura, T. (1990). Microorganisms in Chinese starter 'bubod' and rice wine 'tapuy' in the Philippines. *Journal of Brewing Society of Japan* 85 (11): 818-824.

Krasowska, A. and Sigler, K. (2014). How microorganisms use hydrophobicity and what does this mean for human needs?. *Frontiers In Cellular And Infection Microbiology* 4: 112. doi: 10.3389/fcimb.2014.00112.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA 7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33(7): 1870-1874.

Kumari, A., Angmo, K., Monika, and Bhalla, T.C. (2016). Probiotic attributes of indigenous *Lactobacillus* spp. isolated from traditional fermented foods and beverages of north-western Himalayas using in vitro screening and principal component analysis. *Journal of Food Science and Technology* 53(5): 2463-2475.

Kumari, A., Pandey, A., Ann, A., Raj, A., Gupta, A., Chauhan, A., Sharma, A., Das, A.J., Kumar, A., Attri, B., Neopany, B. *et al.* (2016). *Indigenous Alcoholic Beverages of South Asia.* pp. 501-566, CRC Press, New York.

Kumbhare, S.V., Dhotre, D.P., Dhar, S.K., Jani, K., Apte, D.A., Shouche,Y.S. and Sharma, A. (2015). Insights into diversity and imputed metabolic potential of bacterial communities in the continental shelf of Agatti Island. *Plos One* 10(6). doi: 10.1371/journal.pone.0129864.

Lambert, J.M., Bongers, R.S., de Vos, W.M. and Kleerebezem, M. (2008). Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. *Applied and Environmental Microbiology* 74(15): 4719-4726.

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

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

LeBlanc, J.G., Chain, F., Martín, R., Bermúdez-Humarán, L.G., Courau, S. and Langella, P. (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories* 16(79). doi: 10.1186/s12934-017-0691-z.

Lee, C.H. and Kim, M.L. (2016). History of fermented foods in Northeast Asia. In: *Ethnic Fermented Foods and Alcoholic Beverages of Asia* (Ed: Tamang, J.P.), pp.1-16. Springer, New Delhi.

Lee, C.H. and Lee, S.S. (2002). Cereal fermentation by fungi. *Applied Mycology and Biotechnology* 2: 151-170.

Lee, H.H., Loh, S.P., Bong, C.F.J., Sarbini, S.R. and Yiu, P.H. (2015). Impact of phytic acid on nutrient bioaccessibility and antioxidant properties of dehusked rice. *Journal of Food Science and Technology* 52(12): 7806-7816.

Lemi, B. (2020). Microbiology of Ethiopian traditionally fermented beverages and condiments. *International Journal of Microbiology* 1-8: doi.10.1155/2020/1478536.

Li, H., Jiao, A., Xu, X., Wu, C., Wei, B., Hu, X., Jin, Z. and Tian, Y. (2012). Simultaneous saccharification and fermentation of broken rice: An enzymatic extrusion liquefaction pretreatment for Chinese rice wine production. *Bioprocess Biosystems and Engineering* 36: 10. doi: 1007/s00449-012-0868-0.

Li, P., Aflakpui, F.W.K., Yu, H., Luo, L. and Lin, W. (2015). Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar. *Annals of Microbiology* 65(4): 2019-2027.

Li, Z., Bai Z., Wang D., Zhang W., Zhang M., Lin F., Gao, L., Hui, B. and Zhang, H. (2014). Cultivable bacterial diversity and amylase production in three typical Daqus of Chinese spirits. *International Journal of Food Science and Technology* 49(3): 776-786.

Limtong, S., Sintara, S. and Suwannarit, P. (2002). Yeast diversity in Thai traditional alcoholic starter. *Kasetsart Journal of Natural Science* 36: 149-158.

Liu, S.P., Yu, J.X., Wei, X.L., Ji, Z.W., Zhou, Z.L., Meng, X.Y. and Mao, J. (2016). Sequencing-based screening of functional microorganism to decrease the formation of biogenic amines in Chinese rice wine. *Food Control* 64: 98-104.

Lomer, M.C.E., Parkes, G.C. and Sanderson, J.D. (2008). Lactose intolerance in clinical practice–myths and realities. *Alimentary Pharmacology and Therapeutics* 27(2): 93-103.

Lotong, N. (1998). Koji. *In:Microbiology of Fermented Food* (Ed: Wood, J.B.), pp. 658-695. Vol. 2, 2nd ed, Blackie Academic and Professional, London.

Lu, Y., Lu, X., Chen, X., Jiang, M., Li, C. and Dong, M. (2007). A survey of biogenic amines in Chinese rice wines. *Food Chemistry* 100(4): 1424-1428.

Luana, N., Rossana, C., Curiel, J.A., Kaisa, P., Marco, G. and Rizzello, C.G. (2014). Manufacture and characterization of a yogurt-like beverage made with oat flakes fermented by selected lactic acid bacteria. *International Journal of Food Microbiology* 185: 17-26.

Lv, X.C., Weng, X., Zhang, W., Rao, P.F. and Ni, L. (2012). Microbial diversity of traditional fermentation starters for Hong Qu glutinous rice wine as determined by PCR-mediated DGGE. *Food Control* 28(2): 426-434.

Ly, S., Mith, H., Tarayre, C., Taminiau, B., Daube, G., Fauconnier, M.L. and Delvigne, F. (2018). Impact of microbial composition of Cambodian traditional dried starters (dombea) on flavor compounds of rice wine: combining amplicon sequencing with HP-SPME-GCMS. *Frontiers in Microbiology* 9: 894. doi: 10.3389/fmicb.2018.00894.

Machida, M., Yamada, O. and Gomi, K. (2008). Genomics of *Aspergillus oryzae:* learning from the history of Koji mold and exploration of its future. *DNA Research: An International Journal for Rapid Publication Of Reports On Genes And Genomes* 15(4): 173-183.

Mahdihassan, S. (1979). Distillation assembly of pottery in ancient India with a single item of special construction. *Vishveshvaran and Indological Journal Hoshiarpur* 17: 264-266.

Mahdihassan, S. (1981). Parisrut the earliest distilled liquor of Vedic times or of about 1500 BC. *Indian Journal of History of Science* 16(2): 223-229.

Martín Rosique, R., SoberónMaltos, N.E., Vaneechoutte, M., Flórez García, A.B., Vázquez Valdés, F. and Suárez Fernández, J.E. (2008). Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *International Microbiology* 11: 261-266.

Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G. and Neufeld, J.D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC bioinformatics* 13(1): 31. doi: 10.1186/1471-2105-13-31.

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

McAuliffe, O., Cano, R.J. and Klaenhammer, T.R. (2005). Genetic analysis of two bile salt hydrolase activities in Lactobacillus acidophilus NCFM. *Applied and Environmental Microbiology* 71(8): 4925-4929.

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

McGovern, P.E., Fleming, S.J. and Katz, S.H. (2004). *The Origins and Ancient History of Wine: Food and Nutrition in History and Antropology.* Taylor and Francis, New York.

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

Mo, X., Fan, W. and Xu, Y. (2009). Changes in volatile compounds of Chinese rice wine wheat Qu during fermentation and storage. *Journal of the Institute of Brewing* 115(4): 300-307.

Monteagudo-Mera, A., Rastall, R.A., Gibson, G.R., Charalampopoulos, D. and Chatzifragkou, A. (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. *Applied Microbiology and Biotechnology* 103(16): 6463-6472.

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

Moreno, M.R.F., Callewaert, R., Devreese, B., Van Beeumen, J. and De Vuyst, L. (2003). Isolation and biochemical characterization of enterocins produced by enterococci from different sources. *Journal of Applied Microbiology* 94(2): 214-229.

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

Mukisa, I.M., Byaruhanga, Y.B., Muyanja, C.M.B.K., Langsrud, T. and Narvhus, J.A. (2017). Production of organic flavor compounds by dominant lactic acid bacteria and yeasts from Obushera, a traditional sorghum malt fermented beverage. *Food Science and Nutrition* 5(3): 702-712.

Mulaw, G., Sisay Tessema, T., Muleta, D. and Tesfaye, A. (2019). In vitro evaluation of probiotic properties of lactic acid bacteria isolated from some traditionally fermented Ethiopian food products. *International Journal of Microbiology*. doi: 10.1155/2019/7179514.

Murai, T. (1989). Tane-Koji Konjaku Monogatari (in Japanese). *Shushi-Kenkyu* 7: 39-44.

Murakami, H. (1980). Koji-gaku (in Japanese). Brewing Society of Japan.

Nami, Y., VaseghiBakhshayesh, R., MohammadzadehJalaly, H., Lotfi, H., Eslami, S. and Hejazi, M.A. (2019). Probiotic properties of Enterococcus isolated from artisanal dairy products. *Frontiers in Microbiology* 10: 300. doi: 10.3389/fmicb.2019.00300.

Nandy, S.N., Dhyani, P.P. and Sanal, P.K. (2006). Resources information database of the Indian Himalaya. *ENVIS Monograph* 3: 1-95.

Needham, J. (2000). Biology and biological technology. In: *Science and Civilization in China* (Ed: Huang, H.T.), pp. 149-168. Cambridge University Press: England.

Ness, I.F., Diep, D.B. and Ike, Y. (2014). Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control. In *Enterococci: From commensals to leading causes of drug resistant infection [Internet]*. Massachusetts Eye and Ear Infirmary.

Ngongang, E.F.T., Tiencheu, B., Fossi, B.T., Achidi, A.U., Shiynyuy, D.M., Womeni, H.M. and François, Z.N. (2016). Isolation and identification of cholesterol lowering probiotic bacteria from palm wine (Raffia mambillensis). *Journal of Microbiology Research* 6(5): 93-102.

Nikkuni, S., Karki, T.B., Terao, T. and Suzuki, C. (1996). Microflora of *mana*, a Nepalese rice *koji*. *Journal of Fermentation and Bioengineering* 81(2): 168-170.

Nile, S.H. (2015). The nutritional, biochemical and health effects of makgeolli – a traditional Korean fermented cereal beverage. *Journal of Institute of Brewing* 121(4): 457-463.

Nithya, V. and Halami, P.M. (2013). Evaluation of probiotic characteristics of Bacillus species isolated from different food sources. *Annals of Microbiology* 63(1): 129-137.

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

Nout, M.R. (2009). Rich nutrition from the poorest–Cereal fermentations in Africa and Asia. *Food Microbiology* 26(7): 685-692.

Nuding, D.L., Gough, R.V., Venkateswaran, K.J., Spry, J.A. and Tolbert, M.A. (2017). Laboratory investigations on the survival of *Bacillus subtilis* spores in deliquescent salt mars analog environments. *Astrobiology* 17(10): 997-1008.

Nuraida, L.I.L.I.S. and Krusong, W.A.R.A.W.U.T. (2014). Starter cultures. *In: Indigenous Fermented Foods of South east Asia* (Ed: David Owens, J.), pp.109-124. CRC press, Taylor and Francis, New York.

Oatway, L., Vasanthan, T. and Helm, J.H. (2001). Phytic acid. *Food Reviews International* 17(4): 419-431.

Orji, M.U., Mbata, T.I., Aniche, G.N. and Ahonkhai, I. (2003). The use of starter cultures to produce 'Pito', a Nigerian fermented alcoholic beverage. *World Journal of Microbiology and Biotechnology* 19(7): 733-736.

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

Padmavathi, T., Bhargavi, R., Priyanka, P.R., Niranjan, N.R. and Pavitra, P.V. (2018). Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. *Journal of Genetic Engineering and Biotechnology* 16(2): 357-362.

Park, J.M., Shin, J.H., Lee, J.W., Song, J.C., Suh, H.J., Chang, U.J. and Kim, J.M. (2010). Identification of Lactic Acid Bacteria in Kimchi according to initial and over ripened fermentation using PCR and 16S rRNA gene sequence analysis. *Food Science and Biotechnology* 19(2): 541-546.

Park, K.I., Mheen, T.I., Lee K.H., Chang, C.H., Lee, S.R. and Kwon, T.W. (1977). Korean *yakju* and *takju*. In: *The Proceeding and Symposium on Indigenous Fermented Foods*, GIAMI, Bangkok, November 21-27.

Paula, B.P., Chávez, D.W.H., Lemos Junior, W.J.F., Guerra, A.F., Corrêa, M.F.D,. Pereira, K.S. and Coelho, M.A.Z. (2019). Growth parameters and survivability of *Saccharomyces boulardii* for probiotic alcoholic beverages development. *Frontiers in Microbiology* 10: 2092. doi: 10.3389/fmicb.2019.02092.

Perova, N.V. and Metel'skaia, V.A. (2008). Plant sterols and stanols as the dietary factors lowering hypercholesterolemia by inhibition of intestinal cholesterol absorption. *Kardiologiia* 48(5): 62-69.

Pervez, S., Aman, A., Iqbal, S., Siddiqui, N.N. and UlQader, S.A. (2014). Saccharification and liquefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. *BMC Biotechnology* 14(1): 49. doi: 10.1186/1472-6750-14-49.

Peter-Ikechukwu, A.I., Kabuo, N.O., Alagbaoso, S.O., Njoku, N.E., Eluchie, C.N. and Momoh, W.O. (2016). Effect of wrapping materials on physico-chemical and microbiological qualities of fermented melon seed (*Citrulluscolocynthis* L.) used as condiment. *American Journal of Food Science and Technology* 4 (1): 14-19.

Peyrot des Gachons, C. and Breslin, P.A. (2016). salivary amylase: digestion and metabolic syndrome. *Current Diabetes Reports* 16(10): 102. doi: 10.1007/s11892-016-0794-7.

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

Pichyangkura, S. and Kulprecha, S. (1977). Survey of mycelial moulds in loogpang from various sources in Thailand. *In: The Proceedings and Symposium on Indigenous Fermented Foods*, November 21-27. GIAMI, Bangkok, Thailand.

Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Bzducha-Wróbel, A. and Synowiec, A. (2018). Research on the ability of propionic acid and vitamin B12 biosynthesis by *Propionibacterium freudenreichii* strain T82. *Antonie Van Leeuwenhoek* 111(6): 921-932.

Polak-Berecka, M., Waśko, A., Paduch, R., Skrzypek, T. and Sroka-Bartnicka, A. (2014). The effect of cell surface components on adhesion ability of *Lactobacillus rhamnosus*. *Antonie Van Leeuwenhoek* 106(4): 751-762.

Porto, M.C.W., Kuniyoshi, T.M., Azevedo, P.O.S., Vitolo, M. and Oliveira, R.P.D.S. (2017). *Pediococcus* spp.: an important genus of lactic acid bacteria and pediocin producers. *Biotechnology Advances* 35(3): 361-374.

Prado, F.C., Parada, J.L., Pandey, A. and Soccol, C.R. (2008). Trends in non-dairy probiotic beverages. *Food Research International* 41(2): 111-123.

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

Preet, K., Malhotra, S., Shrivastava, P. *et al.* (2016). Genetic Diversity in Gorkhas: an autosomal STR study. *Scientific Reports* 6: 32494. doi: 10.1038/srep32494.

Pretorius, I.S. (2000). Review: Tailoring wine yeast for the new millennium: novel approaches to the ancient art of wine making. *Yeast* 16(8): 675-729.

Priyodip, P., Prakash, P.Y. and Balaji, S. (2017). Phytases of probiotic bacteria: characteristics and beneficial aspects. *Indian Journal of Microbiology* 57(2): 148-154.

Puerari, C., Magalhaes-Guedes, K.T. and Schwan, R.F. (2015). Physicochemical and microbiological characterization of chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians. *Food Microbiology* 46: 210-217.

Qin, H.Z. (2000). The evolution history and development trend of distilled liquor in China. *Liquor-making Science and Technology* 5(101): 24-30.

Que, F., Mao, L.C. and Pan, X. (2006). Antioxidant activities of five Chinese rice wines and the involvement of phenolic compounds. *Food Research International* 39(5): 581-587.

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

Ramiah, K., Van Reenen, C.A. and Dicks, L.M.T. (2007). Expression of the mucus adhesion genes Mub and MapA, adhesion-like factor EF-Tu and bacteriocin gene plaA of Lactobacillus plantarum 423, monitored with real-time PCR. *International Journal of Food Microbiology* 116(3): 405-409.

Rana, T.S., Datt, B. and Rao, R.R. (2004). *Soor*: A traditional alcoholic beverage in tons valley, Garhwal Himalaya. *Indian Journal of Traditional Knowledge* 3(1): 59-65.

Ray, S., Bagyaraj, D.J., Thilagar, G. and Tamang, J.P. (2016). Preparation of Chyang, an ethnic fermented beverage of the Himalayas, using different raw cereals. *Journal of Ethnic Foods* 3(4): 297-299.

Renfrew, J.W. (1999). Palaeoethnobotany and the archaeology of wine. In: *The Oxford companion to wine* (Ed: Robinson, J.), pp. 508-509. 2nd edition, Oxford, UK: Oxford University Press.

Rezac, S., Kok, C.R., Heermann, M. and Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Frontiers in Microbiology* 9:1785. doi: 10.3389/fmicb.2018.01785.

Risley, H.H. (1928). *The Gazetteer of Sikkim*. D.K. Publishing Distributors (P) Ltd., New Delhi.

Ritchie, H. and Roser, M. (2018). Alcohol consumption. *Our World In Data*. Retrieved from: https://ourworldindata.org/alcohol-consumption.

Robinson, J. (1994). *The Oxford Companion to Wine.* Oxford University Press, Oxford.

Rodrıguez, J.M., Cintas, L.M., Casaus, P., Martınez, M.I., Suárez, A. and Hernández, P.E. (1997). Detection of pediocin PA-1-producing pediococci by rapid molecular biology techniques. *Food Microbiology* 14(4): 363-371.

Roger, D.D., Daoudou, B., James, B. and Etoa, F. (2014). Artisanal production of "kuri" an honey made alcoholic beverage from Adamaoua Cameroon. *Global Journal of Scientific Researches* 2(3): 65-70.

Rogosa, M., Mitchell, J.A. and Wiseman, R.F. (1951). A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *Journal of Bacteriology* 62(1): 132-133.

Roy, B., Kala, C.P., Farooquee, N.A. and Majila, B.S. (2004). Indigenous fermented food and beverages: a potential for economic development of the high altitude societies in Uttaranchal. *Journal of Human Ecology* 15(1): 45-49.

Rudel, L.L. and Morris, M.D. (1973). Determination of cholesterol using o-phthalaldehyde. *Journal of Lipid Research* 14(3): 364-366.

Ruiz, L., Margolles, A. and Sánchez, B. (2013). Bile resistance mechanisms in Lactobacillus and Bifidobacterium. *Frontiers in Microbiology* 4: 396. doi: 10.3389/fmicb.2013.00396

Ryu, D., Choi, B., Kim, E., Park, S., Paeng, H., Kim, C.I., Lee, J.Y., Yoon, H.J. and Koh, E. (2015). Determination of ethyl carbamate in alcoholic beverages and fermented foods sold in Korea. *Toxicological Research* 31(3): 289-97.

Saha, D. (2013). Lesser Himalayan sequences in Eastern Himalaya and their deformation: Implications for Paleoproterozoic tectonic activity along the northern margin of India. *Geoscience Frontiers* 4(3): 289-304.

Sanders M.E. and in't Veld J.H. (1999). Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. *Antonie van Leeuwenhoek* 76: 293-315.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* 74(12): 5463-5467.

Sarkar, A., Mukherjee, A.D., Bera, M.K., Das, B., Juyal, N., Morthekai, P., Deshpande, R.D., Shinde, V.S. and Rao, L.S. (2016). Oxygen isotope in archaeological bioapatites from India: Implications to climate change and decline of Bronze Age Harappan civilization. *Scientific Reports* 6(1): 1-9.

Sarma, P.J. (1939). The art of healing. *Rigveda Annals of Medical History*, 3rd Series: 1, 538.

Sarkar, P.K. and Tamang, J.P. (1995). Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. *Food Microbiology* 12: 317-325.

Saulnier, D.M., Santos, F., Roos, S., Mistretta, T.A., Spinler, J.K., Molenaar, D., Teusink, B. and Versalovic, J. (2011). Exploring metabolic pathway reconstruction and genome-wide expression profiling in *Lactobacillus reuteri* to define functional probiotic features. *PloS one* 6(4): e18783. doi: 10.1371/journal.pone.0018783.

Savic, M., Room, R., Mugavin, J., Pennay, A. and Livingston, M. (2016). Defining "drinking culture": A critical review of its meaning and connotation in social research on alcohol problems. *Drugs: Education, Prevention and Policy* 23(4): 270-282.

Scheers, N., Rossander-Hulthen, L., Torsdottir, I. and Sandberg, A.S. (2016). Increased iron 789 bioavailability from lactic-fermented vegetables is likely an effect of promoting the formation of ferric iron (Fe3+ 790). *European Journal of Nutrition* 55(1): 373-382.

Schiffer, C., Hilgarth, M., Ehrmann, M. and Vogel, R.F. (2019). Bap and cell surface hydrophobicity are important factors in *Staphylococcus xylosus* biofilm formation. *Frontiers in Microbiology* 10: 1387. doi: 10.3389/fmicb.2019.01387.

Schillinger, U. and Lücke, F.K. (1987). Identification of lactobacilli from meat and meat products. *Food microbiology* 4(3): 199-208.

Schmitz, A. and Riesner, D. (2006). Purification of nuclei acids by selective precipitation with polyethylene glycol 6000. *Analytical Biochemistry* 354(2): 311-313.

Sekar, S. and Mariappan, S. (2007). Usage of traditional fermented products by Indian rural folks and IPR. *Indian Journal of Traditional Knowledge* 6(1): 111-120.

Semedo, T., Santos, M.A., Martins, P., Lopes, M.F.S., Marques, J.J.F., Tenreiro, R. and Crespo, M.T.B. (2003). Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. *Journal of Clinical Microbiology* 41(6): 2569-2576.

Sha, S.P., Anupama, A., Pradhan, P., Prasad, G.S. and Tamang, J.P. (2016). Identification of yeasts by polymerase-chain-reaction-mediated denaturing gradient gel electrophoresis in marcha, an ethnic amylolytic starter of India. *Journal of Ethnic Foods* 3(4): 292-296.

Sha, S.P., Jani, K., Sharma, A., Anupma, A., Pradhan, P., Shouche, Y. and Tamang, J.P. (2017). Analysis of bacterial and fungal communities in Marcha and Thiat, traditionally prepared amylolytic starters of India. *Scientific Reports* 7(1): 1-7. doi: 10.1038/s41598-017-11609-y.

Sha, S.P., Suryavanshi, M.S. and Tamang, J.P. (2019). Mycobiome diversity in traditionally prepared starters for alcoholic beverages in India by high-throughput sequencing method. *Frontiers in Microbiology* 10: 348. doi: 10.3389/fmicb.2019.003482237.

Sha, S.P., Suryavanshi, M.V., Jani, K., Sharma, A., Shouche, Y. and Tamang, J.P. (2018). Diversity of yeasts and molds by culture-dependent and culture-independent methods for mycobiome surveillance of traditionally prepared dried starters for the production of Indian alcoholic beverages. *Frontiers in Microbiology* 9: 2237. doi: 10.3389/fmicb.2018.02237.

Shah, N.P. (2007). Functional cultures and health benefits. *International Dairy Journal* 17(11): 1262-1277.

Shang, Y.L., Chen, L.L., Zhang, Z.H. and Lu, J. (2012). A comparative study on the fungal communities of wheat Qu for Qingshuang-type Chinese rice wine. *Journal of the Institute of Brewing* 118(2): 243-248.

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: 1532. doi: 10.1038/s41598-018-19524-6.

Sharma, N., Angural, S., Rana, M., Puri, N., Kondepudi, K.K. and Gupta, N. (2020). Phytase producing lactic acid bacteria: Cell factories for enhancing micronutrient bioavailability of phytate rich foods. *Trends in Food Science and Technology* 96: 1-12.

Sharma, R., Jianchu, X. and Sharma, G. (2007). Traditional agroforestry in the Eastern Himalayan region: Land management system supporting ecosystem services. *Tropical Ecology* 48(2): 189-200.

Shehata, M.G., El Sohaimy, S.A., El-Sahn, M.A. and Youssef, M.M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Annals of Agricultural Sciences* 61(1): 65-75.

Shi, L.H., Balakrishnan, K., Thiagarajah, K., Mohd Ismail, N.I. and Yin, O.S. (2016). Beneficial properties of probiotics. *Tropical Life Sciences Research* 27(2): 73-90.

Shobharani, P. and Halami, P.M. (2016). In vitro evaluation of the cholesterol-reducing ability of a potential probiotic *Bacillus* spp. *Annals of Microbiology* 66(2): 643-651.

Shrestha, H.N.K. and Rati E.R. (2002). Microbiological profile of *murcha* starters and physico-chemical characteristics of *poko*, a rice based traditional food products of Nepal. *Food Biotechnology* 16(1): 1-15.

Shrivastava, K., Greeshma, A.G. and Srivastava, B. (2012). Biotechnology in Tradition-Methods of starter preparation for alcoholic beverages employed by different tribal communities of Arunachal Pradesh. *Indian Journal of Traditional Knowledge* 11: 194-201.

Singh, D., Lee, S. and Lee, C.H. (2019). Fathoming *Aspergillus oryzae* metabolomes in formulated growth matrices. *Critical Reviews in Biotechnology* 39(1): 35-49.

Singh, N.L., Ramprasad Mishra, P.K., Shukla, S.K., Kumar, J. and Singh, R. (2010). Alcoholic fermentation techniques in early Indian tradition. *Indian Journal of History of Science* 45(2): 163-173.

Sivamaruthi, B.S., Kesika, P. and Chaiyasut, C. (2019). Toxins in fermented foods: prevalence and preventions-a mini review. *Toxins* 11(1): 4. doi: 10.3390/toxins11010004.

Soccol1, C.R., de Souza Vandenberghe, L.P., Spier, M.R., Medeiros, A.B.P., Yamaguishi, C.T., Lindner, J.D.D., Pandey, A. and Thomaz-Soccol1, V. (2010). The potential of probiotics: a review. *Food Technology* 48 (4): 413-434.

Sofyan, A., Martin, R.S.H., Laconi, E.B., Jayanegara, A., Julendra, H., Damayanti, E. and Suryani, A.E. (2019). The assays of bacteria-yeast consortia as probiotics candidates and their influences on nutrients utilization of Quails diet. *Tropical Animal Science Journal* 42(3): 196-202.

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

Somasundaram, O., Raghavan, D.V. and Murthy, A.G. (2016). Drinking habits in ancient India. *Indian Journal of Psychiatry* 58(1): 93-96.

Song, S.H., Lee, C., Lee, S., Park, J.M., Lee, H.J., Bai, D.H., Yoon, S.S., Choi, J.B. and Park, Y.S. (2013). Analysis of microflora profile in Korean traditional nuruk. *Journal of Microbiology and Biotechnology* 23(1): 40-46.

Soufleros, E., Bouloumpasi, E., Zotou, A. and Loukou, Z. (2007). Determination of biogenic amines in Greek wines by HPLC and ultraviolet detection after dansylation and examination of factors affecting their presence and concentration. *Food Chemistry* 101(2): 704-716.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. and Massa, S. (2005). A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *Journal of Applied Microbiology* 98 (4): 855-861.

Sreeramulu, G., Srinivasa, D.S., Nand, K. and Joseph, R. (1996). *Lactobacillus amylovorus* as a 813 phytase producer in submerged culture. *Letters in Applied Microbiology* 23(6): 385-388.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S. *et al.* (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS ONE* 10(2): e0117617. doi: 10.1371/journal.pone.0117617.

Steinkraus, K.H. (1996). *Handbook of Indigenous Fermented Food*, 2nd edition. Marcel Dekker, Inc., New York.

Sternes, P.R., Lee, D., Kutyna, D.R. and Borneman, A.R. (2017). A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *Gigascience* 6(7):1-10.

Sujaya, I.N., Amachi, S., Yokota, A., Asano, K. and Tomita, F. (2001). Identification and characterization of lactic acid bacteria in ragi tape. *World Journal of Microbiology and Biotechnology* 17: 349-357.

Surono, I.S. (2016). Ethnic fermented foods and beverages of Indonesia. In: *Ethnic Fermented Foods and Alcoholic Beverages of Asia* (Ed: Tamang, J.P.), pp. 341-382. Springer, New Delhi.

Taira, J., Miyagi, C. and Aniya, Y. (2002). Dimerumic acid as an antioxidant from the mold, *Monascus anka*: the inhibition mechanisms against lipid peroxidation and hemeprotein-mediated oxidation. *Biochemical Pharmacology* 63(5): 1019-1026.

Talon, R., Montel, M.C. and Berdague, J.L. (1996). Production of flavor esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus*. *Enzyme and Microbial Technology* 19(8): 620-622.

Tamang, J.P. (2005). *Food Culture of Sikkim*. Sikkim Study Series volume IV. Information and Public Relations Department, Government of Sikkim, Gangtok, Sikkim.

Tamang, J.P. (2010a). *Himalayan Fermented Foods: Microbiology, Nutrition and Ethnic Values*. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P. (2010b). Diversity of fermented beverages. In: *Fermented Foods and Beverages of the World* (Ed: Tamang J.P. and Kailasapathy, K.), pp. 85-125. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P. (2015). *Health Benefits of Fermented Foods and Beverages*, pp. 636. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P. (2020). History and Culture of Indian Ethnic Fermented Foods and Beverages. In: *Ethnic Fermented Foods and Alcoholic Beverages of India: Science History and Culture* (Ed: Tamang, J.P.), pp. 1-40. Springer Nature, Singapore.

Tamang, J.P. and Fleet, G.H. (2009). Yeasts diversity in fermented foods and beverages. In: *Yeast Biotechnology: Diversity and Applications* (Ed: Satyanarayana, T. and Kunze G.), pp. 169-198. Springer, Dordrecht.

Tamang, J.P. and Samuel, D. (2010). Dietary cultures and antiquity of fermented foods and beverages. *Fermented Foods and Beverages of the World*, pp.1-40. CRC press, Taylor and Francis, New York.

Tamang, J.P. and Sarkar, P.K. (1995). Microflora of murcha: an amylolytic fermentation starter. *Microbios* 81(327): 115-122.

Tamang, J.P. and Thapa, S. (2006). Fermentation dynamics during production of bhaati jaanr, a traditional fermented rice beverage of the Eastern Himalayas. *Food Biotechnology* 20(3): 251-261.

Tamang, J.P., Cotter, P.D., Endo, A., Han, N.S., Kort, R., Liu, S.Q., Mayo, B., Westerik, N. and Hutkins, R. (2020). Fermented foods in a global age: East meets West. *Comprehensive Reviews in Food Science and Food Safety* 19(1): 184-217.

Tamang, J.P., Dewan, S., Tamang, B., Rai, A., Schillinger, U. and Holzapfel, W.H. (2007). Lactic acid bacteria in hamei and marcha of North East India. *Indian Journal of Microbiology* 47(2): 119-125.

Tamang, J.P., Holzapfel, W.H. and Watanabe, K. (2016a). Diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology* 7: 377. doi: 10.3389/fmicb.2016.00377.

Tamang, J.P., Okumiya, K. and Kosaka, Y. (2010). Cultural Adaptation of the Himalayan Ethnic Foods with special reference to Sikkim, Arunachal Pradesh and Ladakh. *Himalayan Study Monographs of RIHN (Kyoto, Japan)* 11: 177-185.

Tamang, J.P., Sarkar, P.K. and Hesseltine, C.W. (1988). Traditional fermented foods and beverages of Darjeeling and Sikkim–a review. *Journal of the Science of Food and Agriculture* 44(4): 375-385.

Tamang, J.P., Shin, D.H., Jung, S.J. and Chae, S.W. (2016b). Functional properties of microorganisms in fermented foods. *Frontiers in Microbiology* 7: 578. doi: 10.3389/fmicb.2016.00578.

Tamang, J.P., Tamang, N., Thapa, S., Dewan, S., Tamang, B.M., Yonzan, H., Rai, A.K., Chettri, R., Chakrabarty, J. and Kharel, N. (2012). Microorganisms and nutritional value of ethnic fermented foods and alcoholic beverages of North East India. *Indian Journal of Traditional Knowledge* 11: 7-25.

Tamang, J.P., Thapa, N. and Bhalla, T.C. (2016c). Ethnic fermented foods and beverages of India. In: *Ethnic Fermented Foods and Alcoholic Beverages of Asia* (Ed: Tamang, J.P), pp. 17-72. Springer, New Delhi.

Tamang, J.P., Thapa, S., Tamang, N. and Rai, B. (1996). Indigenous fermented food beverages of Darjeeling hills and Sikkim: process and product characterization. *Journal of Hill Research* 9(2): 401-411.

Tang, Q., He, G., Huang, J., Wu, C., Jin, Y. and Zhou, R. (2019). Characterizing relationship of microbial diversity and metabolite in Sichuan *Xiaoqu*. *Frontiers in Microbiology* 10: 696. doi: 10.3389/fmicb.2019.00696

Taranto, M.P., de Ruiz Holgado, A.P. and de Valdez, G.F. (1995). Bile salt hydrolase activity in Enterococcus faecium strains. *Microbiologie-Aliments-Nutrition* 13(4): 375-379.

Taranto, M.P., Perdigón, G., Médici, M. and de Valdez, G.F. (2004). Animal model for in vivo evaluation of cholesterol reduction by lactic acid bacteria. In: *Public Health Microbiology,* pp. 417-422. vol 268. Humana Press.

Taylor, B.C., Lejzerowicz, F., Poirel, M., Shaffer, J.P., Jiang, L., Aksenov, A., Litwin, N., Humphrey, G., Martino, C., Miller-Montgomery, S., Dorrestein, P.C., Veiga, P., Song, S.J., McDonald, D., Derrien, M. and Knight, R. (2020). Consumption of fermented foods is associated with systematic differences in the gut microbiome and metabolome. *mSystems* 5:e00901-19. doi: 10.1128/mSystems.00901-19.

Terpou, A., Papadaki, A., Lappa, I.K., Kachrimanidou, V., Bosnea, L.A. and Kopsahelis, N. (2019). Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients* 11(7): 1591. doi: 10.3390/nu11071591.

Teuber, M. (1993). Lactic acid bacteria. *Biotechnology: Biological Fundamentals* 1: 325-366. doi: 10.1002/9783527620821.chapter10.

Thakur, N. and Bhalla, T.C. (2004). Characterization of some traditional fermented foods and beverages of Himachal Pradesh. *Indian Journal of Traditional Knowledge* 3(3): 325-335.

Thakur, N., Saris, P.E. and Bhalla, T.C. (2015). Microorganisms associated with amylolytic starters and traditional fermented alcoholic beverages of North Western Himalayas in India. *Food Bioscience* 11: 92-96.

Thanh, V.N. and Tuan, D.A. (2008). Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *International Journal of Food Microbiology* 128(2): 268-273.

Thapa, N. and Tamang, J.P. (2020). Ethnic Fermented Foods and Beverages of Sikkim and Darjeeling Hills (Gorkhaland Territorial Administration). In: *Ethnic Fermented Foods*

and Alcoholic Beverages of India: Science History and Culture (Ed: Tamang, J.P.), pp. 479-537. Springer Nature, Singapore.

Thapa, S. and Tamang, J.P. (2004). Product characterization of kodo ko jaanr: fermented finger millet beverage of the Himalayas. *Food Microbiology* 21(5): 617-622.

Thesseling, F.A., Bircham, P.W., Mertens, S., Voordeckers, K. and Verstrepen, K.J. (2019). A hands-on guide to brewing and analyzing beer in the laboratory. *Current Protocols in Microbiology* 54: e91. doi: 10.1002/cpmc.91.

Tsuyoshi, N., Fudou, R., Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S. and Tamang, J.P. (2005). Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. *International Journal of Food Microbiology* 99(2): 135-146.

Turpin, W., Humblot, C. and Guyot, J.P. (2011). Genetic screening of functional properties of lactic acid bacteria in a fermented pearl millet slurry and in the metagenome of fermented starchy foods. *Applied Microbiology and Biotechnology* 77(24): 8722-8734.

Turpin, W., Humblot, C., Noordine, M.L., Thomas, M. and Guyot, J.P. (2012). Lactobacillaceae and cell adhesion: genomic and functional screening. *PLoS One*, 7(5): e38034. doi: 10.1371/journal.pone.0038034.

Uchimura, T., Niimura, Y., Ohara, N. and Kozaki, M. (1991). Microorganisms in Luck pang used for vinegar production in Thailand. *Journal of Brewing Society of Japan* 86(1): 62-67.

Väkeväinen, K., Valderrama, A., Espinosa, J., Centurión, D., Rizo, J., Reyes-Duarte, D., Díaz-Ruiz, G., Von Wright, A., Elizaquível, P., Esquivel, K. and Simontaival, A.I. (2018). Characterization of lactic acid bacteria recovered from atoleagrio, a traditional Mexican fermented beverage. *LWT* 88: 109-118.

Varin, C., Reid, N. and Firth, D. (2011). An overview of composite likelihood methods. *Statistica Sinica* 21(1): 5-42.

Versari, A., Parpinello, G.P. and Cattaneo, M. (1999). Leuconostocoenos and malolactic fermentation in wine: a review. *Journal of Industrial Microbiology and Biotechnology* 23(6): 447-455.

Vinderola, C.G and Reinheimer, J.A. (2003). Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International* 36(9-10): 895-904.

Vlasova, A.N., Kandasamy, S., Chattha, K.S., Rajashekara, G. and Saif, L.J. (2016). Comparison of probiotic lactobacilli and bifidobacteria effects, immune responses and rotavirus vaccines and infection in different host species. *Veterinary Immunology and Immunopathology* 172: 72-84.

Wade, M., Strickland, M., Osborne, J. and Edwards, C. (2018). Role of *Pediococcus* in winemaking: *Pediococcus* in winemaking. *Australian Journal of Grape and Wine Research* 25. doi: 10.1111/ajgw.12366.

Wagner, B.D., Grunwald, G.K., Zerbe, G.O., Mikulich-Gilbertson, S.K., Robertson, C.E., Zemanick, E.T. and Harris, J.K. (2018). On the use of diversity measures in longitudinal sequencing studies of microbial communities. *Frontiers in Microbiology* 9: 1037. doi:10.3389/fmicb.2018.01037.

Walker, G.M. and Stewart, G.G. (2016). *Saccharomyces cerevisiae* in the production of fermented beverages. *Beverages* 2: 30. doi: 10.3390/beverages2040030.

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J. and Roos, S. (2007). The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. *Applied and Environmental Microbiology* 73(12): 3924-3935.

Wang, C.L., Shi, D.J. and Gong, G.L. (2008). Microorganisms in Daqu: a starter culture of Chinese Maotai-flavor liquor. *World Journal of Microbiology and Biotechnology* 24(10): 2183-2190.

Wang, J., Wang, G. and Wang, Z. (2004). The innovative technique of Fenjiu Daqu. In: *Fenjiu Daqu (in Chinese)* (Ed: Hao, X. and Wang, H.), pp. 11-23. Shanxi Xinhuacun Fenjiu Group Company: Fen-yang.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73(16): 5261-5267.

Waters, D.M., Mauch, A., Coffey, A., Arendt, E.K. and Zannini, E. (2015). Lactic acid bacteria as a cell factory for the delivery of functional biomolecules and ingredients in cereal-based beverages: a review. *Critical Reviews in Food Science and Nutrition* 55(4): 503-520.

Whitehead, K., Versalovic, J., Roos, S. and Britton, R.A. (2008). Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Applied and Environmental Microbiology* 74(6): 1812-1819.

Wilson, H. (1999). Origins of viticulture. In: *The Oxford companion to wine* (Ed: Robinson, J.), pp. 505-506. 2nd edition. Oxford, UK: Oxford University Press.

Xiang, H., Sun-Waterhouse, D., Waterhouse, G.I.N., Cui, C. and Ruan, Z. (2019). Fermentation-enabled wellness foods: A fresh perspective. *Food Science and Human Wellness* 8(3): 203-243.

Xu, S., Liu, T., Radji, C.A.I., Yang, J. and Chen, L. (2016). Isolation, identification, and evaluation of new lactic acid bacteria strains with both cellular antioxidant and bile salt hydrolase activities in vitro. *Journal of Food Protection* 79(11): 1919-1928.

Yan, Y., Qian, Y., Ji, F., Chen, J. and Han, B. (2013). Microbial composition during Chinese soy sauce*koji*-making based on culture dependent and independent methods. *Food Microbiology* 34(1): 189-195.

Yang, J.G., Dou, X., Han, P.J., Bai, F.Y., Zhou, J., Zhang, S.Y., Quin, H. and Maa, Y.Y. (2017). Microbial diversity in Daqu during production of Luzhou-flavored liquor. *Journal of the American Society of Brewing Chemists* 75(2): 136-144.

Yang, R., Le, G., Li, A., Zheng, J. and Shi, Y. (2006). Effect of antioxidant capacity on blood lipid metabolism and lipoprotein lipase activity of rats fed a high-fat diet. *Nutrition* 22(11-12): 1185-1191.

Yépez, A., Russo, P., Spano, G., Khomenko, I., Biasioli, F., Capozzi, V. and Aznar, R. (2019). In situ riboflavin fortification of different kefir-like cereal-based beverages using selected Andean LAB strains. *Food Microbiology* 77: 61-68.

Yerlikaya, O. and Akbulut, N. (2020). In vitro characterisation of probiotic properties of *Enterococcus faecium* and *Enterococcus durans* strains isolated from raw milk and traditional dairy products. *International Journal of Dairy Technology* 73(1): 98-107.

Zaukuu, J.L.Z., Oduro, I. and Ellis, W.O. (2016). Processing methods and microbial assessment of *pito* (an African indigenous beer), at selected production sites in Ghana. *Journal of Institute of Brewing* 122(4): 736-744.

Zhang, J., Zhu, X., Xu, R., Gao, Q., Wang, D. and Zhang, Y. (2018). Isolation and identification of histamine-producing Enterobacteriaceae from Qu fermentation starter for Chinese rice wine brewing. *International Journal of Food Microbiology* 281: 1-9.

Zheng, X.W., Tabrizi, M.R., Nout, M.J.R. and Han, B.Z. (2011). *Daqu*-a traditional Chinese liquor fermentation starter. *Journal of the Institute of Brewing* 117(1): 82-90.

Zheng, X.W., Yan, Z., Han, B.Z., Zwietering, M.H., Samson, R.A., Boekhout, T. and Nout, M.R. (2012). Complex microbiota of a Chinese "Fen" liquor fermentation starter

(Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiology* 31(2): 293-300.

Zheng, X.W., Yan, Z., Nout, M.J.R., Boekhout, T., Han, B.Z., Zwietering, M.H. and Smid, E.J. (2015). Characterization of the microbial community in different types of *Daqu* samples as revealed by 16S rRNA and 26S rRNA gene clone libraries. *World Journal of Microbiology and Biotechnology* 31(1): 199. doi: 10.1007/s11274-014-1776-z.

Zou, W., Zhao, C. and Luo, H. (2018). Diversity and function of microbial community in Chinese strong-flavor baijiu ecosystem: a review. *Frontiers in Microbiology* 9: 671. doi: 10.3389/fmicb.2018.00671.

CURRICULUM-VITAE

Name: Pooja Pradhan
Email: klebsiella7@gmail.com
Address: Daragaon, Singtam Bazar East Sikkim - 737134
Mobile No: 8016012839

PhD Thesis Title: "Study of Bacterial Diversity in the starter cultures of the Eastern Himalayas"
(Supervisor: Professor Dr. Jyoti Prakash Tamang).

QUALIFICATION

Degree	University	Month and year of joining	Month and year of passing	Marks (%)
MPhil (Microbiology)	Sikkim University	06/2012	12/2014	72.5
MSc (Microbiology)	Bangalore University	06/2009	06/2011	76.5
BSc. (Microbiology)	North Bengal University	06/2006	03/2009	62.2

Research Experience: Expertise in phenotypic and genotypic characterization of bacteria, molecular techniques like Genomic DNA isolation, PCR analysis, Phylogeny Tree Constructions and Bioinformatics. Bacterial diversity using Culture-independent method (High-throughput Amplicon Sequencing).

Awards/Fellowship

1) GATE-qualified (2012)
2) SLET-qualified (2015)
3) ICAR-National Eligibility Test (NET)-qualified (2016) for Lectureship/Assistant Professor conducted by Agricultural Scientists Recruitment Board.

Oral presentations at International Conferences

1) International Conference on "Ethnic Fermented Foods and Beverages: Microbiology and Health Benefits" at Sikkim University, Gangtok: 20-21 Nov, 2015
2) 6th AIST International Imaging Workshop & DAILAB PIKNIKH Series XXXII held at Biomedical Research Institute, AIST, Tsukuba Science City, Japan supported by at DAICENTER, AIST & JST and DBT from January 20-27, 2019
3) 4th International Conference on "Nutraceuticals and Chronic Diseases (INCD)" at Indian Institute of Technology, Guwahati: 23-25 Sep 2019.

Poster Presentations

1) Poster Presentation on "Bacterial Community in some naturally fermented milk products of Arunachal Pradesh and Sikkim analysed by next generation sequencing technique" at International Symposium on Biodiversity and Biobanking (Biodiverse, 2018) organised at IIT Guwahati.

2) Poster Presentation on "Yeast diversity in Marcha, a traditional amylolytic starter of Sikkim by PCR-DGGE method" at International conference on Ethnic fermented foods and beverages: Microbiology and Health benefits. Organized by Sikkim University in association with SASNET.

3) Poster Presentation on "Bacterial Diversity in amylolytic starter cultures of the Eastern Himalayas with Probiotic activities" at 9th India Probiotic Symposium organized by GUT MICROBIOTA and PROBIOTIC SCIENCE Foundation (India).

Workshop/Conferences Attended

1) The 6th AIST international Imaging workshop & DAILAB PIKNIKH series XXXII, organized by AIST, Tsukuba, Japan on January 20-27, 2019.

Publications

1. Sha, S.P., Anupma, A., **Pradhan, P**., Prasad, G.S. and Tamang, J.P. (2016). Identification of yeasts by polymerase-chain-reaction-mediated denaturing gradient gel electrophoresis in *marcha,* an ethnic amylolytic starter of India. *Journal of Ethnic Foods* 3: 292-296. (Impact factor: 2.080).
2. Sha, S.P., Jani, K., Sharma, A., Anupma, A., **Pradhan, P.,** Shouche, Y. and Tamang, J.P. (2017). Analysis of bacterial and fungal communities in *Marcha* and *Thiat*, traditionally prepared amylolytic starters of India. *Scientific Reports* 7: 10967. doi: 10.1038/s41598-017-11609-y. (Impact Factor: 4.525).
3. Anupma, A., **Pradhan, P**., Sha, S.P. and Tamang, J.P. (2018). Traditional skill of ethnic people of the Eastern Himalayas for preservation of microbiota in form of dry amylolytic starters for production of alcoholic beverages. *Indian Journal of Traditional Knowledge* 17: 184-190. (Impact Factor: 0.920).
4. **Pradhan, P**. and Tamang, J.P. (2019). Phenotypic and genotypic identification of bacteria isolated from traditionally prepared dry starters of the Eastern Himalayas. *Frontiers in Microbiology* 10:2526. doi: 10.3389/fmicb.2019.02526 (Impact Factor: 4.259).

J Ethn Foods 3 (2016) 292–296

Contents lists available at ScienceDirect

Journal of Ethnic Foods

journal homepage: http://journalofethnicfoods.net

Original article

Identification of yeasts by polymerase-chain-reaction-mediated denaturing gradient gel electrophoresis in *marcha*, an ethnic amylolytic starter of India

Shankar P. Sha [a], Anu Anupama [a], Pooja Pradhan [a], Gandham S. Prasad [b], Jyoti P. Tamang [a, *]

[a] Department of Microbiology, School of Life Sciences, Sikkim University, Tadong, Sikkim, India
[b] Microbial Type Culture Collection, Council of Scientific & Industrial Research, Institute of Microbial Technology, Chandigarh, India

ARTICLE INFO

Article history:
Received 15 October 2016
Received in revised form
28 November 2016
Accepted 28 November 2016
Available online 2 December 2016

Keywords:
amylolytic starter
denaturing gradient gel electrophoresis
marcha
polymerase chain reaction
Sikkim
Wickerhamomyces anomalus

ABSTRACT

Background: *Marcha* is an ethnic amylolytic starter that is used to ferment boiled cereals to produce alcoholic drinks, commonly called *jaanr*, in the Himalayan Regions of Sikkim and Darjeeling of India.
Methods: The aim of this study was to investigate yeast flora of *marcha* collected from Sikkim in India by phenotypic characterization and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE).
Results: The average load of yeast in *marcha* was 6.0×10^8 colony-forming units/g. The phenotypic characterization of yeast isolates from *marcha* showed the presence of *Candida*, *Pichia*, *Torulospora*, *Schizosaccharomyces*, *Kluveromyces*, *Issatchenki*, and *Saccharomycopsis*. The PCR-DGGE bands showed the dominance of *Wickerhamomyces anomalus* (72%) and *Pichia anomalus* (28%) in *marcha*. *W. anomalus* was reported for the first time from *marcha* using PCR-mediated DGGE.
Conclusion: This is the first report on the yeast community associated with *marcha* analyzed by PCR-mediated DGGE.

1. Introduction

The traditional way of preparation of amylolytic starters is a unique technology of preservation of native microorganisms, consisting of consortia of amylolytic and alcohol-producing yeasts, molds, and some lactic-acid bacteria, with rice or wheat as the base in the form of dry, flattened, or round balls, for alcoholic beverage production in Asia [1]. Amylolytic starters in Asia have different vernacular names such as *marcha* in India, Nepal, and Bhutan, *hamei*, *humao*, and *phab* in India [2–4], *mana* and *manapu* in Nepal [5], *men* in Vietnam [6], *bubod* in the Philippines [7], *chiu/chu* in China and Taiwan [8], *loogpang* in Thailand [9], *ragi* in Indonesia [10], and *nuruk* in Korea [8]. *Marcha* is a nonfood starter culture uses for production of various ethnic alcoholic beverages in the Darjeeling Hills and Sikkim in India, Nepal, and Bhutan [3]. It is a dry, round-to-flattened, creamy to dusty white, solid ball-like starter (Fig. 1). During its preparation, soaked glutinous rice is crushed in a foot-driven heavy wooden mortar, with the addition of the roots of *Plumbago zeylanica* L., leaves of *Buddleja asiatica* Lour, flowers of *Vernonia cinerea* (L.) ginger, red dry chili, and 1% of previously prepared powdered *marcha* for back-sloping fermentation [2]. The mixed dough is kneaded into round or flat cakes of different sizes and shapes that are placed individually on a platform suspended below the bamboo-made ceiling above the earthen kitchen, bedded with fresh fronds of fern *Glaphylopteriolopsis erubescens* (Wall ex Hook.) Ching, and covered with dry fronds of fern and jute bags and are then left to ferment for 1–3 days. Finally, cakes of *marcha* are sun dried for 2–3 days and stored in a dry place at room temperature for > 1 year. Application of polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE), a culture-independent method, is widely applied to study microbial diversity [11–13]. Some species of yeasts such as *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Saccharomycopsis*, *Schizosaccharomyces*, *Torulopsis*, and *Zygosaccharomyces* were previously reported from samples of *marcha* using culture-dependent approaches [7,14,15]. However, a culture-independent method using PCR-DGGE has not

* Corresponding author. Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Tadong 737102, Sikkim, India.
E-mail address: jyoti_tamang@hotmail.com (J.P. Tamang).

http://dx.doi.org/10.1016/j.jef.2016.11.009

Fig. 1. *Marcha*, amylolytic starter of Sikkim in India.

been applied yet in profiling of yeast flora in *marcha*. The present study aimed to profile yeast flora directly from *marcha* samples using PCR-mediated DGGE.

2. Materials and methods

2.1. Sample collection

Ten samples of dry *marcha* were collected from the local market or villages of Sikkim in presterile polythene bags, and were stored in a desiccator at room temperature until analysis.

2.2. Culture-dependent analysis

2.2.1. Isolation of microorganisms

Ten grams of powdered *marcha* was mixed in 90 mL physiological saline (0.85%) and homogenized in a Stomacher Lab-Blender 400 (Seward, Worthing, UK) for 1 minute. Serial dilutions were prepared in sterile diluent and mixed with the molten media and poured into plates. Plates of yeast extract–malt extract agar (M424; HiMedia, Mumbai, India) for enumeration of yeasts were incubated at 30°C for 48 hours. Yeast isolates were purified and preserved at −20°C in yeast extract–malt extract broth (M425; HiMedia) mixed with 20% (v/v) glycerol.

2.2.2. Phenotypic characterization

Cell morphology of yeast isolates was determined using a phase contrast microscope (CH3-BH-PC; Olympus, Tokyo, Japan). Yeast cultures have been characterized on the basis of mycelium type, ascospore type, nitrate reduction, growth at 37°C and 45°C, sugar fermentation, and sugar assimilation following the methods of Kurtzman et al [16].

2.3. Culture-independent analysis

2.3.1. DNA extraction from sample

Ten grams of powdered *marcha* was homogenized in 90 mL of 0.85% w/v sterile physiological saline, and subsequently filtered. The resulting filtered solutions were centrifuged at 14,000 *g* for 10 minutes at 4°C, and pellets were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) after glass bead (0.2–0.5 mm diameter; Sigma-Aldrich, Roth, Germany) beating to rupture the cell walls. The yield and quality of DNA were detected through agarose gel electrophoresis (1.0%), which was stained with ethidium bromide solution.

2.3.2. PCR amplification and DGGE analysis

PCR-DGGE analysis was performed as described previously [17]. Coated PCR with primers sets ITS1-F, ITS4, ITS2, and ITS1F-GC was used to amplify yeast ITS region [18]. A 40-base (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) attached to the 5′ end of the ITS1-F primer was used to stabilize the melting behavior of the DNA fragments during DGGE analysis [19]. The first round of PCRs was carried out in a Mastercycler (Applied Biosystems, Foster City, CA, USA) using 25-μL reaction volumes containing: 1 μL DNA template, 0.25 μL each primer (10μM), 12.5 μL 2× Go Taq Master Mix (Promega), and 11 μL nuclease-free water. PCR cycle was programed as follows: 94°C for 4 minutes followed by 10 cycles of 94°C for 1 minutes, lowering the annealing temperature from 65°C to 55°C in 1°C steps for each cycle for 1 minute, 72°C for 1 minute, and finally 25 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and followed by a final extension at 72°C for 7 minutes. The second PCRs were carried out in a Mastercycler (Applied Biosystems) using 50-μL reaction volumes containing: 1 μL first PCR production, 0.5 μL each primer (10μM), 25 μL 2× Go Taq Master Mix (Promega) and 23 μL nuclease-free water. Cycling parameters were the same as for the first round of PCR. All amplified products were analyzed by electrophoresis in 1.2% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. DGGE analysis was carried out using the PCR products in an universal mutation detection system (DGGEK-1001-220; CBS Scientific, Del Mar, CA, USA) following the procedure described by El Sheikha et al [20]. Samples containing approximately equal amounts of PCR amplicons (30 μL) were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N, N0-methylene bisacrylamide, 37.5/1; Promega) in 1× TAE buffer (40mM Tris–HCl pH 7.4, 20mM sodium acetate, 1.0mM Na_2-EDTA). Electrophoresis was performed at 60°C in a denaturing gradient ranging from 40% to 60% [100% corresponded to 7M urea and 40% (v/v) formamide; Promega], at 20 V for 10 minutes and then at 130 V for 4.5 hours, and the gels were stained for 30 minutes with ethidium bromide and then photographed on a gel documentation unit (GelDoc 1000; Bio-Rad, Hercules, CA, USA).

2.3.3. Identification of bands

Individual DGGE bands were excised, resuspended in 20 μL sterile Tris–EDTA buffer, and stored at 4°C overnight. An aliquot of supernatant was used as a DNA template for PCR reamplification as described above, and electrophoresed with DGGE. Band excision, PCR, and DGGE were repeated until a single band was present. PCR products generated from DGGE bands were amplified with primers ITS2 and ITS-1f (without the GC clamp) for sequencing using DNA sequencer (Applied Biosystems). Sequences of major bands obtained from the DGGE gel fragments were compared with the GenBank database using the web-based nucleotide–nucleotide BLAST search engine hosted by the National Center for Biotechnology Information (Bethesda, MD, USA) for identification (http://www.ncbi.nlm.nih.gov) [21].

2.3.4. Phylogenetic analysis

The BLAST program was used for comparing DNA databases for sequence similarities available on the EzTaxon server (http://eztaxon-e.ezbiocloud.net/). Molecular evolutionary genetics analysis software (MEGA version 6, US National Library of Medicine, National Institution of Health) was used for phylogenetic analyses [22].

Table 1
Characterization of yeasts isolates from *marcha*.

Representative strains*	No. of grouped strains	Pellicle formation	Nitrate reduction	Sugar fermentation†									Tentative genera
				Cellobiose	Arabinose	Ribose	Mannose	Raffinose	Aesculin	Galactose	Trehalodse	Xylose	
GM:Y12	2	+	−	−	−	+	+	+	+	+	−	+	*Saccharomyces*
GM:Y21	2	+	−	−	−	+	1/1	+	+	+	−	−	*Saccharomyces*
GM:Y50	3	+	−	2/1	−	+	+	+	+	2/1	+	+	*Saccharomyces*
GM:Y34	3	−	+	+	+	+	+	+	+	+	−	+	*Pichia*
GM:Y43	2	−	+	+	+	+	+	+	+	+	−	+	*Pichia*
GM:Y7	3	−	−	+	−	−	−	−	−	−	−	2/1	*Candida*
GM:Y37	2	−	−	+	−	−	−	−	−	−	−	+	*Candida*
GM:Y4	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y36	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y10	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y29	4	+	−	+	+	3/1	+	+	+	+	+	−	*Kluveromyces*
GM:46	3	+	−	+	+	2/1	+	+	+	+	+	−	*Kluveromyces*
GM:Y5	3	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:49	2	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:Y15	3	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:Y22	2	+	−	+	1/1	+	+	+	+	+	+	+	*Saccharomycopsis*
GM:Y41	2	+	−	+	1/1	+	+	+	+	+	+	+	*Saccharomycopsis*
GM:Y1	4	+	−	−	+	+	3/1	+	+	+	+	+	*Torulospora*
GM:Y18	4	+	−	−	+	+	3/1	+	+	+	+	+	*Torulospora*

+, all strains positive; −, all strains negative; (../..), number of positive/negative strains.
* All yeast cells were oval to circular, colonies of all strains were smooth and creamy white, showed pseudo mycelia. All strains grew well at 37°C, but not at 45°C, except *Kluveromyces* grew at 45°C.
† All strains fermented sucrose, glucose, fructose, galactose, maltose, and no strains fermented lactose, except *Kluveromyces*, and all strains assimilated sugars except lactose.

3. Results

3.1. Phenotypic characterization of yeasts

The average load of yeast in *marcha* was calculated as 6.0×10^8 colony-forming units/g (data not shown). A total of 50 yeasts isolates were isolated from 10 samples of *marcha*. Characterization of yeasts isolated from different *marcha* samples of Sikkim, which were phenotypically identified on the basis of colony morphology, cell morphology sugar fermentation, and sugar assimilation tests (Table 1). Out of 50 isolates, 19 representative isolates were grouped based on colony appearance, cell shape, type of mycelia and ascospores, pellicle formation, nitrate reduction, and growth at 37°C and 45°C (Table 1). All 50 isolates were tested for sugar fermentation and sugar assimilation for identification up to genus level (Table 1). Tentatively the following yeast genera were identified: *Candida* (18%), *Pichia* (14%), *Torulospora* (14%), *Schizosaccharomyces* (16%), *Kluveromyces* (10%), *Issatchenki* (20%), and *Saccharomycopsis* (8%).

3.2. PCR-DGGE analysis

DNA was directly extracted from *marcha* samples. The results of PCR-DGGE analysis showed the diversity of yeast (Fig. 2). Five bands were identified as *Wickerhamomyces anamolus* and two bands as *Pichia anamolus*. Detected in eight of the 10 samples, *W. anamolus* (DGGE bands MY1, MY3, MY4, MY7, and MY8) was found to be the most abundant yeast species. *P. anamolus* (DGGE bands MY5 and MY6) was detected frequently in *marcha* (Fig. 2). Nevertheless, it is worthwhile to note that some yeast species were only detected by DGGE in some samples, for example, *W. anamolus* was found in *marcha* samples with an intense DGGE band. By contrast, *P. anamolus* was detected both by culture independent (PCR-DGGE) as well as culture-dependent techniques (conventional microbiological method). However, we were not able to identify the minor bands (MY9–MY13) since they could not be excised from the gels due to their low intensity.

The selected seven isolates were identified by partial 18S rRNA gene sequencing and were compared to the EzTaxon server database for their phylogenetic relationship using MEGA 6.06 version software (Fig. 3). Five of the isolates were identified as *W. anamolus* (MY1, MY3, MY4, MY7, and MY8) and two as *P. anomalus* (MY5, MY6). Identification based on BLAST comparison in GenBank of the bands obtained by PCR-DGGE gel using universal primers NL1/LS2 is shown in Table 2.

4. Discussion

The diversity of yeasts associated with amylolytic starters may be closely related to the raw material used and the regional climate

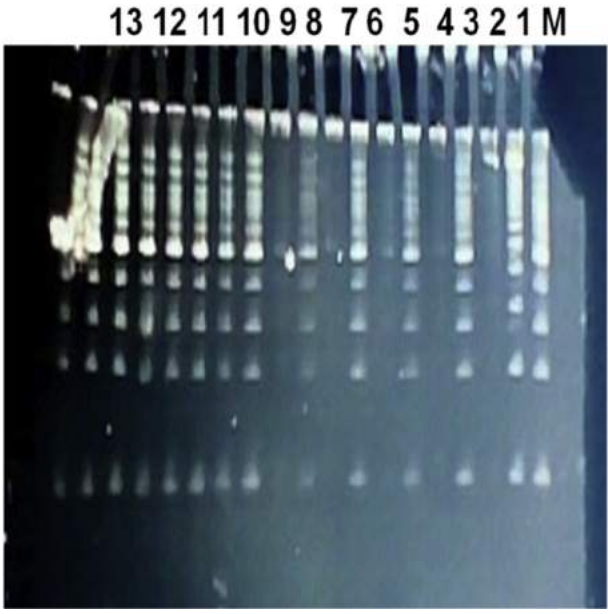


Fig. 2. Denaturing gradient gel electrophoresis profile of bands. 1, *Wickerhamomyces anomalus*; 2, 9, 10, and 11–13 unidentified; 3, *W. anomalus*; 4, *W. anomalus*; 5, *Pichia anomalus*; 6, *P. anomalus*; 7, *W. anomalus*; 8, *W. anomalus*.

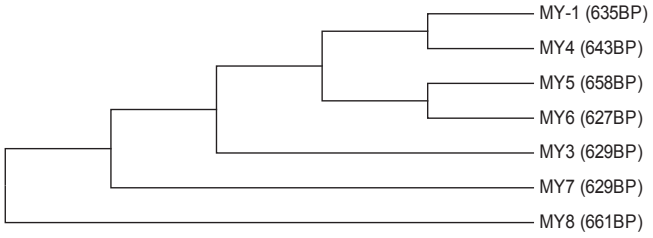


Fig. 3. Phylogenetic tree of yeasts retrieved from bands (MY1–MY8) in denaturing gradient gel electrophoresis profile by neighbor-joining distance tree constructed by MEGA 6 for the seven yeast isolates with the phylogenetic neighbors obtained from EzTaxon server.

Table 2
Identification based on BLAST comparison in GenBank of the bands obtained by polymerase chain reaction–denaturing gradient gel electrophoresis using universal primers NL1/LS2.

Band	Closest relative species	Accession No.	% identity
MY1	*Wickerhamomyces anomalus*	KT175181.1	99
MY3	*W. anomalus*	KT175201.1	99
MY4	*W. anomalus*	G0280811.1	99
MY5	*Pichia anomalus*	E0798697.1	98
MY6	*P. anomalus*	AY349435.1	99
MY7	*W. anomalus*	KT175181.1	99
MY8	*W. anomalus*	KT175181.1	99

where they are produced [23]. *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, *W. anomala*, *Pichia guilliermondii*, and *Candida* sp. are the most common yeasts present in rice-based starters of Asia [24–26]. It is interesting to find that yeast species (*W. anomalus*) could not be detected by conventional media but has been detected by PCR-DGGE analysis. *W. anomalus* has been reported in *hong-qu* and *yao-qu*, traditional amylolytic starters of China [27,28] and *banh men*, a traditional Vietnamese starter [29]. However, we noticed that *P. anomala*, *Issatchenkia*, *S. cerevisiae*, *Torulospora*, *Kluveromyces*, *Candida musae*, *S. fibuligera*, and *Saccharomycopsis* spp., which were detected in *marcha* through culture-dependent methods, were not detected in *marcha* in PCR-mediated DGGE assay. This discrepancy may have been due to the selective amplification, migration of PCR products from different species, and efficiencies of genomic DNA extraction kits for different species [30,31]. Such discrepancies between culturing and DGGE results have also been reported earlier [11,32]. Therefore, it is suggested that the DGGE technique be supplemented with culture-dependent methods, and their combination seems the best strategy to have a complete overview of yeast ecology of traditional alcoholic starters. *W. anomalus*, probably nonculturable yeast, was reported for the first time from *marcha* using PCR-mediated DGGE technique.

This is believed to be the first report on the yeast community associated with *marcha* of India analyzed by PCR-mediated DGGE. The results may enrich our knowledge of nonculturable native microorganisms that may be present in the traditionally prepared starters of Asia.

Conflict of interest

There is no conflict of interest.

Acknowledgments

The authors are grateful to the Department of Biotechnology, Ministry of Science and Technology, Government of India for DBT-Twinning project to J.P.T. and G.S.P.

References

[1] Tamang JP. Diversity of fermented beverages. In: Tamang JP and Kailasapathy K, editors. Fermented foods and beverages of the world. Boca Raton: CRC Press, Taylor and Francis; 2010. p. 85–125.
[2] Tamang JP, Thapa S, Tamang N and Rai B. Indigenous fermented food beverages of Darjeeling hills and Sikkim: process and product characterization. J Hill Res 1996;9:401–11.
[3] Tamang JP. Fermented milks. In: Tamang JP, editor. Himalayan fermented foods: microbiology, nutrition, and ethnic values. Boca Raton: CRC Press, Taylor and Francis; 2010. p. 95–109.
[4] Tamang JP, Tamang N, Thapa S, Dewan S, Tamang BM, Yonzan H, Rai AK, Chettri R, Chakrabarty J and Kharel N. Microorganisms and nutritional value of ethnic fermented foods and alcoholic beverages of North East India. Indian J Traditional Know 2012;11:7–25.
[5] Nikkuni S, Karki TB, Terao T and Suzuki C. Microflora of mana, a Nepalese rice koji. J Fermen Bioeng 1996;81:168–70.
[6] Dung NTP, Rombouts FM and Nout MJR. Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). LWT-Food Sci Technol 2007;40:130–5.
[7] Hesseltine CW and Kurtzman CP. Yeasts in amylolytic food starters. Annls Inst Biol Uni Nac Antón México Ser Bot 1990;60:1–7.
[8] Steinkraus KH. Handbook of indigenous fermented food. 2nd ed. New York: Marcel Dekker, Inc.; 1996.
[9] Vachanavinich K, Kim WJ and Park YI. Microbial study on krachae, Thai rice wine. In: Lee CH, Adler-Nissen J and Bärwald G, editors. Lactic acid fermentation of non-alcoholic dairy food and beverages. Seoul: Ham Lim Won; 1994. p. 233–46.
[10] Uchimura T, Okada S and Kozaki M. Identification of lactic acid bacteria isolated from Indonesian Chinese starter, "ragi". Microorganisms in Chinese starters from Asia (Part 4). J Brew Soc Japan 1991;86:55–61.
[11] Ercolini D, Mauriello G, Blaiotta G, Moschetti G and Coppola S. PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. J Appl Microbiol 2004;96:263–70.
[12] Nielsen DS, Teniola OD, Ban-Koffi L, Owusu M, Andersson TS and Holzapfel WH. The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture-independent methods. Int J Food Microbiol 2007;114:168–86.
[13] Tamang JP, Watanabe K and Holzapfel WH. Review: diversity of microorganisms in global fermented foods and beverages. Frontiers Microbiol 2016;7:377. http://dx.doi.org/10.3389/fmicb.2016.00377.
[14] Tamang JP and Sarkar PK. Microflora of murcha: an amylolytic fermentation starter. Microbios 1995;81:115–22.
[15] Tsuyoshi N, Fudou R, Yamanaka S, Kozaki M, Tamang N, Thapa S and Tamang JP. Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. Int J Food Microbiol 2005;99:135–46.
[16] Kurtzman CP, Fell JW and Boekhout T. The yeasts: a taxonomic study. 5th ed. London: Elsevier; 2011.
[17] Cocolin L, Bisson LF and Mills DA. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol Lett 2000;189:81–7.
[18] Gardes M and Bruns TD. ITS primers with enhanced specificity for basidiomycetes –application to the identification of mycorrhizae and rusts. Mol Ecol 1993;2:113–8.
[19] Sheffield VC, Cox DR, Lerman LS and Myers RM. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 1989;86:232–6.
[20] El Sheikha AF, Condur A, Métayer I, Le Nguyen DD, Loiseau G and Montet D. Determination of fruit origin by using 26S rDNA fingerprinting of yeast communities by PCR-DGGE: an application to *Physalis* fruits from Egypt. Yeast 2009;26:567–73.
[21] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[22] Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.
[23] Jeyaram K, Tamang JP, Capece A and Patrizia Romano P. Geographical markers for *Saccharomyces cerevisiae* strains with similar technological origins domesticated for rice-based ethnic fermented beverages production in North East India. Antonie van Leeuwenhoek 2011;100:569–78.
[24] Lee AC and Fujio Y. Microflora of banh men, a fermentation starter from Vietnam. World J Microbiol Biotechnol 1999;15:57–62.
[25] Xie GF, Li WJ, Lu J, Cao Y, Fang H and Zou HJ. Isolation and identification of representative fungi from Shaoxing rice wine wheat qu using apolyphasic approach of culture-based and molecular-based methods. J Institute Brew 2007;113:272–9.
[26] Jeyaram K, Mohendro Singh W, Capece A and Romano P. Molecular identification of yeast species associated with 'Hamei' – a traditional starter used for rice wine production in Manipur. India. Int J Food Microbiol 2008;124:115–25.
[27] Lv XC, Weng X, Huang RL, Wen Z, Rao PF and Ni L. Research on biodiversity of yeasts associated with Hongqu glutinous rice wine starters and the traditional brewing process. J Chinese Institute Food Sci Technol 2012;12:182–90.

[28] Xu CL, Huang XL, Wen Z, Rao PF and Ni L. Yeast diversity of traditional alcohol fermentation starters for Hong-Qu glutinous rice wine brewing, revealed by culture-dependent and culture-independent methods. Food Control 2013;34:183–90.
[29] Thanh VN, Mai LT and Tuan DA. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR mediated, DGGE. Int J Food Microbiol 2008;128:268–73.
[30] Ishii K and Fukui M. Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Appl Environ Microbiol 2001;67:3753–5.
[31] Katano T and Fukui M. Molecular inference of dominant picocyanobacterial populations by denaturing gradient gel electrophoresis of PCR amplified 16S rRNA gene fragments. Phycol Res 2003;51:71–6.
[32] Kafili T, Razavi SH, Djomeh ZE, Naghavi MR, Álvarez-Martín P and Mayo B. Microbial characterization of Iranian traditional Lighvan cheese over manufacturing and ripening via culturing and PCR-DGGE analysis: identification and typing of dominant lactobacilli. European Food Res Technol 2009;229:83–92.

SCIENTIFIC REPORTS

OPEN

Analysis of bacterial and fungal communities in *Marcha* and *Thiat*, traditionally prepared amylolytic starters of India

Received: 24 May 2017
Accepted: 29 August 2017
Published online: 08 September 2017

Shankar Prasad Sha[1], Kunal Jani[2], Avinash Sharma[2], Anu Anupma[1], Pooja Pradhan[1], Yogesh Shouche[2] & Jyoti Prakash Tamang[1]

Marcha **and** ***thiat*** **are traditionally prepared amylolytic starters use for production of various ethnic alcoholic beverages in Sikkim and Meghalaya states in India. In the present study we have tried to investigate the bacterial and fungal community composition of** ***marcha*** **and** ***thiat*** **by using high throughput sequencing. Characterization of bacterial community depicts phylum** ***Proteobacteria*** **is the most dominant in both** ***marcha*** **(91.4%) and** ***thiat*** **(53.8%), followed by** ***Firmicutes, and Actinobacteria*****. Estimates of fungal community composition showed** ***Ascomycota*** **as the dominant phylum. Presence of** ***Zygomycota*** **in** ***marcha*** **distinguishes it from the** ***thiat*****. The results of NGS analysis revealed dominance of yeasts in** ***marcha*** **whereas molds out numbers in case of** ***thiat*****. This is the first report on microbial communities of traditionally prepared amylolytic starters of India using high throughput sequencing.**

Traditional practice of sub-culturing by back-sloping and preservation of essential native microbiota consisting of consortia of yeasts, molds and bacteria, in the form of dry, flattened, or round balls, for alcoholic beverages production in South-East Asia including the Himalayan regions of India, Nepal, Bhutan, and China is the worth wisdom of the ethnic people for centuries[1]. Some common and uncommon amylolytic starters in Asia are *marcha* of India, Nepal, and Bhutan, *hamei*, *humao*, *thiat*, *phab* of India, *men* of Vietnam, *bubod* of the Philippines, *chiu/chu* of China and Taiwan, *loogpang* of Thailand, *ragi* of Indonesia, *nuruk* of Korea, *mae/dombae/buh/puh*in Cambodia, etc.[2–7] Traditionally prepared Asian amylolytic starters have consortia of mixed micrbiota representing filamentous molds, yeast and bacteria[1–3], hence many researchers have studied the fungal, yeast and bacterial populations in Asian starter cultures, commonly based on culture-dependent techniques including phenotypic and 16S rRNA sequencing, and isolated and identified filamentous molds *Absidia corymbifera*, *Amylomyces rouxii*, *Botryobasidium subcoronatum*, *Mucor circinelloides* forma *circinelloides*, *Mucor hiemalis*, *Rhizopus oryzae*, *Rhi. microsporus*, *Rhi. chinensis*, and *Rhi. stolonifer*, *Xeromyces* bisporus[5,8,9]; yeasts *Candida glabrata*, *C. tropicalis*, *Clavispora lusitaniae*, *Issatchenkia* sp., *Pichia anomala*, *P. ranongensis*, *P. burtonii*, *Saccharomycopsis fibuligera*, *Sm. capsularis*, *Saccharomyces cerevisiae*, *Sacch. Bayanus*[5,9–13]; and bacteria *Acetobacter orientalis*, *A. pasteurianus*, *Bacillus amyloliquefaciens*, *B. circulans*, *B. sporothermodurans*, *B. subtilis*, *Pediococcus pentosaceus*, *Lactobacillus bifermentans*, *Lb. brevis*, *Lb. plantarum*, *Weissella confusa*, *W. paramesenteroides*[5,14–16].

Introduction of culture-independent methods and it's applicability in food microbiology[7,17], has been a motivation for few researchers to profile the microbial community structure of some Asian starter cultures using PCR-DGGE, pyrosequencing, etc. which is suggestive to provide more insight into the microbial diversity of ethnic starters[3,5,18–22]. Rapid evolution in next generation sequencing (NGS) technologies has enabled researchers to have increased accuracy, throughput, with reasonably low cost and in relatively short period of time[17,23]. However, there are still a limited number of studies, characterizing the microbial community composition of fermented foods such as cheese[24–26], kefir grains[27], some ethnic Asian fermented foods[28–31]. Furthermore, the information on the community composition of Asian starter culture is rudimentary and needs in depth exploration using cutting edge technologies[7].

[1]DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, 737102, India. [2]National Centre for Microbial Resource, National Centre for Cell Science, Pune, 411021, India. Correspondence and requests for materials should be addressed to J.P.T. (email: jyoti_tamang@hotmail.com)

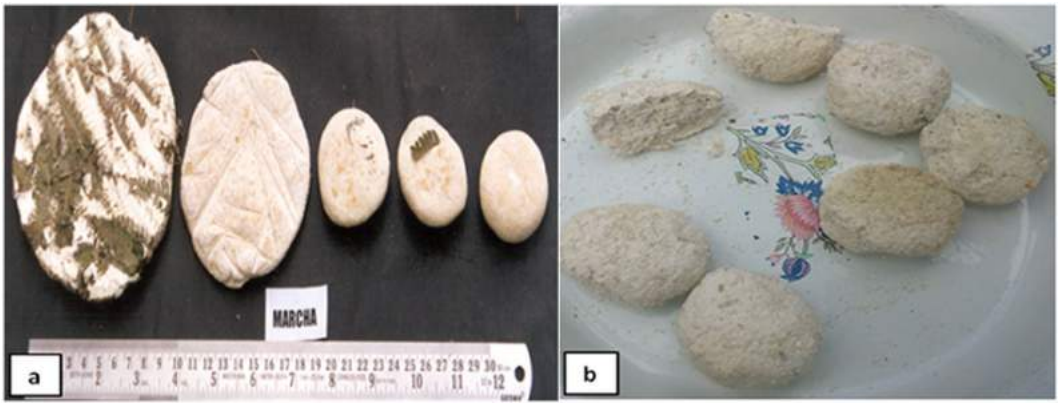


Figure 1. Traditionally prepared amylolytic starter cultures (**a**) *Marcha* and (**b**) *Thiat*.

	Chao1	Goods coverage	Shannon	Simpson
a) Bacterial				
Marcha	1520.925	0.998902539	4.01115959	0.866763863
Thiat	4256.838	0.997475969	5.489325073	0.940199394
b) Fungal				
Marcha	5.25	0.75	2.25	0.78125
Thiat	5	1	1.802366931	0.671398892

Table 1. Alpha diversity estimation. Non parametric alpha diversity was calculated for ethnic amylolytic starter cultures *marcha* and *thiat*.

In present study, we attempted to profile the microbial community composition of *marcha* and *thiat*, traditionally prepared ethnic starter cultures of India using targeted amplicon sequencing. We selected two different traditionally prepared amylolytic starter cultures from two regions in India, *marcha* (Fig. 1a) from Sikkim (www.sikkim.gov.in) and *thiat* (Fig. 1b) from Meghalaya (www.megtourism.gov.in). *Marcha* is prepared from soaked rice with some wild herbs ((*Plumbago zeylanica*, *Buddleja asiatica* and *Vernonia cinerea*), ginger and red dry chili, 1–2 % of previously prepared *marcha* powder as an inoculum, crushed in a wooden mortal by wooden pestle, mised and dough are made into round to flatted cakes of different size and shape. Cakes are covered with fern fronds (*Glaphylopteriolopsis erubeseens*), fermented at room temperature for 24 h, sun dried for 3–5 days and are used as amylolytic starters for production of cereal-based ethnic fermented beverages such as *kodo ko jaanr*, *bhaati jaanr*, *raksi*, etc.[2] During *thiat* preparation, soaked glutinous rice is grinded with leaves and roots of wild plant *Amomum aromaticum*, 1–2% of old *thiat*, mixed and made into a dough by adding water. Flat to round balls are made and fermented for 1–3 days. The freshly prepared *thiat* balls are sun dried for 3–5 days. It is used to ferment alcoholic beverage locally called *kiad* in Meghalaya[2]. Fermentation process involved in preparation of these starters is unconditional and may harbor both bacterial and fungal communities as consortia. Therefore, we aimed to explore the bacterial and fungal (filamentous molds and yeasts) communities in *marcha* and *thiat*. This is the first report on complete microbial community profile of traditionally prepared amylolytic starters of India using NGS technique.

Results

Characterizing microbial diversity. High throughput sequencing and quality trimming of 16S rRNA and ITS gene yielded ~0.85and ~0.29 million quality reads in both *marcha* and *thiat*, respectively, which was used for subsequent analysis. Taxonomic assignment of sequences with the reference database resulted into 5,015 operational taxonomic units (OTUs). The average Good's coverage of both the samples of *marcha* and *thiat* for 16Sr RNA amplicon sequencing was found to be 99.08% ± 0.1% (mean ± SD) whereas for ITS region was recorded as 87.5% ± 17.6% (mean ± SD) indicating majority of the diversity was captured.

The estimates of alpha diversity indices revealed significant differences between *marcha* and *thiat* when computed for both the bacterial and fungal diversity (Table 1a and b). The bacterial species richness was found to be higher in *thiat* (4256.83) than *marcha* (1520.92), in contrast, fungal species richness depicts higher in *marcha* (5.25) over *thiat* (5.0). Significant variations were also noticed in non-parametric shannon index for bacterial communities in *thiat* (5.48) and *marcha* (4.01). Shannon index for fungal communities follow the reverse trend with *marcha* (2.25) and *thiat* (1.80). This observation is suggestive of higher bacterial diversity in *thiat* while *marcha* showed higher fungal diversity.

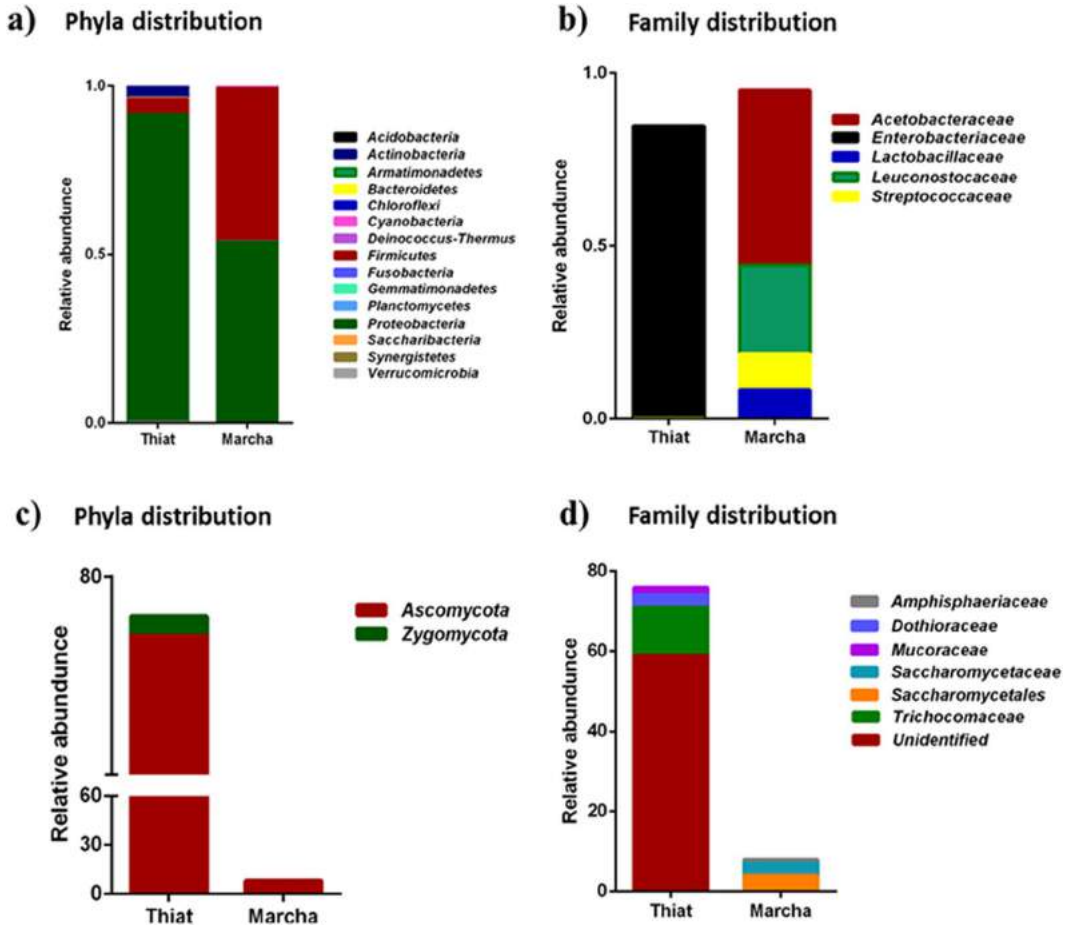


Figure 2. Taxa distributions of phylum and family at different phylogenetic level in *thiat* and *marcha*. (**a**) bacterial phyla; (**b**) bacterial family; (**c**), fungal phyla and (**d**) fungal family.

Bacterial community profile of *thiat* and *marcha*. 16S rRNA gene amplicon sequencing yielded 15 bacterial phyla in *thiat* and *marcha*, respectively (Fig. 2a). In *thiat* bacterial phyla distributions were *Proteobacteria* (91.4%), *Actinobacteria* (4%), *Firmicutes* (4%) and the rest (0.6%) constituted the minor phyla *Cyanobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Fusobacteria*, *Planctomycetes*, *Deinococcus-Thermus*, *Chloroflexi*, *Synergistetes*, *Acidobacteria*, *Saccharibacteria*, *Gemmatimonadetes*, *Armatimonadetes*. In *marcha* the phyla distributions of bacteria were *Proteobacteria* (53.8%), *Firmicutes* (45.4%) and other minor phyla were 0.8% constituting *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Fusobacteria*, *Planctomycetes*, *Deinococcus-Thermus*, *Chloroflexi*, *Synergistetes*, *Acidobacteria*, *Saccharibacteria*, *Gemmatimonadetes*, and *Armatimonadetes*. The abundance of thirteen minor phyla was very less hence percentage of composition was not shown in Fig. 2a. Bacterial phylum *Proteobacteria* was found to outnumber other bacterial phyla in *thiat* whereas *marcha* was found to constitute *Proteobacteria* and *Firmicutes* as major phyla.

At family level, OTUs with $\geq$1% abundance were filtered which differed quantitatively between *thiat* and *marcha* (Fig. 2b). The family level distributions of bacteria in *thiat* were *Enterobacteriaceae* (84.6%), *Microbacteriaceae* (3.24%), *Enterococcaceae* (2.47%), *Clostridiaceae* (1.13%) *Neisseriaceae* (0.87%) and *Oxalobacteraceae* (0.59%) (Fig. 2b). Whereas the family level of bacterial distributions in *marcha* were *Acetobacteraceae* (50.6%), *Leuconostocaceae* (25.5%), *Streptococcaceae* (10.5%), *Lactobacillaceae* (8.38%), *Burkholderiaceae* (2.13%) and *Staphylococcaceae* (0.54%) (Fig. 2b).

At the genus level, OTUs with $\geq$1% abundance were filtered (Fig. 3a,b), which retained 18 differentially abundant genera in both samples of *marcha* and *thiat*. Distribution of bacterial genera in *marcha* were *Acetobacter* (52.6%), *Fructobacillus* (21.1%), *Lactococcus* (10.3%), *Lactobacillus* (8.4%), *Leuconostoc* (4.0%) (Fig. 3a), *Burkholderia* (2.1%) and *Gluconacetobacter* (1.4%). Genera in *thiat* were *Pantoea* (32.4%), *Cronobacter* (21.4%), *Escherichia-Shigella* (15.5%), *Enterobacter* (13.1%), *Citrobacter* (4.2%) (Fig. 3b), *Salmonella* (3.2%), *Serratia* (2.8%), *Enterococcus* (2.5%), *Curtobacterium* (2.2%), *Kluyvera* (1.6%) and *Clostridium* (1.1%). The composition percentage of bacterial genera which was less than 3.9% was not shown in Fig. 3a,b.

Fungal (filamentous molds and yeasts) composition in *thiat* and *marcha*. Fungal ITS gene sequencing and taxonomic analysis demonstrated the predominance of yeast phylum *Ascomycota* (98.6%) in *thiat*, whereas the distribution of filamentous phyla *Zygomycota* was only 1.4% (Fig. 2c). However, in *marcha* only yeast phylum *Ascomycota* constituted the fungal diversity (Fig. 2c). Filamentous mold phylum was not detected

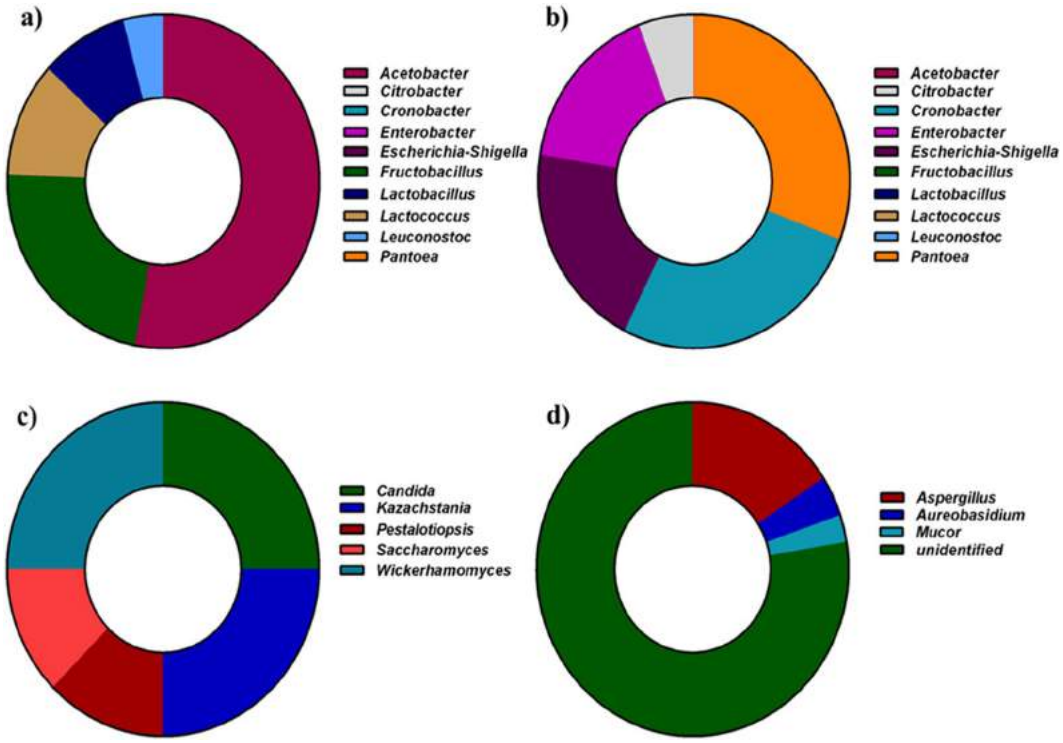


Figure 3. Taxa distributions of genus at different phylogenetic level. (**a**) bacterial genera in *marcha*; (**b**) bacterial genera in *thiat*; (**c**) fungal genera in *marcha* and (**d**) fungal genera in *thiat*.

in *marcha*. Distributions of fungi (filamentous molds and yeasts) at the family level in *thiat* were *Trichocomaceae* (15.7%), *Dothioraceae* (3.94%), *Mucoraceae* (2.63%) and unidentified fungi (77.73%). Whereas the distributions of yeasts at the order/family level in *marcha* were *Saccharomycetales* (50%), *Saccharomycetaceae* (37.5%) and *Amphisphaeriaceae* (12.5%). (Fig. 2d). Distributions of yeasts genera in *marcha* were *Wickerhamomyces* (25%), *Candida* (25%), *Kazachstania* (25%), *Saccharomyces* (12.5%) and *Pestalotiopsis* (12.5%) (Fig. 3c). The filamentous mold genera distribution in *thiat* were *Aspergillus* (15.7%), *Aureobasidium* (3.9%) and *Mucor* (2.7%) and unidentified genera (77.7%) (Fig. 3d). The unidentified genera represented the yeast phylum *Ascomycota* in *thiat*. The sequence reads showed the species of filamentous molds were *Aspergillus penicillioides*, *Aureobasidium pullulans* and *Mucor circinelloides*, whereas the yeasts species were *Wickerhamomyces anomalus*, *Candida quercitrus* and *Kazachstania exigua* (data not shown).

Discussion

Our study provides comprehensive microbial diversity analysis using deep sequencing approach of ethnic amylolytic starter from India. Quantitative differences were noted for the presence of bacterial and fungal taxa among *marcha* and *thiat*; which could be the consequence of differences in the preparation, incubation period and most importantly the type of preservations. Alpha diversity estimation of amylolytic starters *marcha* and *thiat* using species richness and non-parametric Shannon index suggested higher bacterial diversity in *thiat* while *marcha* shows the higher assemblage of fungal diversity with dominance of yeast phylum *Ascomycota*. Persistence of higher fungal diversity in *marcha* is determinant factor suggesting the higher acidic conditions of *marcha*; in contrast, higher bacterial diversity of *thiat* depicts the faster turnover from acidic to alkali with the presence of acid neutralizing bacterial taxa[32].

Acetobacter, *Fructobacillus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Burkholderia*, and *Gluconacetobacter* were the predominant bacterial genera in *marcha*. Higher proportion of *Acetobacter* was possibly due to its retention and enrichment during fermentation. We observed relatively lower proportion of *Streptococcus* and *Lactococcus* than *Lactobacilli*; as *Lactobacilli* have high acid tolerance over former two[33]. Though some species of *Lactococcus* have low acid tolerance, however, they could be isolated from raw milk and were found flourishing during the early stage of fermentation[24]. This supports the lower abundance of *Lactococcus* than *Lactobacillus* as seen in our samples. Another interesting observation was absence of *Pediococcus* in bacterial community profile which was otherwise present as a one of the dominant genus in earlier report by culture dependent methods in *marcha*[10,16]. Furthermore, since there is no earlier report on microbial composition based on culture dependent or culture-independent methods of *thiat* the present study describe microbial diversity of *thiat* using NGS method as its first report. *Pantoea*, *Cronobacter*, *Escherichia*, *Shigella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Serratia*, and *Enterococcus* depicts most dominant bacterial genera of *thiat* each comprised over 0.1% of total bacterial sequences. Significantly varied microbial composition among *thiat* and *marcha* is a clear indication of differences in amylolytic starters. Genus *Enterobacter* was also detected in Mexican alcoholic beverages speculated

to originate from the bacterial contamination in raw milk and they subsequently decreased during the fermentation process[34]. The lactic acid bacterium such as *Lb. plantarum* seemed to be one factor for the good quality of the alcoholic beverages, as it can perform malolactic fermentation to decrease wine acidity[32] and also produces bacteriocins[35].

Exploration of fungal diversity of ethnic amylolytic starters suggested higher abundance of yeast in *marcha* and *thiat* constitutes for 32.33-fold yeast to the filamentous molds. This observation was in coherence with the earlier report of culture-dependent studies showing the dominance of *Mucor* and *Rhizopus* genera of *Mucorales* in *marcha*[8]. Interestingly no filamentous molds were detected in *marcha* using the applied high throughout sequencing method; the exact reasons for the observed variation in the microbiota have not been identified. This may be due to lower abundance of molds, limited sample size and/or age of the sample and finally also due to inadequate cell lysis which may prevent the release of nucleases[36]. Our study was in accordance to the previous reports describing the exposure of cheese to different external environments such as manufacturing process; geographical region, etc have varied impact on the microbial composition of the end products[28]. Thus, we speculate that the factor of geographic environment including altitudes and climate play a more significant role over the manufacturing process in resulting in the different microbial compositions of the starter culture under study. Some other crucial factors that may affect the composition of microbial communities in fermented amylolytic starters are level of hygiene, quality of the glutinous rice, water, as well as the back slopping technique. In this study three dominant yeasts in *marcha* were *Wickerhamomyces anomalus*, *Candida quercitrus* and *Kazachstania exigua*, followed by *Saccharomyces* and *Pestalotiopsis* which also accompany the findings of ref. 21 by PCR-DGGE method. ITS gene sequences analysis of the *thiat* revealed the existence of *Aspergillus penicillioides*, *Aureobasidium pullulans* and *Mucor circinelloides* as the most dominant filamentous molds in *thiat*. At family level *thiat* shows *Trichocomaceae*, *Dothioraceae*and *Mucoraceae*as the major constituents of fungal community composition emphasizing the significant differences between *thiat* and *marcha* viz differences in starter substrates, preparations, inoculums, consortia, geography, hygiene, preservation technique, caloric values etc.

In the present study *Ascomycota* was dominant in starter cultures of India like in Korean and Chinese starters cultures, which was also reported earlier, based on NGS tools, in Korean alcoholic beverages[3] and in Chinese liquors[37]. We could also expect similar observation in case of *marcha* as it has higher abundance of lactic acid bacteria. *Aspergullus oryzae* has strong secretion of amylases including alpha-amylase, which may accelerate the degradation of grains and provide more nutrients for microbes in alcoholic fermentation[38].

Amylolytic starter culture-making technology reflects the traditional knowledge of the ethnic Indian people on sub-culturing desirable inocula from previous batch to new culture using rice as base substrates by back-sloping method. This technique preserves the consortia of microbial community ranging from filamentous molds, yeasts and bacteria which were co-existed in traditionally prepared amylolytic and alcohol producing starters[7], and also preserves vast biological genetic resources, otherwise, which may be forced to disappear. Fermented beverages produced by using amylolytic starters in India are generally mild-alcoholic (4–5%), sweet taste with several health benefits to the local consumers as high source of calories, some vitamins and minerals[2]. Ethnic fermented beverages and alcoholic drinks have the potential to grow beverage industry if proper scientific and technical support are applied to the existing indigenous practices of home based alcoholic fermentation.

Materials and Methods

Sample collection. Samples of sun-dried amylolytic starters *marcha* and *thiat* were collected immediately after the preparation from local people of Gangtok and Shillong in Sikkim and Meghalaya states of India, respectively. Dry samples were transferred to sterile containers, sealed, and stored at desiccator at room temperature for the further analysis.

Community DNA Extraction. The total community DNA was extracted using ProMega DNA kit (ProMega). 1g of amylolytic starter culture sample was suspended in lysis solution and incubated at 65 °C for 15 min. Subsequently, the RNA was eliminated from the cellular lysate by administering the RNase solution following incubation at 35 °C for 15 min. The residual proteins were removed by adding protein precipitation solution and centrifuged at max speed. Finally, the DNA was precipitated by adding isopropanol, which was purified with two washes of 70% ethanol. The quality of DNA was checked on 0.8% agarose gel and concentration was measured using Nano-DropND-1000 spectrophotometer (Nano Drop technologies, Willington, USA) as described by ref. 39. The DNA was stored at −20 °C until further processing.

Amplicon sequencing. Amongst the nine hypervariable regions of bacterial 16S rRNA gene, we have targeted V4 hyper-variable region[40] to investigate bacterial diversity of *marcha* and *thiat*. The universal 16S rRNA gene primer sets F515 and 806R[41] was used for the amplification of V4 hyper-variable region. Similarly, fungal Internal Transcribed Spacer (ITS) II region was targeted for taxonomic profiling amylolytic starters, which was subjected to amplification using ITS1 and ITS2 primers. The library preparation of both the 16S rRNA and ITS gene amplicons were in accordance with the protocols of Illumina (USA). These amplicon libraries were further processed for sequencing using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA). The resultant product was screened with the LabChip GX (Perkin Elmer, Waltham, MA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Subsequently, the 16S rRNA and ITS gene library were sequenced on the Illumina MiSeq platform using 2x 250bp chemistry. The sequences obtained from high throughput sequencing effort were submitted to National Center for Biotechnology Information (NCBI) which are available under BioProject ID PRJNA376467.

Bioinformatics analysis. The raw sequences generated from MiSeq platform was assembled using FLASH tool (Fast Length Adjustment of Short reads) a Paired end assembler for DNA sequences[42]. The assembled reads were subjected to quality filtering using via Quantitative Insights into Microbial Ecology (QIIME) 1.8[42]. Sequence

reads were assigned to bacterial and fungal operational taxonomic units (OTUs) by a closed reference-based OTU picking approach by using SILVA and UNITE reference databases, respectively. The OTU picking was carried out using UCLUST method with similarity threshold of 97%[43]. Taxonomic assignments were performed using RDP naïve bayesian classifier[44]. Alpha diversity indices like Chao, Shannon and Simpson were calculated via QIIME after rarefying all samples to the same sequencing depth[45,46].

Data availability. The sequences obtained from high throughput sequencing effort, was submitted to NCBI which are available under Bio Project ID PRJNA376467.

References

1. Tamang, J. P. & Kailasapathy, K. *Fermented foods and beverages of the world*. CRC Press (Eds.), Taylor and Francis Group, New York (2010).
2. Tamang, J. P. *Himalayan fermented foods: microbiology*, *nutrition and ethnic value*, CRC Press, Taylor and Francis Group, New York (2010).
3. Jung, M. J., Nam, Y. D., Roh, S. W. & Bae, J. W. Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food Microbiol.* **30**, 112–23 (2012).
4. Sujaya, I. *et al*. Identification and characterization of yeasts in brem, a traditional Balinese rice wine. *World J. Microbiol. Biotechnol.* **20**, 143–150 (2004).
5. Thanh, V. N., Mai, L. T. & Tuan, D. A. Microbial diversity of traditional Vietnamese alcohol fermentation starters (*banh men*) as determined by PCR-mediated DGGE. *Int. J. Food Microbiol.* **128**, 268–273 (2008).
6. Yamamoto, S. & Matsumoto, T. Rice fermentation starters in Cambodia: cultural importance and traditional methods of production. *Southeast Asian Studies.* **49**, 192–213 (2011).
7. Tamang, J. P., Watanabe, K. & Holzapfel, W. H. Review: Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* **7**, 377, doi:10.3389/fmicb.2016.00377 (2016).
8. Tamang, J. P., Sarkar, P. K. & Hesseltine, C. W. Traditional fermented foods and beverages of Darjeeling and Sikkim - a review. *J. Sci. Food Agric.* **44**, 375–385 (1988).
9. Dung, N. T. P., Rombouts, F. M. & Nout, M. J. R. Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. *Food Microbiol.* **23**, 331–340 (2006).
10. Tamang, J. P. & Sarkar, P. K. Microflora of murcha: an amylolytic fermentation starter. *Microbios.* **81**, 115–122 (1995).
11. Tsuyoshi, N. *et al*. Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. *Int. J. Food Microbiol.* **99**, 135–146 (2005).
12. Jeyaram, K., Mohendro Singh, W., Capece, A. & Romano, P. Molecular identification of yeast species associated with 'Hamei"- a traditional starter used for rice wine production in Manipur, India. *Int. J. Food Microbiol.* **124**, 115–125 (2008).
13. Jeyaram, K., Tamang, J. P., Capece, A. & Romano, P. P. Geographical markers for *Saccharomyces cerevisiae* strains with similar technological origins domesticated for rice-based ethnic fermented beverages production in North East India. *Antonie van Leeuwenhoek.* **100**, 569–578 (2011).
14. Dung, N. T. P., Rombouts, F. M. & Nout, M. J. R. Characteristics of some traditional Vietnamese starch-based rice wine starters (*Men*). *LWT-Food Sci. Technol.* **40**, 130–135 (2007).
15. Hesseltine, C. W. & Ray, M. L. Lactic acid bacteria in murcha and ragi. *J. Appl. Bacteriol.* **64**, 395–401 (1988).
16. Tamang, J. P. *et al*. Lactic acid bacteria in *Hamei* and *Marcha* of North East India. *Indian J. Microbiol.* **47**, 119–125 (2007).
17. Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. & Rantsiou, R. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int. J. Food Microbiol.* **167**, 29–43 (2013).
18. Chao, S. H. *et al*. Microbial diversity analysis of fermented mung beans (Lu-Doh-Huang) by using pyrosequencing and culture methods. *PLOS ONE.* **8**(5), e63816 (2013).
19. Chen, B., Wu, Q. & Xu, Y. Filamentous fungal diversity and community structure associated with the solid state fermentation of Chinese *Maotai*-flavor liquor. *Int. J. Food Microbiol.* **179**, 80–84 (2014).
20. Lv, X. C., Huang, X. L., Zhang, W., Rao, P. F. & Ni, L. Yeast diversity of traditional alcohol fermentation starters for Hong Qu glutinous rice wine brewing, revealed by culture-dependent and culture-independent methods. *Food Control.* **34**, 183–190 (2013).
21. Sha, S. P., Anupma, A., Pradhan, P., Prasad, G. S. & Tamang, J. P. Identification of yeasts by PCR-mediated DGGE in *marcha*, an ethnic amylolytic starter of India. *J. Ethnic Foods.* **3**, 292–296 (2016).
22. Zhu, L. *et al*. BOX-PCR and PCR-DGGE analysis for bacterial diversity of a naturally fermented functional food (Enzyme). *Food Bioscience.* **5**, 115–122 (2014).
23. Mayo, B. *et al*. Impact of next generation sequencing techniques in food microbiology. *Curr. Genomics.* **15**(4), 293–309 (2014).
24. Alegría, Á., Szczesny, P., Mayo, B., Bardowski, J. & Kowalczyk, M. Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and-independent approaches. *Appl. Environ. Microbiol.* **78**, 1890–8 (2012).
25. Quigley, L. *et al*. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Appl. Environ. Microbiol.* **78**, 5717–23 (2012).
26. Ercolini, D., De Filippis, F., La Storia, A. & Iacono, M. A "remake" of the microbiota involved in the production of water buffalo mozzarella cheese by high throughput sequencing. *Appl. Environ. Microbiol.* **78**, 8142–8145 (2012).
27. Bourrie, B. C. T., Willing, B. P. & Cotter, P. D. The microbiota and health promoting characteristics of the fermented beverage kefir. *Front. Microbiol.* **7**, 647 (2016).
28. Nam, Y. D., Lee, S. Y. & Lim, S. I. Microbial community analysis of Korean soy- bean pastes by next-generation sequencing. *Int. J. Food Microbiol.* **155**, 36–42 (2012).
29. Romi, W., Ahmed, G. & Jeyaram, K. Three-phase succession of autochthonous lactic acid bacteria to reach a stable ecosystem within 7 days of natural bamboo shoot fermentation as revealed by different molecular approaches. *Mol. Ecol.* **24**, 3372–3389 (2015).
30. Keisam, S., Romi, W., Ahmed, G. & Jeyaram, K. Quantifying the biases in metagenome mining for realistic assessment of microbial ecology of naturally fermented foods. *Sci. Rep.* **6**, 34155 (2016).
31. Zhang, J. *et al*. Metagenomic approach reveals microbial diversity and predictive microbial metabolic pathways in Yucha, a traditional Li fermented food. *Sci. Rep.* **6**, 32524 (2016).
32. Kosseva, M., Beschkov, V., Kennedy, J. F. & Lloyd, L. L. Malolactic fermentation in Chardonnay wine by immobilized. *Lactobacillus casei* cells. *Process Biochem.* **33**, 793–797 (1998).
33. Rogosa, M., Mitchell, J. A. & Wiseman, R. F. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bacteriol.* **62**, 132 (1951).
34. Escalante, A. *et al*. Analysis of bacterial community during the fermentation of pulque, a traditional Mexican alcoholic beverage, using apolyphasic approach. *Int. J. Food Microbiol.* **124**, 126–34 (2008).
35. Navarro, L., Zarazaga, M., Ruiz-Larrea, F., Torres, C. & Saenz, J. Bacteriocin production by lactic acid bacteria isolated from Riojared wines. *J. Appl. Microbiol.* **88**, 44–51 (2000).

36. Dolci, P., Alessandria, V., Rantsiou, K. & Cocolin, L. Advanced methods for the identification, enumeration, and characterization of microorganisms in fermented foods. In *Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits*. Elsevier (2015).
37. Li, X. R. *et al*. Bacterial and fungal diversity in the traditional Chinese liquor fermentation process. *Int. J. Food Microbiol.* **146**, 31–7 (2011).
38. Li, H. Simultaneous saccharification and fermentation of broken rice: an enzymatic extrusion liquefaction pretreatment for Chinese rice wine production. *Bioprocess Biosyst. Eng.* **36**, 1141–1148 (2013).
39. Kumbhare, S. V. *et al*. Insights into diversity and imputed metabolic potential of bacterial communities in the continental shelf of Agatti Island. *PLoS One.* **10**, 0129864 (2015).
40. Bartram, A. K., Lynch, M. D., Stearns, J. C., Moreno-Hagelsieb, G. & Neufeld, J. D. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl. Environ. Microbiol.* **77**, 3846–3852 (2011).
41. Caporaso, J. G. *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* **7**, 335–336 (2010).
42. Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D. PANDA seq: paired-end assembler for illumina sequences. *BMC Bioinformatics* **13**, 31 (2012).
43. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* **26**, 2460–2461 (2010).
44. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**, 5261–5267 (2007).
45. Bokulich, N. A., Lucy Joseph, C. M., Allen, G., Benson, A. K. & Mills, D. A. Next-generation sequencing reveals significant bacterial diversity of Botrytized wine. *PLoS ONE.* **7**, e36357 (2012).
46. Blaalid, R. *et al*. ITS1 versus ITS2 as DNA metabarcodes for fungi. *Mol. Ecol. Resour.* **13**, 218–224 (2013).

Acknowledgements

Authors gratefully acknowledged the financial support of Department of Biotechnology, New Delhi for research project and DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre of DBT.

Author Contributions

S.P.S., A.A. and P.P. contributed to this present work as co-authors that are a part of their research work. K.J. and A.S. helped and assisted in all the molecular work and NGS (Bioinformatics and statistical) analyses. A.S., Y.S. 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 approval of J.P.T.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indian Journal of Traditional Knowledge
Vol. 17 (1), January 2018, pp. 184-190

Traditional skill of ethnic people of the Eastern Himalayas and North East India in preserving microbiota as dry amylolytic starters

Anu Anupma, Pooja Pradhan, Shankar Prasad Sha & Jyoti Prakash Tamang*

DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre, Department of Microbiology, School of Life Sciences Sikkim University, Tadong 737 102, Sikkim, India;
*Department of Microbiology, Sikkim University, Tadong 737 102, Sikkim, India
E-mail: jyoti_tamang@hotmail.com

Received 09 March 2017, revised 28 July 2017

Preparation of ethnic fermented beverages using dry amylolytic starters is an integral part of socio-cultural practice of different ethnic groups of people residing in the Eastern Himalayan region of Nepal, Bhutan and India including North-East India. Alcoholic beverages are produced by traditional fermentation using specific amylolytic starters, which are prepared in different ways by diverse ethnic groups. This study is aimed at documenting the traditional skill of various ethnic groups of people of North East India and the Eastern Himalayas in preserving microbiota as dry amylolytic starters generally used for preparation of alcoholic beverages.

Keywords: Alcoholic beverage, Amylolytic starter, Ethnic groups, Eastern Himalayas, North East India.

IPC Int. Cl.[8]: C12G, C12C 12/04, C12G 3/08, C12H 3/00, G01N 33/14, C12C 12/00, C12C 5/00

The geographical extent of Eastern Himalayas comprises eastern Nepal, Indian states of Sikkim, Arunachal Pradesh and Darjeeling hills, and a mountainous country Bhutan[1]. North East India is comprised of the cluster of eight states namely Assam, Nagaland, Sikkim and Tripura. More than 137 different ethnic groups reside in these regions[1]. The consumption of alcoholic beverages, prepared by fermentation using amylolytic starter inocula commonly referred to as amylolytic starter, is a common practice among the people of North East India and the Eastern Himalayas. The amylolytic starter is a round to flattened ball of mixed dough containing various wild and domesticated plant parts[2] and amylolytic and alcohol-producing yeasts, starch degrading moulds and lactic acid bacteria[3,4]. Varieties of ethnic amylolytic starters are prepared such as *marcha* of Nepal, Sikkim and the Darjeeling hills in India, *pho* of Bhutan, *emao/humao* of Assam; *xaaz pitha* and *modor pitha* of Assam, *hamei* of Manipur, *thiat* of Meghalaya, *chowan* of Tripura, *kherie/khekhrii* of Nagaland, *Pee*, *paa*, *phut* and *phab* of Arunachal Pradesh, and *dawdim* of Mizoram (Fig. 1). This paper aims to give extensive information on the indigenous knowledge of preparation process of various amylolytic starters found in different regions of the Eastern Himalayas and North East India.

*Corresponding author

Locations of documentation and data collection

Documentation work was carried out for a period of 12 months from December 2015 till November 2016. Data collection was done based on structured questionnaire, interviewing the people involved in traditional preparation of amylolytic starters, personally analyzing the preparation procedures, collection of plant samples and their identification from Botanical Survey of India, Sikkim circle, Gangtok. The study was done in Basilakha village of Sikkim; Dharan, Dhankutta and Hiley districts of Eastern Nepal; Napchey village of Dhonakha, Bhutan; Kokrajhar, Jorhat, Sivsagar, and Moran district of Assam; Kohima of Nagaland and West Siang and Nirjuli district of Arunachal Pradesh. The interviewees were local people of the villages visited and had proper knowledge of preparation of the starter cultures.

Results and discussion

Traditional preparation process of amylolytic starters

Marcha

Marcha is a dry flat creamy white solid ball-like starter of different size and shape traditionally prepared in Sikkim and the Darjeeling hills in India **(**Fig. 1A), Bhutan (Fig. 1B) and Nepal (Fig. 1C),

Fig. 1 — Amylolytic starter culture of the Eastern Himalayas and North East India- (A): *Marcha* of Sikkim and the Darjeeling hills; B): *Marcha* of Bhutan; (C): *Marcha* of Nepal; (D): *Pho* of Bhutan; (E): *Emao/humao* of Assam; (F): *Xaaz pitha* of Assam; (G): *Modor pitha* of Assam; (H): *Hamei* of Manipur; (I): *Thiat* of Meghalaya; (J): *Chowan* of Tripura; (K): *Kherie/Khekhrii* of Nagaland; (L): *Pee* of Arunachal Pradesh; (M): *Phut* of Arunachal Pradesh; (N): *Paa* of Arunachal Pradesh; (O): *Phab* of Arunachal Pradesh; (P): *Dawdim* of Mizoram.

which is used to ferment starchy material into various ethnic fermented beverages and alcoholic drinks. In the South and eastern parts of Bhutan, *marcha* is predominantly prepared and used by the ethnic *Nepali* of Bhutan. The preparation process of *marcha* in these regions is almost same.

Indigenous knowledge of preparation of marcha

Glutinous rice (*Oryza sativa)* is soaked in water for 8-10 h (overnight) at an ambient temperature. After soaking rice is crushed in a foot driven heavy wooden mortar pestle. Wild herbs such as roots of *guliyo jara* or *chitu* (*Plumbago zeylanica)* leaves of *bheemsen paate* (*Buddleja asiatica*), flower of *sengreknna* (*Vernonia cinerea*), ginger and red dry chili (2-3 pieces) are crushed and added to the powdered rice. Mixtures are then mixed with water to make a thick paste or dough, from which dough balls of different sizes are made. These balls are then dusted with the old *marcha* which are used as an inoculum. The freshly prepared flattened balls are kept on the leaves of fern *Glaphylopteriolopsis erubescens* (commonly known as *pirey uneu*) and covered with the ferns and fermented at room temperature for 24 h. After fermentation, ferns are removed and balls are collected and dried in the sun or kept at room temperature for about 5 days (Fig. 2). The dried *marcha* balls are then sold in the market.

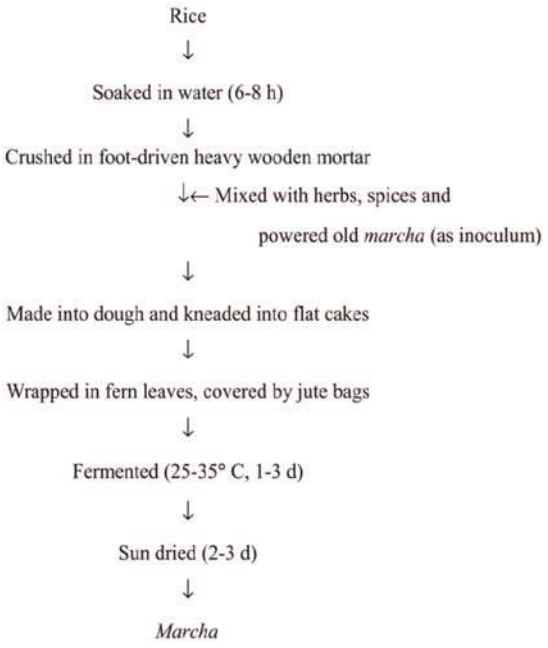


Fig. 2 — Preparation of *marcha* in Sikkim

Mana/Manapu

Mana is a black colored amylolytic starter of Nepal. *Newari* community of Nepal specifically prepares this amylolytic starter to ferment alcoholic distilled liquor, *ayela*, which is very popular alcoholic drinks and is used in special occasions and rituals in

Nepal. Owing to a much easier process of preparation, the use and preparation of *marcha* is replacing the use of *mana* in Nepal, due to which the practice of making *mana* has almost come to an extinction point.

Indigenous knowledge of preparation of mana

During preparation of *mana*, wheat grains are soaked in water over night, boiled for 30 min, drained off excess water, cooled, added dried leaves of *harrah* plant, old *mana* powder and ground in a traditional mortar and pestle. Dough mixture is made into small balls, placed on paddy straw spread on a clean floor, and again covered by paddy straw or straw mat, and fermented for 6-7 days till green moulds appear on the ball. It is dried in the sun to get a black-coloured *mana* and stored for future use.

Pho

Pho or *phab* (Fig. 1D) is a flat dark brown colored cake prepared from powdered maize. This amylolytic starter is prepared indigenously by *Drukpa* community of Bhutan residing in the North Western side of Bhutan. *Pho* is used in preparing the fermented alcoholic drink *Ara*, which is popularly used during the festive seasons in Bhutan.

Indigenous knowledge of preparation of *pho*

The main ingredient used for preparing *pho* is a flower of *bhagham* plant. This flower blooms during October and is white in color and almost 8 inches long (20 cm). The flowers of this plant are sun dried and powdered. During the preparation, powdered maize, rice husks and dried powdered flowers of *bhagham* are taken in a specific ratio of 2:4:3, respectively. Previously prepared *pho* is used as inoculum by back-sloping method and added to the mixtures (Fig. 3). *Pho* stored for more than one year is usually not preferred to be used as a starter culture.

Emao/humao

Emao (Fig. 1E) is amylolytic starter prepared by *Bodo* community of Assam. *Dimasa,* another ethnic community in Assam calls it *humao*. *Emao* is a ball like starter used to ferment glutinous rice to prepare alcoholic beverages of Assam like *zu*/*jou*/*judima*.

Indigenous knowledge of preparation of *emao*

Glutinous rice (*Oryza sativa)* is soaked in water for about 2-3 h and mixed with plants like leaves of banana, *lwkwna*, *dong-phang-rakhep* (*Scoparia dulcis*) and dry barks of *Albizia myriophylla*. These ingredients are then ground together in a wooden mortar pestle (this set of apparatus is locally called *gaihen* and *ual*). The powder is then sieved in a *sandri* (traditional sieve made of bamboo) to which little amount of water is added to make thick paste or dough. Different sizes of small round cakes are made from this dough which are then dusted with the old *emao*, basically used as an inoculum, covered with rice straw and fermented for 2-3 days. After the incubation balls are sundried.

Xaaz pitha

Xaaz pitha (Fig. 1F) is an amylolytic starter produced by *Ahom* community of Assam. *Xaaz pitha* is a ball like starter used to ferment glutinous rice into fermented beverages. Unlike addition of fresh leaves of wild plants during preparation of *emao*, dried leaves are added in preparation of *xaaz pitha*.

Indigenous knowledge of preparation of *xaaz pitha*

Xaaz pitha is traditionally prepared mixed amylolytic dough inocula used as a starter for the preparation of various ethnic alcoholic beverages. Local rice is soaked for 10-12 h and dried leaves and stems of wild herbs are added to the soaked rice. The mixture is then grounded together in an wooden mortar by a pestle. A thick dough is prepared by adding water to the above powdered mixture, from this fresh oval–shaped balls are made and placed on fern leaves (Fig. 4). The freshly prepared *xaaz pitha* is

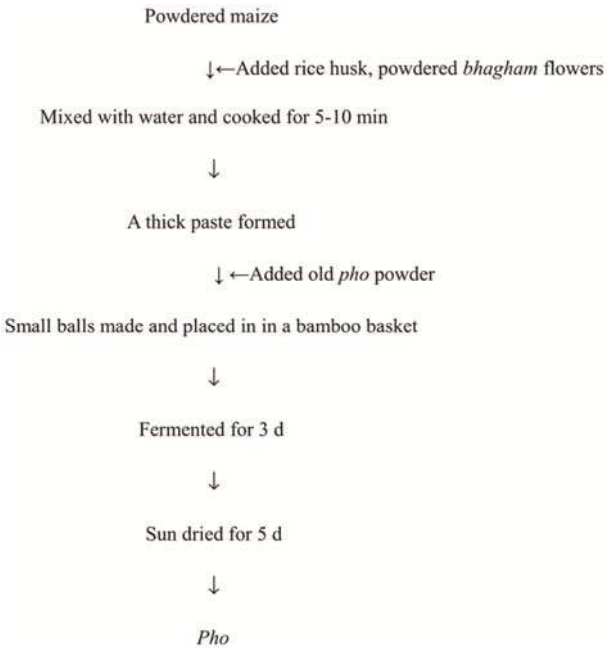


Fig. 3 — Preparation of *pho in* Bhutan

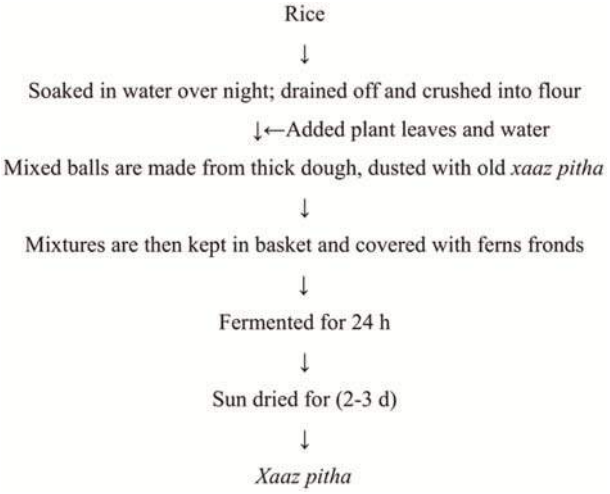


Fig. 4 — Preparation of *xaaz pitha* in Assam

then dried either in the sun or over the fireplace. After a period of about five days they become hard and ready for use.

Modor pitha

Modor pitha (Fig. 1G) is also a starter prepared by *Kachari* community of Assam to ferment rice into alcoholic beverages such as *xaaz pani*.

Indigenous knowledge of preparation of *modor pitha*

Glutinous rice (*Oryza sativa)* is soaked in water for about 2-3 h and mixed with the dried leaves, stem and roots of different types of plants. The above mixture is crushed together in a foot driven wooden mortar pestle (this set of apparatus is locally called *dekhi*). A little amount of water is added to make thick paste, made into small round cakes, dusted with powdered old *modor pitha*, placed on rice straw and then covered with ferns, and fermented for 2-5 days. Once the fermentation process is completed *modor pitha* ball starts to swell. Finally, it becomes hard and is ready to be used as starters for alcoholic beverages.

Hamei

Hamei (Fig. 1H) is a dry, round to flatten, solid ball-like mixed dough inocula used as starter cultures to prepare *atingba*, an alcoholic beverage in Manipur.

Indigenous knowledge of preparation of *hamei*

Hamei is prepared from local varieties of rice which is either soaked in water or used directly without soaking. The rice is mixed with powdered bark of *yangli* (*Albizia myriophylla* Benth.), 1-2 % of old *hamei*, grinded and dough is prepared by adding a small amount of water. The dough is pressed into flat cakes and kept over paddy husk in a bamboo basket, covered by sack clothes and fermented for 2-3 days at room temperature, and then sun dried for 2-3 days (Fig. 5), and is sold in local markets.

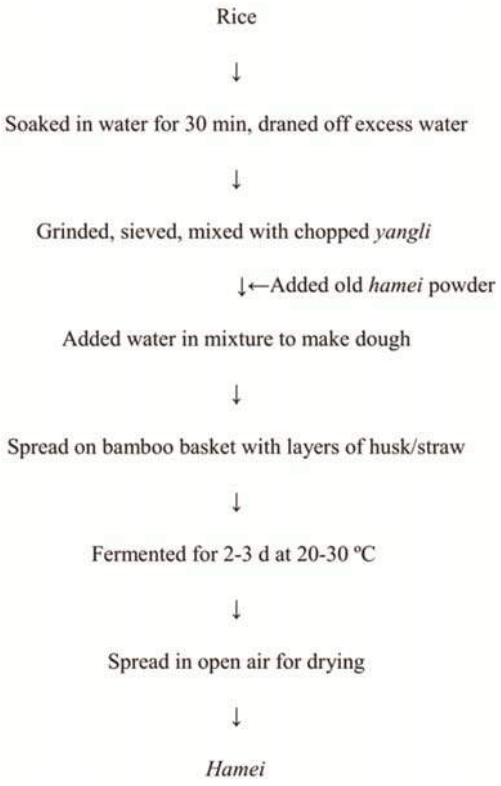


Fig. 5 — Preparation of *hamei in* Manipur

Thiat

Thiat (Fig. 1I) is an amylolytic starter culture used for the preparation of fermented alcoholic beverages in Meghalaya.

Indigenous knowledge of preparation of *thiat*

Glutinous rice is used as a substrate for the preparation of *thiat.* Rice is soaked in water overnight after which it is sun dried for a short period of time to drain off the excess water. Dried leaves and roots of herbs *khaw-iang* (*Amomum aromaticum*) are added to the soaked rice and, and then mixture is made into thick dough by adding water. Flat to round balls are made from the dough and fermented for 1-3 days (Fig. 6). The freshly prepared *thiat* balls are sun dried for 3-5 days.

Chowan

Chowan/chuwan beleb) (Fig. 1J) is a traditionally prepared starter culture of different ethnic tribes in

Fig. 6 — Preparation of *thiat in* Meghalaya

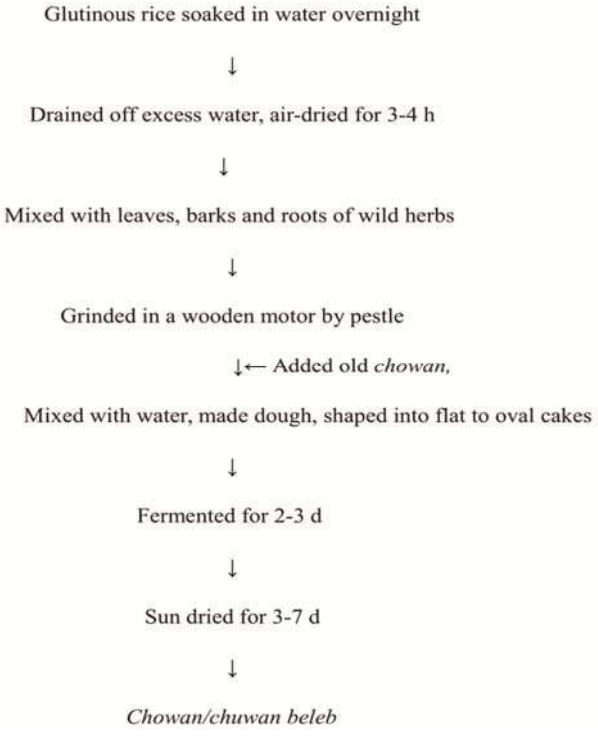


Fig. 7 — Preparation of *chowan* in Tripura

Tripura, and is used for production of local alcoholic beverage *gora bwtwk* and distilled alcoholic drinks *chuwak*.

Indigenous knowledge of preparation of *chowan*

During *chowan* preparation, soaked glutinous rice is mixed with roots, barks and leaves of locally available herbs and powdered old *chowan*. Use of wild herbs during preparation of *chowan* varies from one tribe to other. The mixture is then made into paste by adding water and kneaded into flat and oval cakes of varying sizes and shapes, and fermented for 2-3 days over earthen oven in kitchen (Fig. 7). These freshly prepared *chowan* cakes are then sun dried for 3-7 days and used.

Khrie/Khekhrii

Khrie/khekhrii (Fig. 1K) is an amylolytic starter culture prepared by germinated sprouted rice grains in Nagaland. This is the only amylolytic starter in North-East India which is not prepared by using the old starter through back-sloping method, rather it is prepared by fermenting germinated sprouted-rice grains and then sun-dried to use as dry starters to prepare the local alcoholic drink called *zutho*.

Indigenous knowledge of preparation of *khrie/ khekhrii*

Unhulled glutinous rice (*dhan*) is washed with water two to three times and then soaked into water for 2-5 days. It is then kept and covered with *khreihenyii* leaves and allowed to germinate for 2-3 days in summer and 4-5 days in winter. After germination when the rice sprout is about half an inch in length, the sprouted rice is sun dried and powdered (Fig. 8).

Phab

Phab (Fig. 1O) is a flat white colored cake, which is prepared using rice flour. *Tagin* community of Arunachal Pradesh prepares this amylolytic starter traditionally. *Apatani* community and *Nyshing* community of Arunachal Pradesh also prepare similar starter cultures called *pee* (Fig. 1L) and *paa* (Fig. 1N), respectively.

Indigenous knowledge of preparation of *phab*

During *phab* preparation soaked rice is dried and mixed with leaves of some plants *nakail* (*Cinnamomum glanduliferum* Mesissn.), *ctuepatti* (*Cissampelos pareira* L.), *khanoba (Clerodenderon viscosum* Vent.), 5-7 balls of old *phab* are added, made into fine powder, added water and mixed

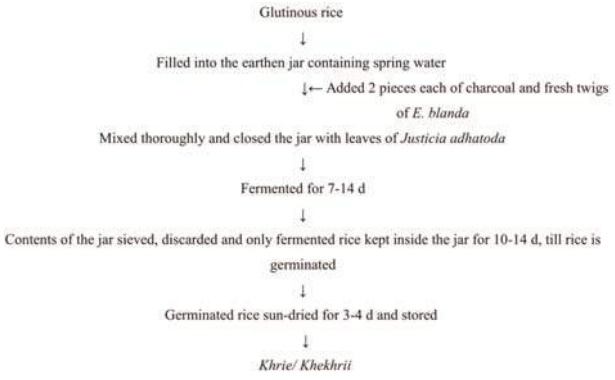


Fig. 8 — Preparation of *khrie/khekhrii* in Nagaland

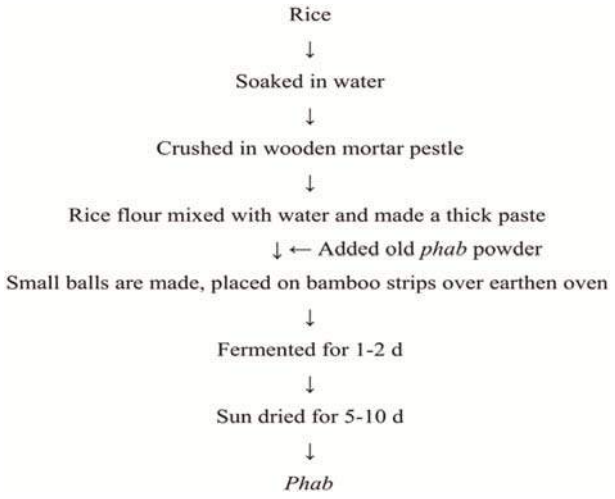


Fig. 9 — Preparation of *phab* in Arunachal Pradesh

thoroughly. The mixture is made into a paste and then small round cakes, wrapped in fresh leaves and placed in bamboo strips over the fireplace and fermented for 1-2 days. Cakes are sun dried for 5-10 days and store up to 6 months (Fig. 9). The preparation process of *phab*, *pee* and *paa* is almost same except for *paa* where rice is boiled without soaking. No herbs are added during the preparation of *pee*.

Phut

Phut (Fig. 1M) is a flat cake prepared by glutinous rice flour. *Mongpa* community of Arunachal Pradesh prepares *phut* traditionally.

Indigenous knowledge of preparation of *phut*

During *phut* preparation soaked rice is dried and crushed into a fine powder using wooden mortar and pestle. Handful leaves of plant along with cakes of old *phut* are made into fine powder, added to rice flour and mixed thoroughly. The mixture paste is made into

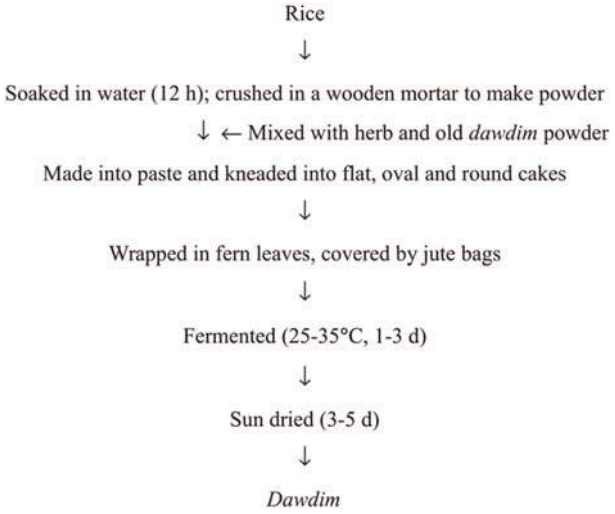


Fig. 10 — Preparation of *dawdim* in Mizoram

small round cakes, wrapped in straw and fermented for 1-2 days. Cakes are sun dried for 3-7 days.

Dawdim

Dawdim (Fig. 1P) is a traditionally prepared amylolytic starter of Mizoram used in preparation of local alcoholic beverages.

Indigenous knowledge of preparation of *dawdim*

During preparation, soaked local varieties of rice are crushed to make fine rice flour, mixed with local leaves of herbs with addition of 2 % old *dawdim*. The mixture is then made into paste by adding water and kneaded into flat and oval cakes of varying sizes and shapes, wrapped in fern leaves in bamboo-made baskets, and then covered by jute bags and kept for fermentation above the earthen kitchen oven for 1-3 days. These freshly prepared cakes are sun dried for 3-5 days (Fig. 10).

Socioeconomic importance

The present documentation focuses on the preparation of amylolytic starters, which are used to make fermented beverages. It was observed that the substrate (rice) used for making the amylolytic starters is almost same among the different ethnic groups, except for the *Drukpa* community of Bhutan who used maize instead of rice as the starchy substrate. Amylolytic starters are not just prepared at household level but also at a commercial scale in small villages of North-East states of India and the Eastern Himalayan regions of Nepal and Bhutan. From these small villages the starter cultures are supplied to the local markets and sold at various

prices. Some ethnic groups of people are economically dependent on the preparation of these amylolytic starter cultures. The mountain women have been storing and culturing the functional microorganisms for alcoholic fermentation in the form of dry starter cultures for more than 1000 of years[1]. The producers earn about 60-70 % profit by selling these starters and are one of the major sources of income in the village areas contributing to local economy.

The consortium of microbiota consisting of filamentous moulds, amylolytic and alcohol producing yeasts and species of lactic acid acid is preserved in cereals as starchy bases, as a source of starch, together with the use of glucose-rich wild herbs to supplement the carbon sources for growing microorganisms[1]. Starter culture-making technology reflects the traditional method of sub-culturing desirable inocula from previous batch to new culture using rice as base substrates. This technique preserves the microbial diversity for beverages production. Saccharifying activities are mostly shown by filamentous moulds *Rhizopus* spp. and *Mucor* spp., and yeast *Saccharomycopsis fibuligera* whereas liquefying activities are shown by *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*[5]. *Rhizopus* spp. and *Sm. fibuligera* degrade cereal starch and produce glucose, and then alcohol-producing yeasts species of *Saccharomyces* and *Pichia* rapidly convert glucose into ethanol[5]. Lactic acid bacteria present in amylolytic starters impart flavor, antagonism and acidification of the substrates[6]. Fermented beverages produced by using amylolytic starters are generally mild-alcoholic (4-5 %), sweet taste with several health benefits to the local consumers as high source of calories, some vitamins and minerals[1,7].

Conclusion

It has been noticed that majority of the young generations do not know ethnic foods, their culinary practices and processing method. Native microorganisms with vast biological genetic resources, which are associated with ethnic fermented foods, are forced to disappear. These fermented food and beverages have the potential to grow in a small medium sized industry if proper scientific and technical support is extended to the existing indigenous practices of home based fermentation.

References

1 Tamang JP, *Himalayan Fermented Foods: Microbiology, Nutrition and Ethnic value*, (CRC Press, Taylor and Francis Group, USA, New York), 2010.

2 Tanti B, Gurung L, Sarma HK & Buragohain AK, Ethnobotany of starter cultures used in alcohol fermentation by a few ethnic ethnic groups of Northeast India, *Indian J Tradit Knowle*, 9 (3) (2010) 463-466.

3 Tamang JP, Tamang N, Thapa S, Dewan S, Tamang BM, Yonzan H, Rai AK, Chettri R, Chakrabarty J & Kharel N, Microorganisms and nutritional value of ethnic fermented foods and alcoholic beverages of North East India, *Indian J Tradit Knowle*, 11 (1) (2012) 7-25.

4 Jeyaram K, Singh M, Capece A & Romano P, Molecular identification of yeast species associated with '*Hamei*"- a traditional starter used for rice wine production in Manipur, India, *Int J Food Microbiol*, 124 (2008) 115-125.

5 Thapa S & Tamang JP, Product characterization of *kodo ko jaanr*: fermented finger millet beverage of the Himalayas, *Food Microbiol*, 21 (2004) 617-622.

6 Tamang JP & Thapa S, Fermentation dynamics during production of bhaati jaanr, a traditional fermented rice beverage of the Eastern Himalayas, *Food Biotechnol*, 20 (2006) 251-261.

7 Tamang JP, Dewan S, Tamang B, Rai A, Schillinger U & Holzapfel WH, Lactic acid bacteria in *Hamei* and *Marcha* of North East India, *Indian J Microbiol*, 47 (2007) 119-125.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 November 2019
doi: 10.3389/fmicb.2019.02526

Phenotypic and Genotypic Identification of Bacteria Isolated From Traditionally Prepared Dry Starters of the Eastern Himalayas

*Pooja Pradhan and Jyoti Prakash Tamang**

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

Preparation of dry starters for alcohol production is an age-old traditional technology in the Eastern Himalayan regions of east Nepal, the Darjeeling hills, Sikkim, and Arunachal Pradesh in India, and Bhutan. We studied the bacterial diversity in 35 samples of traditionally prepared dry starters, represented by *marcha* of Nepal, Sikkim, the Darjeeling hills, and Bhutan, *phab* of Bhutan, and *paa, pee*, and *phut* of Arunachal Pradesh, respectively. Populations of bacteria in these starters were 10^5 to 10^8 cfu/g. A total of 201 bacterial strains were isolated from starter samples, phenotypically characterized, and their identities confirmed by the 16S rRNA sanger sequencing method. The dominant phylum was *Firmicutes* (85%), followed by *Proteobacteria* (9%), and *Actinobacteria* (6%). Lactic acid bacteria (LAB) (59%) formed the most abundant group, followed by non-LAB (32%) and Gram-negative bacteria (9%). Based on the 16S rRNA gene sequencing result, we identified LAB: *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*; non-LAB: *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans,* and *Micrococcus yunnanensis*; Gram-negative bacteria: *Pseudomonas putida, Klebsiella pneumoniae, Enterobacter hormaechei* subsp. *xiangfangensis, E. hormaechei* subsp. *steigerwaltii*, and *Stenotrophomonas maltophilia*. We characterized diversity indexes of the bacterial community present in traditionally prepared dry starters. This is the first report on the bacterial diversity of traditionally dry starters of the Eastern Himalayas by sanger sequencing.

Keywords: Eastern Himalayas, starters, 16S rRNA sequencing, bacterial diversity, lactic acid bacteria

OPEN ACCESS

Edited by:
Vincenzina Fusco, Italian National Research Council (ISPA-CNR), Italy

Reviewed by:
Carmen Wacher, National Autonomous University of Mexico, Mexico
Lixin Luo, South China University of Technology, China

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

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 August 2019*
Accepted: *21 October 2019*
Published: *05 November 2019*

Citation:
Pradhan P and Tamang JP (2019) Phenotypic and Genotypic Identification of Bacteria Isolated From Traditionally Prepared Dry Starters of the Eastern Himalayas. Front. Microbiol. 10:2526. doi: 10.3389/fmicb.2019.02526

INTRODUCTION

The Himalayas, well known for high mountains with natural beauty and rich biological resources, extend from peak Nanga Parbat in Pakistan to peak Namcha Barwa across India, Nepal, and Bhutan (Le Fort, 1975). Based on geo-morphology and demography, the Himalayas are divided into three regions, the Western, Central, and Eastern Himalayas (Nandy et al., 2006). The geographical

location of the Eastern Himalayas extends from eastern Nepal, North East India (Darjeeling hills, Sikkim, and Arunachal Pradesh), Bhutan, and Tibet Autonomous Regions in China (Saha, 2013). Agrarian and pastoral types of mountain farming dominate the agriculture and animal husbandry systems in the Eastern Himalayas, and these are practiced by diverse ethnic communities (Sharma et al., 2007; Bhasin, 2013). Many major and rare types of ethnic fermented foods and beverages are traditionally produced from locally available plant and animal resources and are made into a wide variety of flavorsome cuisine that is consumed as staple diets, side-dishes, curries, soups, condiments, and alcoholic drinks by ethnic people of the Eastern Himalayas (Tamang, 2010; Tamang et al., 2012). The majority of ethnic Himalayan people drink home-made traditional alcoholic beverages and distilled liquor prepared from cereals (rice, finger millets, and maize) as per socio-compulsion but also for enjoyment. Vinification, malting, and brewing processes for alcohol production are completely unknown in the food culture of the Himalayan people; instead, rice or finger millets are fermented into mildly alcoholic (∼4%) beverages (Thapa and Tamang, 2004) by using dry starters, which are unique to these regions.

The Himalayan people have been practicing the art of starter-making using indigenous technology for centuries by using overnight-soaked and pounded rice flours mixed with wild herbs, spices, and 1–2% of previously prepared dry starters in powder form to make doughs. Doughs mixtures with desirable shapes and sizes are placed in fresh fern leaves and allowed to ferment for 2–3 days at room temperature, and the freshly fermented doughs are then sun dried for 2–3 days to get dry starters (Thakur et al., 2015; Anupma et al., 2018). Every ethnic community in the Western, Central, and Eastern Himalayas prepare amylase and alcohol-producing starters with slight variation in the use of substrates, such as rice or wheat, and wrapping materials, such as fern fronds, paddy straw, or plant leaves. In local languages, these are termed *marcha* in Nepal, the Darjeeling hills, and Sikkim in India (Shrivastava et al., 2012; Thakur et al., 2015; Anupma et al., 2018), *mana* and *manapu* in Nepal (Nikkuni et al., 1996), *phab* in Bhutan (Tamang, 2010), *chowan* in Tripura, *dawdim* in Mizoram, *humao, modor pitha* in Assam, *hamei* in Manipur, *khekhrii* in Nagaland, and *phut* in Arunachal Pradesh (Anupma et al., 2018) in India. Similar types of alcohol-producing starters are also prepared in South East Asia by ethnic Asian communities, such as the Vietnamese *benh* (Dung et al., 2007), Korean *nuruk* (Jung et al., 2012), Indonesian *ragi* (Surono, 2016), Philippine *bubod* (Kozaki and Uchimura, 1990), Chinese *daque* or *chiu* or *chu* (Chen et al., 2014), Thai *loogpang* (Limtong et al., 2002), and Cambodian *dombea* (Ly et al., 2018). The most remarkable advent in the traditional preparation of starter cultures is the practice of the "back-slopping method" (terminology in modern food microbiology) used by ethnic Asians irrespective of their geographical locations for sub-culturing the desirable and essential microbiota.

Traditionally prepared dry starters show coexistence of mixed microbiota represented by different genera and species of filamentous molds (Hesseltine et al., 1988; Tamang et al., 1988; Sha et al., 2019), yeasts (Hesseltine and Kurtzman, 1990; Jeyaram et al., 2008, 2011; Sha et al., 2017, 2018, 2019), and bacteria (Hesseltine and Ray, 1988; Tamang et al., 2007; Sha et al., 2017) for saccharification (Lee and Lee, 2002; Thapa and Tamang, 2004), liquefaction (Pervez et al., 2014), and ethanol production (Tsuyoshi et al., 2005; Zheng et al., 2011) to produce traditional alcoholic beverages and distilled liquor in many South East Asian countries, including Nepal, India, and Bhutan in the Himalayas. Filamentous molds (species of *Rhizopus, Mucor, Aspergillus*), and yeasts (species of *Saccharomyces, Pichia, Sacharomycopsis, Candida*) are involved in saccharification and liquefaction; they produce amylolytic enzymes for degrading starch into sugars, and the main alcohol-producing yeasts are *Saccharomyces* for alcohol production (Nout and Aidoo, 2002; Thapa and Tamang, 2004; Li et al., 2012; Nile, 2015). Besides the saccharifying and alcohol-producing ability of mycelia molds and yeasts, some bacterial species present in starters also contribute by imparting flavor, antagonism, and acidification onto the fermenting substrates (Tamang et al., 2007; Huang et al., 2017). Extensive profiling of the diversity of yeasts and mycelial molds in various traditionally prepared dry starters collected from different places of North East India have been reported earlier (Tamang et al., 1988; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Bora et al., 2016; Sha et al., 2017, 2018, 2019). Samples of *marcha* collected from the Darjeeling hills and Sikkim were analyzed earlier and reported few species of bacteria: *Pediococcus pentosaceus* (Tamang and Sarkar, 1995), *Pediococcus pentosaceus* and *Lb. brevis* (Tamang et al., 2007), *Acetobacter*, *Fructobacillus, Lactococcus*, *Lactobacillus, Leuconostoc*, *Burkholderia*, and *Gluconacetobacter* (Sha et al., 2017). However, no published reports on bacterial diversity associated with *marcha* in Nepal and Bhutan, *phab* in Bhutan, and *paa, pee*, and *phut* in Arunachal Pradesh are available to date. *Marcha* (**Figures 1A–D**) is a dry rice-based starter, prepared by the Gorkha/Nepali community in the Darjeeling hills and Sikkim in India, east Nepal, and south Bhutan, to ferment boiled finger-millets into a sweet-sour, mildly alcoholic beverage called *kodo ko jaanr* or *chyang* (Tamang et al., 1996). *Marcha* is prepared

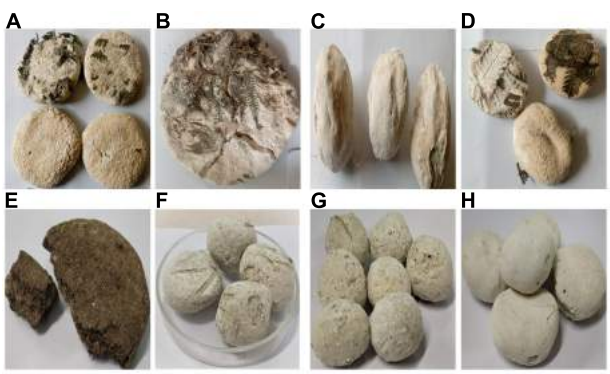


FIGURE 1 | Different types of dry starters from the Eastern Himalayas: **(A)** *Marcha* from Nepal, **(B)** *Marcha* from Darjeeling, **(C)** *Marcha* from Sikkim, **(D)** *Marcha* from Bhutan, **(E)** *Phab* from Bhutan, **(F)** *Paa* from Arunachal Pradesh, **(G)** *Pee* from Arunachal Pradesh, and **(H)** *Phut* from Arunachal Pradesh.

from soaked and pounded rice flours mixed with some wild herbs, few spices, 1–2% of previously prepared powdered *marcha* by the back-slopping method to make doughs that are placed in fresh ferns leaves, are allowed to ferment for 2–3 days, and are then sun dried for 2–3 days to get dry starters. *Phab* or *pho* (**Figure 1E**) is a dark brown, flattened, cake-like starter prepared from powdered maize by the Drukpa community in Bhutan to produce a home-made distilled alcoholic drink called *ara* from barley and finger millets (Anupma et al., 2018). *Paa* (**Figure 1F**), *pee* (**Figure 1G**), and *phut* (**Figure 1H**) are dry starters prepared from rice by the Nyshing, Apatani, and Mongpa communities of Arunachal Pradesh, respectively (Anupma et al., 2018). *Pee* is used to ferment rice into a mildly alcoholic beverage called *opo* by the Nyshing tribes, a mildly alcoholic drink called *apong* by the Apatani, and *phut* is used to prepare a sweet-sour, mildly alcoholic beverage called *themsing* by the Mongpa tribes of Arunachal Pradesh (Shrivastava et al., 2012). Preparation of *marcha, phab, paa, pee*, and *phut* is more or less similar except for some variation in the use of substrates, such as rice in the case of *marcha, phut, paa*, and *pee* and maize-rice husk in *phab*, and wrapping materials, of which fern leaves are used for fermenting rice flour during *marcha* preparation, dry paddy straws are used for *phab* preparation, and locally available plant leaves are used for the preparation of *paa, pee*, and *phut*. We collected dry samples of *marcha, pee, paa, phut*, and *phab* from different places in the Eastern Himalayan regions of Nepal, India, and Bhutan to profile the bacterial diversity as information on yeasts and the mycelial molds community is already available (Sha et al., 2017, 2018, 2019). The present study aimed to profile bacterial diversity isolated from *marcha, pee, paa, phut*, and *phab* based on phenotypic and biochemical tests that use the 16S rRNA gene sequencing method.

MATERIALS AND METHODS

Samples

A total of 35 samples of traditionally prepared dry starters were collected in pre-sterile poly bags from different places located in the Eastern Himalayas viz *marcha* (8 samples) from Nepal, *marcha* (5) from the Darjeeling hills, *marcha* (8) from Sikkim, *marcha* (5) from Bhutan, *paa* (2), *pee* (3), and *phut* (2) from Arunachal Pradesh, and *phab* (2) from Bhutan (**Table 1**). Collected samples were transported and kept in a desiccator at room temperature since traditionally sun-dried starters are stored in a dry place for more than a year (Tamang et al., 1996).

Analysis of Moisture and pH

The moisture content of the samples was estimated by a moisture analyzer (OHAUS/MB-45, United States). The pH of the samples was determined by homogenizing 1 g of sample in 10 mL of distilled water, and the readings were taken using a digital pH-meter (Orion 910003, Thermo Fisher Scientific, United States).

Microbiological Analysis

Dry starter samples were taken from a desiccator, coarsely crushed by a sterile spatula, and 10 g of the powered sample was then homogenized with 90 mL of 0.85% physiological saline in a stomacher lab blender 40 (Seward, United Kingdom) for 2 min. The homogenized samples were serially diluted in the same diluents, and 1 mL of appropriate diluents was then plated using specific media by the pour plate method. Nutrient agar (MM102, HiMedia, Mumbai, India) for aerobic mesophilic bacterial count, MRS (Man-Rogosa-Sharpe) agar (M641, HiMedia, Mumbai, India) and M17 Agar Base (M929, HiMedia, Mumbai, India)

TABLE 1 | Bacterial load of dry starters from the Eastern Himalayas.

Sample	Region	Sample Collection Site	Altitude (Meter)	Latitude	Longitude	Moisture content (%)	pH	cfu/g ($\times 10^7$)
Marcha	Nepal	Dharan	371	26°48′ N	87°17′ E	12.5	5.6	2.1
		Dhankuta	1154	26°53′ N	87°8 ′ E	(9.6–17.0)	(5.5–5.9)	(1.1–2.9)
		Hiley	857	27°02′ N	87°24′ E			
		Hathikharka	1394	27°01′ N	87°32′ E			
Marcha	Darjeeling hills	Darjeeling	2059	27°04′ N	88°26′ E	13.1	5.4	15.3
		Kalimpong	1176	27°07′ N	88°47′ E	(12.9–13.3)	(5.2–5.6)	(11.0–19.6)
Marcha	Sikkim	Pakyong	1341	27°24′ N	88°59′ E	11.8	5.7	18.5
		Gangtok	1637	27°32′ N	88°61′E	(10.0–13.4)	(5.6–5.9)	(10.2–26.5)
		Recab	1072	27°21′ N	88°50′E			
		Basilakha	906	27°22′ N	88°60′ E			
Marcha	Bhutan	Gedumari	1045	26°90′ N	89°39′E	13.76	5.7	0.01
		Thimphu	2401	27°47′ N	89°62′ E	(11.8–15.72)	(5.5–5.9)	(0.01–0.02)
Paa	Arunachal Pradesh	Lower Subansiri	661	27°8′ N	93°6′E	11.7	5.1	2.3
						(11–12)	(5–5.2)	(2.0–2.6)
Pee		Ziro valley	1576	27°53′ N	93°81′E	12.1	5.5	17.6
						(11–13)	(5.2–5.8)	(16.8–18.4)
Phut		Upper Subansiri	1816	28°3′ N	94°E	11.6	5.2	11.5
						(11.4–11.8)	(5.1–5.3)	(9.8–13.2)
Phab	Bhutan	Dhonakha	2311	27°66′ N	89°70′ E	6.17	5.2	0.03
						(6.13–6.2)	(5.0–5.4)	(0.02–0.04)

for lactic acid bacteria (LAB), and VRBGA (violet red bile glucose agar) (M581, HiMedia, Mumbai, India) for Gram-negative bacteria were used for the enumeration of bacteria in respective plates. Nutrient agar plates and VRBGA plates were incubated at 37°C for 24 h, and MRS plates and M17 plates were incubated at 30°C for 24–48 h aerobically. The number of colonies was counted as colony forming unit cfu/g. The purity of colonies was maintained by re-streaking them into fresh medium, and this was further confirmed by microscopic examination. The pure colonies were then preserved in 50% glycerol at −20°C for further identification and analysis.

Phenotypic and Biochemical Characterization

Bacterial isolates were phenotypically characterized for their presumptive identification, and groupings were done on the basis of cell morphology, Gram's reaction, colony morphology, catalase test, sporulation tests, gas production from glucose, and ammonia production from arginine (Holt et al., 1994). The physiological tests including growth at different pHs, temperatures, and salt tolerance were performed (Tamang et al., 2007). Biochemical characterization of isolates such as sugar fermentation tests, IMViC (Indole, Methyl red; Voges-Proskauer and Citrate) tests specifically for Gram-negative isolates, nitrate reduction tests, and urease tests were also performed using the method of Hammes and Hertel (2003).

Genotypic Characterization

Genomic DNA Extraction

The genomic DNA of each bacterial isolate was extracted by the standard phenol/chloroform method of Cheng and Jiang (2006) with slight modifications. A total of 1 ml of culture grown overnight in MRS broth (M369, HiMedia, Mumbai, India) at 30°C was centrifuged at 8,000 rpm for 10 min. The pellets were centrifuged at 3,000 rpm, suspended in 40 μl 1× TE buffer, and freshly prepared 15 μl lysozyme and 15 μl RNAse enzyme were added to the pellets and incubated at 37°C for 3 h. After incubation, 15 μl of 20% SDS (sodium dodecyl sulfate) and 15 μl of proteinase-K were added and further incubated at 55°C for 3 h. An equal volume of phenol-chloroform solution (49:48) was added to the above mixture, centrifuged at 10,000 rpm for 15 min, and the aqueous upper layer formed was transferred to a fresh vial containing chloroform-isoamyl solution (48:1). It was centrifuged again at 10,000 rpm for 15 min, and the upper aqueous layer formed was transferred to a fresh vial containing 15 μl of 3M sodium acetate and 400 μl of cold absolute alcohol and kept at −20°C for 1 h. The mixture was again centrifuged at 10,000 rpm for 30 min, and the pellets were washed with 70% ethanol and further centrifuged at 10,000 rpm for 30 min. The pellets were then collected, air dried, and suspended in 30 μl 1× TE buffer and stored at −20°C for further analysis. The quality of the genomic DNA was checked by electrophoresis in 0.8% agarose gel and quantified using a NanoDrop spectrometer (ND-1000 spectrometer, NanoDrop technologies, Willington, CT, United States) (Kumbhare et al., 2015).

PCR Amplification

The PCR of the 16S rRNA gene from the isolated genomic DNA was amplified using a universal oligonucleotide primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) in a Thermal cycler (Applied Biosystems-2720, United States). The reaction mixture, conditions, and protocol for the polymerase chain reaction amplification were performed following the method of Chagnaud et al. (2001). PCR amplification was performed in a mixture containing a final volume of 50 μl of Go green Taq master mix (1×) (NEB), 10 μM of F primer, 10 μM of R primer, and nuclease-free water (NEB). The PCR reaction program was set under the following PCR conditions: 94°C for 10 min; 94°C for 1 min, 65°C for 1 min, 72°C for 30 s for 35 cycles, and 72°C for 7 min. PCR products were detected by electrophoresis using 1% agarose, and the bands were stained with 7 μl/100 mL of ethidium bromide (RM813, HiMedia, Mumbai, India) and visualized in UV source Gel-Doc 1000 (Bio-Rad, 97-0186-02, United States). A standard 100 base pair DNA ladder (HiMedia, Mumbai, India) was used for the verification of amplicon size.

Purification of the PCR Amplicons

The amplified PCR products were then purified using PEG (polyethylene glycol)-NaCl (sodium chloride) precipitation (20% w/v of PEG, 2.5 M NaCl) precipitation method with little modifications of method described by Schmitz and Riesner (2006). About 0.6 volume of 20% PEG-NaCl was added to final volume of PCR products and incubated at 37°C for 30 min. After centrifugation at 12,000 rpm for 30 min, the aqueous solution was discarded, the pellet was washed twice with freshly prepared ethanol (70%) by centrifugation at 12,000 rpm for 30 min. The collected pellet was then air-dried overnight and 20 μl of nuclease-free water was added, and the final purified product was loaded in 1% agarose gel.

16S rRNA Gene Sequencing

PCR products were set up in 5 μl volume for single primer amplification with the same universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) for separate reactions for each primer. PCR reaction was set as follows: denaturation for (96°C, 10 s), annealing (50°C, 5 s), and elongation (60°C, 2 min) with a stop reaction at 4°C. The amplicons were then precipitated with 1 μl sodium acetate (3M, pH 5.2) and 24 μl of absolute alcohol, mixed briefly in vortex and incubated at room temperature for 15 min, spun at 12,000 rpm for 20 min, further washed with 70% ethanol, air-dried, and suspended in 10 μl formamide. Sequencing of the amplicons was performed by the Sanger Sequencing method or the Chain-termination DNA (Sanger et al., 1977), the automation of a modified Sanger method that is commonly used to check the sequence of the templates (Heather and Chain, 2016), was carried out in an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, United States).

Bioinformatics

The sequence quality was checked by Sequence Scanner v.1.0 (Applied Biosystems, Foster City, CA, United States). After checking the sequence quality, the sequences were assembled using a ChromasPro 1.5 (McCarty, 1998). The orientation of the assembled sequences was checked using an orientation checker v.1.0. The identity of bacterial isolates was assigned by comparing their DNA sequences with those available in the GenBank NCBI (National Center for Biotechnology Information) database using a BLAST (basic local alignment search tool) 2.0 program (Altschul et al., 1990). The sequences were then aligned by pairwise alignment using clustalW, and the phylogenetic tree was constructed using MEGA7.0 software by the neighbor joining method (Gascuel and Steel, 2006; Kumar et al., 2016). Diversity indices were calculated using a PAST (PAleontological STatistics) v.3.25, which is a comprehensive statistics package used in many fields of life sciences, economics, earth science, engineering, and paleontology (Hammer et al., 2001). The Chao 1 value for species richness was calculated following the method of Chao and Chiu (2016).

Data Availability

The sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-NCBI under the nucleotide accession number: MK748250-MK748278, MK202997-MK203032, and MK752675-MK752677.

RESULTS

Microbial Population

Populations of bacteria in 35 samples of traditionally prepared dry starters collected from different regions of the Eastern Himalayas were 1.0×10^5 to 2.7×10^8 cfu/g (**Table 1**). The moisture contents of all samples analyzed were 10%–17% except for *phab* of Bhutan in which the moisture content was comparatively low (<6%). Average pH of all samples was 5.5 (**Table 1**).

Phenotypic Characterization

We isolated 201 total bacterial isolates from 35 different samples of traditionally prepared starters collected from the Eastern Himalayas, which were represented by 139 isolates from *marcha* (Sikkim 49; Darjeeling 38; Nepal 34, Bhutan 18), 12 isolates from *paa* (Arunachal Pradesh), 17 isolates from *pee* (Arunachal Pradesh), 11 isolates from *phut* (Arunachal Pradesh), and 22 isolates from *phab* (Bhutan). All 201 bacterial isolates were phenotypically characterized based on various biochemical and physiological parameters (**Table 2**). A total of nine different bacterial genera including unidentified group were presumptively identified based on phenotypic results following Bergey's manual of bacteriological classification (Holt et al., 1994), which were mostly represented by Gram-positive bacteria (*Pediococcus, Lactobacillus, Enterococcus, Leuconostoc, Bacillus*, and *Staphylococcus*) and two Gram-negative bacteria (*Enterobacter* and *Citrobacter*). We randomly grouped 201 isolates into 68 representative bacterial strains based on phenotypic characterization results (data not shown).

Molecular Identification of Bacterial Isolates

The genomic DNA of each isolate of all 68 representative bacteria strains was extracted and PCR products were prepared for identification by 16S rRNA gene sequence using the Sanger method. DNA sequences of bacterial isolates were assigned by comparing them with those available in the GenBank NCBI database using a BLAST 2.0 program (Altschul et al., 1990) for identification. The phylogenetic trees of the nucleotide sequences of 68 bacteria isolates from samples of *marcha, paa, pee, phut*, and *phab* were constructed using the Neighbor-joining method with 1,000 bootstrap value replicates (**Figure 2**). The 16S rRNA sequencing results showed three bacterial phyla represented by *Firmicutes* (85%), *Proteobacteria* (9%), and *Actinobacteria* (6%). The phylum distribution of the *marcha* samples from Nepal showed *Firmicutes* (80%) followed by *Actinobacteria* (20%); Darjeeling showed *Firmicutes* (100%); Sikkim showed *Firmicutes* (92%), and *Actinobacteria* (8%); Bhutan showed *Firmicutes* (100%). In starters from Arunachal Pradesh the variable distribution pattern in phyla level was observed. Samples of *paa* showed *Firmicutes* (80%), and *Proteobacteria* (20%), *pee* showed *Firmicutes* (67%), *Proteobacteria* (16%), and *Actinobacteria* (17%), and *phut* showed *Firmicutes* (75%), and *Proteobacteria* (25%). Similarly, phylum distribution in *phab* from Bhutan showed *Firmicutes* (57%) and *Proteobacteria* (43%). Based on results of the 16S rRNA gene sequencing, 15 different genera viz. *Leuconostoc, Enterococcus, Bacillus, Staphylococcus, Lactobacillus, Enterobacter, Klebsiella, Pseudomonas, Pediococcus, Stenotrophomonas, Kocuria, Brevibacterium, Lysinibacillus, Weissella*, and *Micrococcus* with 32 species from starters of the Eastern Himalayas were identified (**Tables 3**, **4**). A wide diversity of bacteria (mainly LAB) was reported for the first time in traditionally prepared dry starters of the Eastern Himalayas (**Table 5**). The dominance of species of LAB was observed with 59% of total isolates in samples over non-LAB isolates (31%) (**Figure 3**). *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria* were lactic acid bacterial species found in starter samples. *Enterococcus durans* (54.5%) was the most dominant species present in *marcha* samples from India (Darjeeling), whereas *Pediococcus pentocaseus* (5.8%) showed the lowest prevalence in *marcha* samples from Bhutan (**Figure 4**). LAB were found in all samples with highest occurrence in *marcha* samples of Darjeeling (91%). Non-LAB species were also recovered in many samples of starters, which were represented by *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans,* and *Micrococcus yunnanensis.* Interestingly, we detected few Gram-negative bacteria in

TABLE 2 | Phenotypic characterization of bacterial isolates from dry starters from the Eastern Himalayas.

Presumptive Identification (Total number of isolates)	Sugar fermentation													Tolerance							IMViC test					
														NaCl		pH			Temperature(°C)			Indole	MR	VP	Citrate	Urease	Nitrate
	Cellobiose	Raffinose	Sorbitol	Arabinose	Melibiose	Xylose	Lactose	Ribose	Melizitose	Glucose	Sucrose	Mannitol	Rhamnose	5%	10%	3.6	9.6	10.6	15	10	45						
Leuconostoc (15)	+(6) −(9)	+(7) −(8)	+(5) −(10)	+(4) −(11)	+(9) −(6)	−(10) +(5)	+(9) −(5) v(1)	−(6) +(8) v(1)	+(10) −(5)	+	+(8) −(6) v(1)	+(12) −(3)	+(4) −(10) v(1)	+	+(1) −(14)		+		+(5) −(10)	+(9) −(6)	+(12) −(3)	IMViC test was not determined for Gram-positive bacteria.					
Enterococcus (41)	+(18) −(23)	+(29) −(12)	+(23) −(18)	+(14) −(27)	+(34) −(7)	+(18) −(24)	+(34) −(6) v(1)	+(34) −(7)	+(33) −(3) v(5)	+	−(7) +(34)	+(7) −(34)	+(23) −(17) v(1)	+(36) −(5)	+(6) −(30) v(5)	+(6) −(35)	+(35) −(6)		+(13) −(28)	+(32) −(4) v(5)	+(35) −(6)						
Pediococcus (57)	+(19) −(38)	+(27) −(30)	+(19) −(38)	+(23) −(26) v(8)	+(28) −(29)	+(23) −(29) v(5)	+(36) −(12) v(9)	+(49) −(5) v(3)	+(32) −(24) v(1)	+	+(33) −(24)	+(13) −(44)	+(38) −(18) v(1)	+(48) v(9)	+(4) −(51) v(2)	+(3) −(51) v(3)	+(40) −(16) v(1)	+(5) −(52)	+(7) −(44) v(6)	+(38) −(13) v(6)	+(46) −(9) v(2)						
Lactobacillus (15)	+(12) −(3)	+(5) −(10)	+(10) −(5)	+(5) −(10)	+(10) −(5)	+(5) −(10)	+	+(12) −(3)	+(4) −(11)	+	+(12) −(3)	+(10) −(2) v(3)	+(4) −(11)	+	+(7) −(8)	+(10) −(5)	+(3) −(6) v(6)		+	+(9) −(6)	+(5) −(5) v(5)						
Bacillus (21)	+(17) −(4)	+(3) −(18)	+(3) −(18)	+(3) −(18)	+(3) −(18)	+(6) −(15)	+(15) −(6)	+(17) −(4)	+(9) −(12)	+(18) −(3)	−(11) +(10)	+(15) −(6)		+	+(7) −(14)	+(6) −(15)	+(17) v(4)		+	+(19) v(2)	+						
Staphylococcus (13)	+(8 −(5)	+(6) −(5) v(2)	+(5) −(8)	+(4) −(9)	+(6) −(7)	+(5) −(6) v(2)	+(9) −(2) v(2)	+(5) −(8)	+(3) −(10)	+	+(8) −(4) v(1)	+(8) −(5)	+(8) −(3) v(2)	+(6) −(2) v(5)	+(2) −(9) v(2)	+(2) −(9) v(2)	+(5) −(7) v(1)		+(2) −(10) v(1)	+(5) −(7) v(1)	+(9) −(3) v(1)						
Unidentified (21)	+(12) −(9)	+(7) −(14)	+(4) −(17)	+(11) −(8) v(2)	+(8) −(13)	+(9) −(10) v(2)	+(12) −(6) v(3)	+(18) −(3)	+(12) −(9)	+	+(11) −(10)	+(3) −(18)	+(12) −(9)	+	+(2) −(19)	+(1) −(19) v(1)	+(14) −(6) v(1)	+(3) −(17) v(1)	+(11) −(8) v(2)	+(15) −(3) v(3)	+(15) −(4) v(2)						
Enterobacter (8)	+	−	+(2) −(6)	+	−	+	−	+(6) −(2)	−	+	+	+	+	Physiological tests were not done for Gram–negative bacteria								−	−	+	+	+	+
Citrobacter (10)	+(5) −(5)	-	−	+(5) −(5)			+(8) −(2)	+(7) −(3)		+(8) −(2)	−(7) +(3)	−(5) v(5)	−(7) +(3)									−	−	−	+(6) −(4)	−(6) +(4)	+(6) −(4)

+,Positive; −,Negative; v, Variable; Numbers in parenthesis indicates number of isolates; All strains of Gram-negative bacteria were tested for IMViC either +/−. +,Positive; −,Negative; v, Variable; Numbers in parenthesis indicates number of isolates; All strains of Gram-negative bacteria were tested for IMViC either ±.

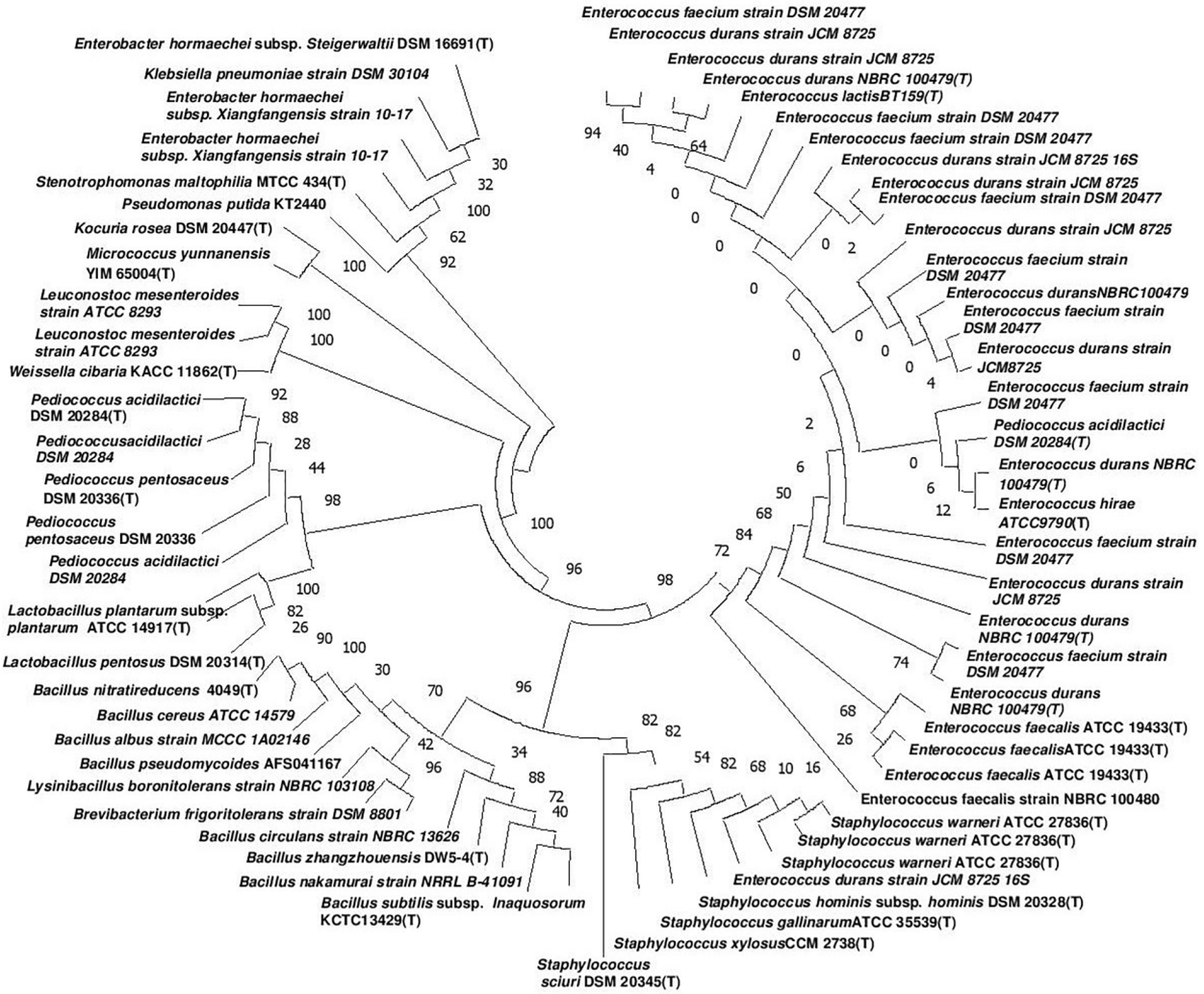


FIGURE 2 | Phylogenetic tree of the nucleotide sequences of 68 bacteria isolates from 35 different samples of dry starter from the Eastern Himalayas based on 16S rRNA sequencing. The tree was constructed by using the Neighbor-joining method (Gascuel and Steel, 2006) with bootstrap values for 1,000 replicates shown at the nodes of the tree using MEGA-7 (Kumar et al., 2016). The optimal tree with the sum of branch length = 0.98855936 is shown. The evolutionary distances were computed by the Maximum Composite Likelihood method (Varin et al., 2011) and are expressed in the units of the number of nucleotide substitutions per site. All positions containing gaps and missing data were eliminated. There were 308 total positions in the final dataset.

some of the starter cultures from Arunachal Pradesh such as *Stenotrophomonas maltophilia* in *paa, Klebsiella pneumoniae* in *pee, Pseudomonas putida* in *phut,* and *Enterobacter hormaechei* subsp. *xiangfangensis,* and *E. hormaechei* subsp. *steigerwaltii* in some samples of *phab* from Bhutan (**Table 5**).

Diversity indexes of bacterial communities of different starter cultures were characterized by the Shannon diversity index *H*, the Simpson's index, and the Dominance and Chao1 index (**Table 6**). The Shannon diversity index *H* for evaluating bacterial diversity recorded highest in *marcha* from Sikkim (*H*:2.305) and lowest in *marcha* from Darjeeling (*H*:1.121). Simpson's diversity index (1-*D*) values were 0.8878, 0.8711, 0.86, and 0.8374 for starters from Sikkim, Arunachal Pradesh, Nepal, and Bhutan, respectively. An estimation of species richness based on abundance was shown by the Chao 1 index. The dominance *D*-values were recorded as being highest for *marcha* samples from Darjeeling and lowest for *marcha* samples from Sikkim.

DISCUSSION

In this study five types of traditionally prepared dry starters (*marcha, pha, paa, pee*, and *phut*) were collected from different regions of the Eastern Himalayas, and they were analyzed for microbial load, pH, and moisture. The average bacterial population of all samples was 10^8 cfu/g, which was not reported earlier except for *marcha* from the Darjeeling hills and Sikkim (Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Tamang et al., 2007). The bacterial load of *marcha* from Sikkim was 10^6 to 10^8 cfu/g (**Table 1**), which was almost the same as that

TABLE 3 | Identification of LAB isolates from dry starters from the Eastern Himalayas based on 16S rRNA gene sequencing.

Isolate code	Sample (Place)	Identity	Type species (% similarity)	GenBank Accession No.	Size (base pair)
AKB6	*Marcha* (Darjeeling)	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.54%)	MK748250	1315
BPB18	*Marcha*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 16S (99.52%)	MK748251	1254
DMB4	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.55%)	MK748252	1325
SMB13	*Marcha* (Sikkim)	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.55%)	MK748253	1339
AKB3	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.62%)	MK748254	833
AOB14	*Pee* (Arunachal Pradesh)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.62%)	MK748255	1333
AOB15	*Pee* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.11%)	MK748256	1430
AOB25	*Phut* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.71%)	MK748258	1406
AOB4	*Paa* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.38%)	MK748259	1460
BPB11	*Marcha*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.91%)	MK748260	1476
BPB31	*Phab*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.28%)	MK748264	1390
BPB33	*Phab*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.51%)	MK748265	1432
DMB11	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.78%)	MK748267	1342
DMB12	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.59%)	MK748268	1462
DMB6	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.38%)	MK748269	1443
MBV14	*Pee* (Arunachal Pradesh)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.86%)	MK748270	1436
SMB15	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.86%)	MK748274	1447
SMB21	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.64%)	MK748276	1400
SMB5	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.78%)	MK748277	1391
SMB7	*Marcha* (Sikkim)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (98.71%)	MK748278	1158
AOB5	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.86%)	MK202997	1421
BPB13	*Marcha*(Bhutan)	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.73%)	MK203008	1456
BPB21	*Phab*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.79%)	MK203010	1430
BPB4	*Marcha*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.65%)	MK203013	1437
DMB3	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203015	1441
AOB24	*Phut* (Arunachal Pradesh)	*Enterococcus hirae*	*Enterococcus hirae* ATCC 9790(T) (99.86%.)	MK202998	1411
DMB13	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.58%)	MK203017	1433
DMB14	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.52%)	MK203018	1461
DMB11	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.66%)	MK203019	1456
DMB15	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203020	1437
NMB3	*Marcha* (Nepal)	*Lactobacillus pentosus*	*Lactobacillus pentosus* DSM 20314(T) (97.44%)	MK203022	1276
NMB8	*Marcha* (Nepal)	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) (99.65%)	MK203024	1441
AOB26	*Phut* (Arunachal Pradesh)	*Enterococcus lactis*	*Enterococcus lactis* BT159 (T) (98.0%)	MK202999	1398
NMB7	*Marcha* (Nepal)	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) (100%)	MK203027	1435
SMB9	*Marcha* (Sikkim)	*Weissella cibaria*	*Weissella cibaria* KACC 11862(T) (99.66%)	MK203028	1455
SMB13	*Marcha* (Sikkim)	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.79%)	MK203029	1433
AOB2	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.79%).	MK203002	1420
AOB11	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.58%).	MK203003	1421
SMB11	*Marcha* (Sikkim)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (96.44%)	MK752677	1432
SMB3	*Marcha* (Sikkim)	*Enterococcus faecalis*	*Enterococcus faecalis* NBRC 100480 (97.42%)	MK752675	1123

ATCC, American Type Cell Culture; JCM, Japan Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen; MCCC, Microbial Culture Collection; NBRC, Biological Resource Centre, NITE; CCM, Czech collection of microorganisms; KACC, Korean Agricultural Culture Collection; YIM, Yunnan Institute of Microbiology; KCTC, Korean Collection for Type Cultures; NRRL, Agricultural Research Service Culture Collection.

of populations of yeasts and filamentous molds in *marcha* from Sikkim (Tsuyoshi et al., 2005). This shows that bacterial populations in traditionally prepared starters of the Eastern Himalayas may have co-existed equally with filamentous molds and yeasts (Hesseltine et al., 1988; Zheng et al., 2015). The moisture content of all starters was low due to the sun-drying process that followed immediately after fermentation, the step necessary to maintain the potency of traditionally prepared starters to be able to be stored in a dry place at room temperature for future use. The pH of all samples was mildly acidic, which may be due to the dominance of LAB ($\sim 10^8$ cfu/g) in dry starters (Tamang and Sarkar, 1995).

TABLE 4 | Identification of non-LAB and Gram-negative bacteria from dry starters from the Eastern Himalayas based on 16S rRNA gene sequencing.

Isolate code	Sample (Place)	Identity	Type species (% similarity)	GenBank Accession No.	Size (base pair)
AOB48	*Phut* (Arunachal Pradesh)	*Pseudomonas putida*	*Pseudomonas putida* KT2440 (99.85%)	MK203004	1379
AOB18	*Pee* (Arunachal Pradesh)	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104 (99.3%)	MK748257	1439
BPB23	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *xiangfangensis*	*Enterobacter hormaechei subsp. Xiangfangensis* 10−17 (99.58%)	MK748261	1431
BPB27	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *Xiangfangensis*	*Enterobacter hormaechei* subsp. *Xiangfangensis* 10−17 (98.88%)	MK748263	1446
BPB26	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *steigerwaltii*	*Enterobacter hormaechei* subsp. *Steigerwaltii* DSM 16691(T) (99.23%)	MK203011	1422
AOB9	*Paa* (Arunachal Pradesh	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* MTCC 434(T) (99.79%)	MK203000	1416
NMB10	*Marcha* (Nepal)	*Bacillus zhangzhouensis*	*Bacillus zhangzhouensis* DW5−4(T) (99.58%)	MK203023	1432
NMB23	*Marcha* (Nepal)	*Staphylococcus xylosus*	*Staphylococcus xylosus* CCM 2738(T) (99.86%)	MK203021	1426
BPB24	*Phab* (Bhutan)	*Bacillus albus*	*Bacillus albus* MCCC 1A02146 (99.02%)	MK748262	1437
BPB8	*Marcha* (Bhutan)	*Bacillus circulans*	*Bacillus circulans* NBRC 13626 (98.64%)	MK748266	1412
NMB11	*Marcha* (Nepal)	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (100%)	MK748271	1460
NMB12	*Marcha* (Nepal)	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (99.72%)	MK748272	1426
NMB13	*Marcha* (Nepal)	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (100%)	MK748273	1388
SMB19	*Marcha* (Sikkim)	*Lysinibacillus boronitolerans*	*Lysinibacillus boronitolerans* NBRC 103108 (99.59%)	MK748275	1220
BPB1	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203006	1437
BPB10	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.79%)	MK203007	1432
BPB17	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203009	1429
BPB3	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (98.92%)	MK203012	1490
BPB7	*Marcha* (Bhutan)	*Bacillus nitratireducens*	*Bacillus nitratireducens* 4049(T) (99.36%)	MK203014	1404
DMB5	*Marcha* (Darjeeling)	*Staphylococcus hominis* subsp. *hominis*	*Staphylococcus hominis* subsp. *hominis* DSM 20328(T) (99.93%)	MK203016	1425
NMB20	*Marcha* (Nepal)	*Staphylococcus gallinarum*	*Staphylococcus gallinarum* ATCC 35539(T) (99.86%)	MK203025	1437
NMB22	*Marcha* (Nepal)	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345(T) (99.65%)	MK203026	1439
SMB22	*Marcha* (Sikkim)	*Micrococcus yunnanensis*	*Micrococcus yunnanensis* YIM 65004(T) (99.64%)	MK203030	1379
SMB1	*Marcha* (Sikkim)	*Bacillus subtilis* subsp. *inaquosorum*	*Bacillus subtilis* subsp. *Inaquosorum* KCTC 13429(T) (99.65%)	MK203031	1425
SMB8	*Marcha* (Sikkim)	*Bacillus pseudomycoides*	*Bacillus pseudomycoides* AFS041167 (99.93%)	MK203032	1407
AOB19	*Pee* (Arunachal Pradesh)	*Kocuria rosea*	*Kocuria rosea* DSM 20447(T) (99.79%.)	MK203001	1399
AOB20	*Pee* (Arunachal Pradesh)	*Bacillus subtilis* subsp. *inaquosorum*	*Bacillus subtilis* subsp. *Inaquosorum* KCTC 13429(T) (99.79%)	MK203005	1431
SMB14	*Marcha* (Sikkim)	*Bacillus nakamurai*	*Bacillus nakamurai* NRRL B-41091 (96.65%)	MK752676	1103

First, we phenotypically characterized all 201 bacterial strains isolated from samples of *marcha, paa, pee, phut*, and *phab* and presumptively identified four genera of LAB- *Enterococcus, Pediococcus, Leuconostoc*, and *Lactobacillus,* two genera of non-LAB-*Bacillus* and *Staphylococcus*, and two Gram-negative bacterial genera, *Enterobacter* and *Citrobacter.* We grouped 201 isolates into 68 representative bacterial strains on the basis of phenotypic and biochemical tests for confirmation of their identity and assigned the taxonomical nomenclature by using 16S rRNA gene sequencing. In our study, we found a dominance of phylum *Firmicutes* (85%) over *Proteobacteria* (9%) and *Actinobacteria* (6%) in starters from the Eastern Himalayas. *Firmicutes* was also reported as the major abundant phylum in *daqu,* a starter for Chinese strongly flavored

TABLE 5 | Bacterial diversity in dry starters from the Eastern Himalayas.

Country/place	Starter	Bacterial species	
Nepal	*Marcha*	LAB:	*Lactobacillus pentosus, Lb. plantarum* subsp. *plantarum*
		Non-LAB:	*Bacillus cereus, B. zhangzhouensis, Brevibacterium frigoritolerans, Staphylococcus xylosus, S. gallinarum, S. sciuri*
		Gram-ve bacteria:	NR
India (Darjeeling hills)	*Marcha*	LAB:	*Enterococcus durans, Pediococcus acidilactici, Leuconostoc mesenteroides*
		Non-LAB:	*Staphylococcus hominis* subsp. *hominis*
		Gram-ve bacteria:	NR
India (Sikkim)	*Marcha*	LAB:	*Pediococcus pentosaceus, Leuconostoc mesenteroides, Enterococcus faecium, E. faecalis, E. durans, Weissella cibaria*
		Non-LAB:	*Lysinibacillus boronitolerans, Micrococcus yunnanensis, Bacillus subtilis* subsp. *inaquosorum, B. pseudomycoides, B. nakamurai*
		Gram-ve bacteria:	NR
India (Arunachal Pradesh)	*Paa*	LAB:	*Enterococcus faecalis, E. faecium*
		Non-LAB:	NR
		Gram-ve bacteria:	*Stenotrophomonas maltophilia*
	Pee	LAB:	*Enterococcus faecalis, E. durans, E. faecium*
		Non-LAB:	*Kocuria rosea, Bacillus subtilis* subsp. *inaquosorum*
		Gram-ve bacteria:	*Klebsiella pneumoniae*
	Phut	LAB:	*Enterococcus hirae, E. lactis, E. faecium*
		Non-LAB:	NR
		Gram-ve bacteria:	*Pseudomonas putida*
Bhutan	*Marcha*	LAB:	*Pediococcus pentosaceus, Enterococcus durans, E. faecium*
		Non-LAB:	*Staphylococcus warneri, Bacillus nitratireducens, B. circulans*
		Gram-ve bacteria:	NR
	Phab	LAB:	*Enterococcus durans, E. faecium*
		Non-LAB:	*Bacillus albus*
		Gram-ve bacteria:	*Enterobacter hormaechei* subsp. *xiangfangensis, Enterobacter hormaechei* subsp. *steigerwaltii*

LAB, lactic acid bacteria; NR, not recovered.

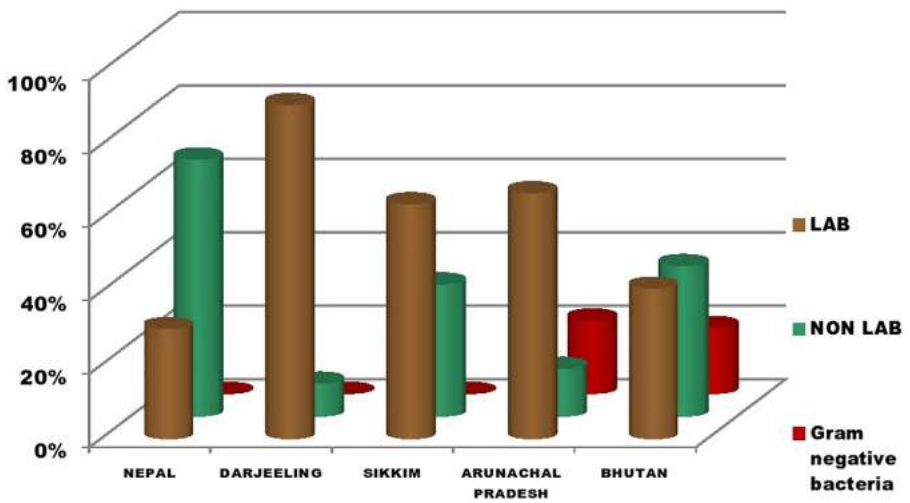


FIGURE 3 | Distribution of LAB, non-LAB, and Gram-negative bacteria in dry starters from the Eastern Himalayas.

liquor (Zou et al., 2018; He et al., 2019), and in *nuruk*, a starter from Korean used to produce *makgeolli,* a Korean alcoholic beverage (Jung et al., 2012). The sequence data based on a constructed phylogenetic tree revealed a dominance of LAB (59%) with five different genera and 11 species represented by *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*. Only two genera of LAB represented by *Pediococcus pentosaceus* and *Lactobacillus brevis* were reported

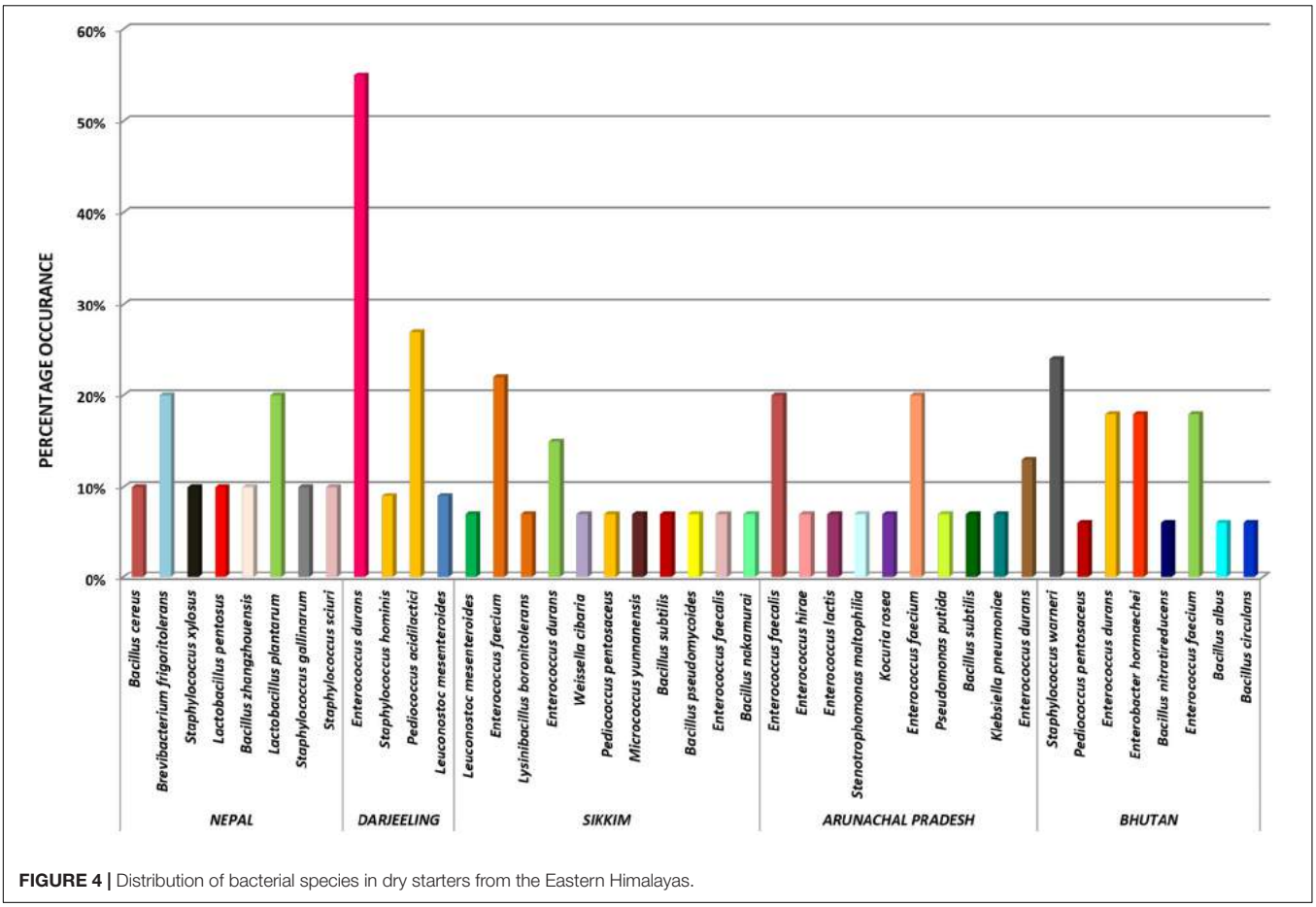


FIGURE 4 | Distribution of bacterial species in dry starters from the Eastern Himalayas.

earlier from *marcha* samples from Sikkim and the Darjeeling hills (Tamang and Sarkar, 1995; Tamang et al., 2007). However, in this study we found a wide diversity of LAB in samples of *marcha* collected from the Darjeeling hills and Sikkim in India, which included *Pediococcus pentosaceus, P. acidilactici, Enterococcus faecium, E. durans, E. faecalis, Leuconostoc mesenteroides*, and *Weissella cibaria*, whereas, *Lactobacillus pentosus* and *Lb. plantarum* subsp. *plantarum* were found only in *marcha* samples from Nepal. Variations in altitude and other geographical factors may affect the composition of microbiota in dry starters (Jeyaram et al., 2011; Lv et al., 2012). Traditional methods of preparation of *marcha, phab, paa, pee*, and *phut* are more or less similar except for some variations that were observed in the use of substrates, such as rice for *marcha, phut, paa*, and *pee*, and maize-rice husk for *phab* from Bhutan, and also wrapping materials for fermenting substrates such as fern leaves (*Glaphylopteriolopsis erubeseens*) for *marcha* preparation, dry paddy straws for *phab*, and locally available plant leaves for the preparation of *paa, pee*, and *phut*. Bacterial diversity in dry starters from the Eastern Himalayas may be influenced by hygienic conditions, quality of cereal substrates, wrapping materials, and sources of natural or tap water during traditional methods of preparation (Peter-Ikechukwu et al., 2016; Gonelimali et al., 2018; Sha et al., 2019).

The bacterial profile of *marcha* from Nepal and Bhutan, *paa, pee*, and *phut* of Arunachal Pradesh, and *phab* from Bhutan has been reported for the first time in our study. A similar type of dry starter for Assam in North East India called *xaj-pitha* also contained several species of LAB such as *Lactobacillus plantarum, Lb. brevis, Weissella cibaria, W. paramesenteroides, W. confusa, Lactococcus lactis, Lactobacillus casei group, Leuconostoc lactis, Leuconostoc pseudomesenteroides, Pediococcus pentosaceus, Lactococcus garvieae,* and *Enterococcus* sp. (Bora et al., 2016). Thanh et al. (2008) reported many species of LAB in Vietnamese *banh men*, which included *Pediococcus pentosaceus, Lactobacillus plantarum, Lb. brevis, Lb. fermentum, Lb. agilis, W. confusa, W. paramesenteroides,* and *Lactococcus lactis*. *Enterococcus faecium, Lactobacillus*

TABLE 6 | Diversity indices of different dry starters from the Eastern Himalayas.

Country/Region	Diversity indices			
	Simpson's index (1-*D*)	Shannon's index (*H*)	Dominance (*D*)	Chao-1
Nepal	0.86	2.025	0.14	13
India (Darjeeling hills)	0.6116	1.121	0.3884	5
India (Sikkim)	0.8878	2.305	0.1122	29
India (Arunachal Pradesh)	0.8711	2.176	0.1289	20.5
Bhutan	0.8374	1.925	0.1626	14

plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weissella paramesenteroides, and *W. cibaria*, were reported in *nuruk* from Korea (Hoon et al., 2013). Several species of LAB in Cambodian *dombea* were also reported: *Weissella cibaria, Lactobacillus plantarum, Lactococcus lactis, Pediococcus pentosaceus*, and *Enterococcus durans* (Ly et al., 2018). This indicates that species of LAB predominate the microbial composition of traditionally prepared dry starters in Asia, including the Eastern Himalayas. LAB have been considered as favorable bacteria in cereal-based beverages due their ability to improve protein digestibility, enhance organoleptic quality, and increase nutritional bioavailability (Luana et al., 2014). Species of *Weissella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Enterococcus* are known for flavor development, the production of organic acids, and antimicrobial activities in Chinese *daqu* used for liquor production (Gou et al., 2015). *Enterococcus* sp. has been reported to produce enterocins, which play a major role in preventing the growth of foodborne and spoilage-causing pathogens (Javed et al., 2011).

Non-LAB species formed the next abundant group (32%) in starters from the Eastern Himalayas with the dominance of *Bacillus* spp. The abundance of *Bacillus* sp. may be due to its ability to survive in environments with low moisture and high temperature (Nuding et al., 2017). Also, the *Bacillus* species are important sources of amylase and protease enzymes, which are involved in saccharification and flavor production (Beaumont, 2002). A dominance of *Bacillus* sp. was also reported in *daqu* from China (Wang et al., 2008; Zheng et al., 2012) and *banh men* from Vietnam (Thanh et al., 2008). The next most abundant bacterium was *Staphylococcus* spp., found in the Himalayan starters, which secretes amylase (Li et al., 2014) and protease in Chinese *daqu* (Yang et al., 2017) and also produces lipases for the production of esters for flavor (Talon et al., 1996); thus, this group of bacteria probably plays a major role in the flavor enhancement of the final product. The prevalence of phylum *Actinobacteria* in some starters of the Eastern Himalayas was only 6%, represented by *Kocuria rosea*, *Micrococcus yunnanensis*, and *Brevibacterium frigoritolerans*. The presence of *Actinobacteria* has been reported in Chinese *daqu* (Zou et al., 2018) and Indian *marcha* and *thiat* (Sha et al., 2017).

Few species of opportunist pathogens and environmental contaminants such as *Micrococcus*, *Stenotrophomonas*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* were detected, and they were found only in samples of *paa, pee,* and *phut* from Arunachal Pradesh, and *phab* from Bhutan. However, both the prevalence and populations of these contaminants were low and it is presumed that these organisms might have contaminated the samples during the traditional method of preparation from substrates, herbs, water, utensils, wrapping materials, etc., Gram-negative bacteria were not detected in any samples of *marcha* collected from Nepal, India, or Bhutan. In our previous study on *marcha*, no Gram-negative bacteria were found at the genus level, and this was discovered through an analysis using a high-throughput sequencing method (Sha et al., 2019). Although most of these bacteria are opportunists and probable foodborne pathogens, some of them, such as *Enterobacter* sp., are involved in the production of amylases and lipases and also the formation of flavor in *daqu* (Li et al., 2015). The presence of LAB inhibits the growth of pathogenic and spoilage microorganisms in foods (Cizeikiene et al., 2013; Castellano et al., 2017) and produces flavor compounds (Mukisa et al., 2017).

A diversity index, or phylogenetic metric, is a quantitative measure to show phylogenetic relations within different species in a community (Birtel et al., 2015). We characterized diversity indexes of the bacterial community present in starters from the Eastern Himalayas by using the Shannon diversity index *H*, Simpson's index, and Dominance and Chao1 index (**Table 6**). The Shannon diversity index *H* for evaluating bacterial diversity was recorded as being highest in *marcha* from Sikkim (*H*:2.305) and lowest in *marcha* from Darjeeling (*H*:1.121), indicating a higher bacterial diversity in *marcha* from Sikkim as compared to other starters. The Simpson's diversity index (1-*D*) index, which considers both the number of species as well as the relative abundance of each species for evaluating diversity, showed the highest values for *marcha* from Sikkim. The dominance *D*-values were recorded as being highest for *marcha* samples from Darjeeling and lowest for *marcha* samples from Sikkim, which supports the above inference regarding bacterial diversity. The dominance *D*-value ranged between 0–1, where the value 0 indicated that all taxa were equally present and value 1 indicated the dominance of one taxon over the whole community (Wagner et al., 2018). Thus, the values near zero indicate a highly diverse ecosystem and values near 1 indicate a less diverse or homogenous ecosystem (Lv et al., 2012). Hence, the phylogenetic matric of the bacterial community present in dry starters from the Eastern Himalayas showed high diversity within the community. The Eastern Himalayas are known for their rich floral and faunal diversity within a wide ecosystem (Chettri et al., 2010). Our findings thus highlight the richness of microbial diversity in the food ecosystem of the Eastern Himalayas.

The microbial communities and their interactions in starters are extremely important for proper fermentation, which may determine the productivity and flavor quality of the final alcoholic beverage (Cai et al., 2018). There has been an increasing amount of concern regarding the safety of fermented beverages due to the presence of ethyl carbamate, which is considered to be carcinogenic (Ryu et al., 2015), biogenic amines (Liu et al., 2016), mycotoxin (Sivamaruthi et al., 2018), and contamination by opportunistic microbial pathogens (Hong et al., 2016). All these considerations mandate a deep understanding of the microbial community in starters. Also, the profile of native microbiota in these starters opens a possibility of finding novel strain(s) with functional properties for industrial purposes. This study also records the bacterial diversity of *phab* from Bhutan, which is found to be produced rarely by a few ethnic people of Bhutan. This is probably due to their preference for commercial *marcha,* similar to *phab*, which is sold in local markets. Bacteria present in traditionally prepared dry starters have no amylolytic activities (Thapa and Tamang, 2004); however, they may contribute to the acidification of fermenting substrates and impart flavor with a mildly acidic and sour taste to traditional alcoholic beverages (*kodo ko jaanr, opo, apong*, and *themsing*) preferred by the Himalayan people (Thapa and Tamang, 2006; Tamang et al., 2007).

CONCLUSION

Information on the microbial composition of traditionally prepared dry starters of the Eastern Himalayan regions of India, Nepal, and Bhutan viz. *phab, paa, pee*, and *phut,* was unknown except for *marcha* from Sikkim in India. These traditional starters are used by the Himalayan people to ferment cereals into various alcoholic beverages for home consumption. The main objective of this study was to profile and assign the taxonomical identity of bacteria isolated from these traditional starters of the Eastern Himalayas based on 16S rRNA sequencing. *Firmicutes* was the most dominant phylum in all starters and was represented by several genera and species of LAB and also by some non-LAB. Interestingly our study showed high diversity within the bacterial community in traditionally prepared starters of the Eastern Himalayas, which may supplement the richness of microbial conservation in the food ecosystem of the regions. Besides diversity, some bacteria isolated from these traditional starters may have commercial and industrial importance. This is the first report on the bacterial diversity of dry starters of the Eastern Himalayas by Sanger sequencing.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-NCBI under the nucleotide accession number: MK748250-MK748278, MK202997-MK203032, and MK752675-MK752677.

AUTHOR CONTRIBUTIONS

PP performed the majority of the experiments. JT supervised the experiments and finalized the manuscript.

FUNDING

The authors are grateful to the Department of Biotechnology (DBT), Government of India, for financial support. PP is grateful to DBT for the award of Traineeship in DBT-funded Bioinformatics Centre of Sikkim University sanctioned to JT.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anupma, A., Pradhan, P., Sha, S. P., and Tamang, J. P. (2018). Traditional skill of ethnic people of the Eastern Himalayas and North East India in preserving microbiota as dry amylolytic starters. *Indian J. Tradit. Knowl.* 17, 184–190.

Beaumont, M. (2002). Flavouring composition prepared by fermentation with *Bacillus* spp. *Int. J. Food Microbiol.* 75, 189–196. doi: 10.1016/S0168-1605(01)00706-1

Bhasin, V. (2013). Pastoralists of Himalayas. *J. Biodiver.* 4, 83–113. doi: 10.1080/09766901.2013.11884746

Birtel, J., Walser, J. C., Pichon, S., Bürgmann, H., and Matthews, B. (2015). Estimating bacterial diversity for ecological studies: methods, metrics, and assumptions. *PLoS One* 10:e0125356. doi: 10.1371/journal.pone.0125356

Bora, S. S., Keot, J., Das, S., Sarma, K., and Barooah, M. (2016). Metagenomics analysis of microbial communities associated with a traditional rice wine starter culture (Xaj-pitha) of Assam, India. *3 Biotech*. 6:153. doi: 10.1007/s13205-016-0471-1

Cai, H., Zhang, T., Zhang, Q., Luo, J., Cai, C., and Mao, J. (2018). Microbial diversity and chemical analysis of the starters used in traditional Chinese sweet rice wine. *Food Microbiol.* 73, 319–326. doi: 10.1016/j.fm.2018.02.002

Castellano, P., Pérez Ibarreche, M., Blanco Massani, M., Fontana, C., and Vignolo, G. M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: a focus on meat ecosystems and industrial environments. *Microorganisms* 5:38. doi: 10.3390/microorganisms5030038

Chagnaud, P., Machinis, K., Coutte, L. A., Marecat, A., and Mercenier, A. (2001). Rapid PCR –based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. *J. Microbiol. Methods* 44, 139–148. doi: 10.1016/S0167-7012(00)00244-X

Chao, A., and Chiu, C. (2016). *Nonparametric Estimation and Comparison of Species Richness*. Chichester: John Wiley & Sons, doi: 10.1002/9780470015902.a0026329

Chen, B., Wu, Q., and Xu, Y. (2014). Filamentous fungal diversity and community structure associated with the solid state fermentation of Chinese Maotai-flavor liquor. *Int. J. Food Microbiol.* 179, 80–84. doi: 10.1016/j.ijfoodmicro.2014.03.011

Cheng, H. R., and Jiang, N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol. Lett.* 28, 55–59. doi: 10.1007/s10529-005-4688-z

Chettri, N., Sharma, E., Shakya, B., Thapa, R., Bajracharya, B., Uddin, K., et al. (2010). *Biodiversity in the Eastern Himalayas: Status, Trends and Vulnerability to Climate Change*. Technical Report 2. Kathmandu: ICIMOD.

Cizeikiene, D., Gražina, J., Paškevičius, A., and Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control* 31, 539–545. doi: 10.1016/j.foodcont.2012.12.004

Dung, N. T. P., Rombouts, F. M., and Nout, M. J. R. (2007). Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). *LWT Food Sci. Technol.* 40, 130–135. doi: 10.1016/J.LWT.2005.08.004

Gascuel, O., and Steel, M. (2006). Neighbor-Joining revealed. *Mol. Biol. Evol.* 23, 1997–2000. doi: 10.1093/molbev/msl072

Gonelimali, F. D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M., et al. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. *Front. Microbiol.* 9:1639. doi: 10.3389/fmicb.2018.01639

Gou, M., Wang, H., Yuan, H., Zhang, W., Tang, Y., and Kida, K. (2015). Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production. *J. Inst. Brew.* 121, 620–627. doi: 10.1002/jib.272

Hammer, Ø, Harper, D. A. T., and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* 4:9.

Hammes, W. P., and Hertel, C. (2003). "The genus *Lactobacillus*," in *The Prokaryotes an Electronic Resource for the Microbiological Community*, eds M. Dworkin, S. Flakow, E. Rosenberg, K. H. Schleifer, and E. Stackbrandt, (New York, NY:: Springer-Verlag).

He, G., Huang, J., Zhou, R., Wu, C., and Jin, Y. (2019). Effect of fortified daqu on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Front. Microbiol.* 10:56. doi: 10.3389/fmicb.2019.00056

Heather, J. M., and Chain, B. (2016). The sequence of sequencers: the history of sequencing DNA. *Genomics* 107, 1–8. doi: 10.1016/j.ygeno.2015.11.003

Hesseltine, C. W., and Kurtzman, C. P. (1990). Yeasts in amylolytic food starters. *Anal. Inst. Biol. Univ. Nac. Autón. México, Ser. Bot.* 60, 1–7. doi: 10.1016/B978-0-444-52149-1.00187-7

Hesseltine, C. W., and Ray, M. L. (1988). Lactic acid bacteria in *murcha* and *ragi*. *J. Appl. Microbiol.* 64, 395–401. doi: 10.1111/j.1365-2672.1988.tb05096.x

Hesseltine, C. W., Rogers, R., and Winarno, F. G. (1988). Microbiological studies on amylolytic Oriental fermentation starters. *Mycopathology* 101, 141–155. doi: 10.1002/jsfa.2740440410

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th Edn. Baltimore, MD: Williams & Wilkins, 965–1599.

Hong, X. T., Chen, J., Liu, L., Wu, H., Tan, H. Q., Xie, G. F., et al. (2016). Metagenomic sequencing reveals the relationship between microbiota composition and quality of Chinese rice wine. *Sci. Rep.* 6:26621. doi: 10.1038/srep26621

Hoon, S. S., Lee, C., Lee, S., Park, J. M., Lee, H. J., Bai, D. H., et al. (2013). Analysis of microflora profile in Korean traditional nuruk. *J. Microbiol. Biotechnol.* 23, 40–46. doi: 10.4014/jmb.1210.10001

Huang, Y., Yi, Z., Jin, Y., Zhao, Y., He, K., Liu, D., et al. (2017). New microbial resource: microbial diversity, function and dynamics in Chinese liquor starter. *Sci. Rep.* 7:14577. doi: 10.1038/s41598-017-14968-8

Javed, A., Masud, T., Ain, Q. U., Imran, M., and Maqsood, S. (2011). Enterocins of *Enterococcus faecium*, emerging natural food preservatives. *Ann. Microbiol.* 61, 699–708. doi: 10.1007/s13213-011-0223-8

Jeyaram, K., Singh, W., Capece, A., and Romano, P. (2008). Molecular identification of yeast species associated with "Hamei" — A traditional starter used for rice wine production in Manipur. *Indian Int. J. Food Microbiol.* 124, 115–125. doi: 10.1016/j.ijfoodmicro.2008.02.029

Jeyaram, K., Tamang, J. P., Capece, A., and Romano, P. (2011). Geographical markers for *Saccharomyces cerevisiae* strains with similar technological origins domesticated for rice-based ethnic fermented beverages production in North East India. *Antonie Van Leeuwen.* 100, 569–578. doi: 10.1007/s10482-011-9612-z

Jung, M. J., Nam, Y. D., Roh, S. W., and Bae, J. W. (2012). Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food Microbiol.* 30, 112–123. doi: 10.1016/j.fm.2011.09.008

Kozaki, M., and Uchimura, T. (1990). Microorganisms in Chinese starter 'bubod' and rice wine 'tapuy' in the Philippines. *J. Brewing Soc. Japan* 85, 818–824. doi: 10.6013/jbrewsocjapan1988.85.818

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA 7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Kumbhare, S. V., Dhotre, D. P., Dhar, S. K., Jani, K., Apte, D. A., Shouche, Y. S., et al. (2015). Insights into diversity and imputed metabolic potential of bacterial communities in the continental shelf of Agatti Island. *PLoS One* 10:e0129864. doi: 10.1371/journal.pone.0129864

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

Le Fort, P. (1975). Himalayas: the collided range. Present knowledge of the continental arc. *Am. J. Sci.* 275A, 1–44.

Lee, C. H., and Lee, S. S. (2002). Cereal fermentation by fungi. *Appl. Mycol. Biotechnol.* 2, 151–170. doi: 10.1016/S1874-5334(02)80009-0

Li, H., Jiao, A., Xu, X., Wu, C., Wei, B., Hu, X., et al. (2012). Simultaneous saccharification and fermentation of broken rice: an enzymatic extrusion liquefaction pretreatment for Chinese rice wine production. *Bioprocess Biosyst. Eng.* 36, 1141–1148. doi: 10.1007/s00449-012-0868-0

Li, P., Aflakpui, F. W. K., Yu, H., Luo, L., and Lin, W. (2015). Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar. *Ann. Microbiol.* 65, 2019–2027. doi: 10.1007/s13213-015-1040-2

Li, Z., Bai, Z., Wang, D., Zhang, W., Zhang, M., Lin, F., et al. (2014). Cultivable bacterial diversity and amylase production in three typical Daqus of Chinese spirits. *Int. J. Food Sci. Technol.* 49, 776–786. doi: 10.1111/ijfs.12365

Limtong, S., Sintara, S., and Suwannarit, P. (2002). Yeast diversity in Thai traditional alcoholic starter. *Kasetsart J. Natural Sci.* 36, 149–158.

Liu, S. P., Yu, J. X., Wei, X. L., Ji, Z. W., Zhou, Z. L., Meng, X. Y., et al. (2016). Sequencing-based screening of functional microorganism to decrease the formation of biogenic amines in Chinese rice wine. *Food Control* 64, 98–104. doi: 10.1016/j.foodcont.2015.12.013

Luana, N., Rossana, C., Curiel, J. A., Kaisa, P., Marco, G., and Rizzello, C. G. (2014). Manufacture and characterization of a yogurt-like beverage made with oat flakes fermented by selected lactic acid bacteria. *Int. J. Food Microbiol.* 185, 17–26. doi: 10.1016/j.ijfoodmicro.2014.05.004

Lv, X. C., Weng, X., Zhang, W., Rao, P. F., and Ni, L. (2012). Microbial diversity of traditional fermentation starters for Hong Qu glutinous rice wine as determined by PCR-mediated DGGE. *Food Control* 28, 426–434. doi: 10.1016/j.foodcont.2012.05.025

Ly, S., Mith, H., Tarayre, C., Taminiau, B., Daube, G., Fauconnier, M. L., et al. (2018). Impact of microbial composition of Cambodian traditional dried starters (dombea) on flavor compounds of rice wine: combining amplicon sequencing with HP-SPME-GCMS. *Front. Microbiol.* 9:894. doi: 10.3389/fmicb.2018.00894

McCarty, C. (1998). *CHROMASPRO 1.34: Free Program*. Available at: http://www.technelysium.com.au/chromas.html

Mukisa, I. M., Byaruhanga, Y. B., Muyanja, C. M. B. K., Langsrud, T., and Narvhus, J. A. (2017). Production of organic flavor compounds by dominant lactic acid bacteria and yeasts from Obushera, a traditional sorghum malt fermented beverage. *Food Sci. Nutr.* 5, 702–712. doi: 10.1002/fsn3.450

Nandy, S. N., Dhyani, P. P., and Sanal, P. K. (2006). Resources information database of the Indian Himalaya. *ENVIS Monogr.* 3, 1–95.

Nikkuni, S., Karki, T. B., Terao, T., and Suzuki, C. (1996). Microflora of *mana*, a Nepalese rice *koji*. *J. Ferment.Bioeng.* 81, 168–170. doi: 10.1016/0922-338x(96)87597-0

Nile, S. H. (2015). The nutritional, biochemical and health effects of makgeolli – a traditional Korean fermented cereal beverage. *J. Inst. Brew.* 121, 457–463. doi: 10.1002/jib.264

Nout, M. J. R., and Aidoo, K. E. (2002). "Asian Fungal Fermented Food," in *Mycota, a Comprehensive Treatise On Fungi as Experimental Systems and Applied Research, Industrial Applications*, Vol. 10, ed. H. D. Osiewacz, (Berlin: Springer-Verlag), 23–47. doi: 10.1007/978-3-662-10378-4_2

Nuding, D. L., Gough, R. V., Venkateswaran, K. J., Spry, J. A., and Tolbert, M. A. (2017). Laboratory investigations on the survival of *Bacillus subtilis* spores in deliquescent salt mars analog environments. *Astrobiology* 17, 997–1008. doi: 10.1089/ast.2016.1545

Pervez, S., Aman, A., Iqbal, S., Siddiqui, N. N., and Ul Qader, S. A. (2014). Saccharification and liquefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. *BMC Biotechnol.* 14:49. doi: 10.1186/1472-6750-14-49

Peter-Ikechukwu, A. I., Kabuo, N. O., Alagbaoso, S. O., Njoku, N. E., Eluchie, C. N., and Momoh, W. O. (2016). Effect of wrapping materials on physico-chemical and microbiological qualities of fermented melon seed (*Citrullus colocynthis* L.) used as condiment. *Am. J. Food Sci. Technol.* 4, 14–19. doi: 10.12691/ajfst-4-1-3

Ryu, D., Choi, B., Kim, E., Park, S., Paeng, H., Kim, C. I., et al. (2015). Determination of ethyl carbamate in alcoholic beverages and fermented foods sold in Korea. *Toxicol Res.* 31, 289–297. doi: 10.5487/TR.2015.31.3.289

Saha, D. (2013). Lesser Himalayan sequences in Eastern Himalaya and their deformation: implications for Paleoproterozoic tectonic activity along the northern margin of India. *Geosci. Front.* 4, 289–304. doi: 10.1016/j.gsf.2013.01.004

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nati. Acad. Sci. U.S.A.* 74, 5463–5467. doi: 10.1073/pnas.74.12.5463

Schmitz, A., and Riesner, D. (2006). Purification of nuclei acids by selective precipitation with polyethylene glycol 6000. *Anal. Biochem.* 354, 311–313. doi: 10.1016/j.ab.2006.03.014

Sha, S. P., Jani, K., Sharma, A., Anupma, A., Pradhan, P., Shouche, Y., et al. (2017). Analysis of bacterial and fungal communities in Marcha and Thiat, traditionally prepared amylolytic starters of India. *Sci. Rep.* 7:10967. doi: 10.1038/s41598-017-11609-y

Sha, S. P., Suryavanshi, M. S., and Tamang, J. P. (2019). Mycobiome diversity in traditionally prepared starters for alcoholic beverages in India by high-throughput sequencing method. *Front. Microbiol.* 10:348. doi: 10.3389/fmicb.2019.003482237

Sha, S. P., Suryavanshi, M. V., Jani, K., Sharma, A., Shouche, Y. S., and Tamang, J. P. (2018). Diversity of yeasts and molds by culture-dependent and culture-independent methods for mycobiome surveillance of traditionally prepared dried starters for the production. *Front. Microbiol.* 9:2237. doi: 10.3389/fmicb.2018.02237

Sharma, R., Jianchu, X., and Sharma, G. (2007). Traditional agroforestry in the eastern Himalayan region: land management system supporting ecosystem services. *Tropical Ecol.* 48, 189–200.

Shrivastava, K., Greeshma, A. G., and Srivastava, B. (2012). Biotechnology in tradition – a process technology of alcoholic beverages practiced by different

tribes of Arunachal Pradesh, North East India. *Indian J. Trad. Knowl.* 11, 81–89.

Sivamaruthi, B. S., Kesika, P., and Chaiyasut, C. (2018). Toxins in fermented foods: prevalence and preventions—a mini review. *Toxins* 11:E4. doi: 10.3390/toxins11010004

Surono, I. S. (2016). "Ethnic fermented foods and beverages of Indonesia," in *Ethnic Fermented Foods and Alcoholic Beverages of Asia*, ed. J. P. Tamang, (New Delhi: Springer), 341–382.

Talon, R., Montel, M. C., and Berdague, J. L. (1996). Production of flavor esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus*. *Enzyme Microb. Technol.* 19, 620–622. doi: 10.1016/S0141-0229(96)00075-0

Tamang, J. P. (2010). *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic Values*. New York, NY: CRC Press, 295.

Tamang, J. P., Dewan, S., Tamang, B., Rai, A., Schillinger, U., and Holzapfel, W. H. (2007). Lactic acid bacteria in hamei and marcha of North East India. *Indian J. Microbiol.* 47, 119–125. doi: 10.1007/s12088-007-0024-8

Tamang, J. P., and Sarkar, P. K. (1995). Microflora of murcha: an amylolytic fermentation starter. *Microbios* 81, 115–122.

Tamang, J. P., Sarkar, P. K., and Hesseltine, C. W. (1988). Traditional fermented foods and beverages of Darjeeling and Sikkim–a review. *J. Sci. Food Agric.* 44, 375–385. doi: 10.1002/jsfa.2740440410

Tamang, J. P., Tamang, N., Thapa, S., Dewan, S., Tamang, B. M., Yonzan, H., et al. (2012). Microorganisms and nutritional value of ethnic fermented foods and alcoholic beverages of North East India. *Indian J. Tradit. Knowl.* 11, 7–25.

Tamang, J. P., Thapa, S., Tamang, N., and Rai, B. (1996). Indigenous fermented food beverages of Darjeeling hills and Sikkim: a process and product characterization. *J. Hill Res.* 9, 401–411.

Thakur, N., Saris, P. E., and Bhalla, T. C. (2015). Microorganisms associated with amylolytic starters and traditional fermented alcoholic beverages of North Western Himalayas in India. *Food Biosci.* 11, 92–96. doi: 10.1016/j.fbio.2015.05.002

Thanh, V. N., Mai Le, T., and Tuan, D. A. (2008). Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int. J. Food Microbiol.* 128, 268–273. doi: 10.1016/j.ijfoodmicro.2008.08.020

Thapa, S., and Tamang, J. P. (2004). Product characterization of kodo ko jaanr: fermented finger millet beverage of the Himalayas. *Food Microbiol.* 21, 617–622. doi: 10.1016/j.fm.2004.01.004

Thapa, S., and Tamang, J. P. (2006). Microbiological and physico-chemical changes during fermentation of kodo ko jaanr, a traditional alcoholic beverage of the Darjeeling hills and Sikkim. *Indian J. Microbiol.* 46, 333–341.

Tsuyoshi, N., Fudou, R., Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S., et al. (2005). Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. *Int. J. Food Microbiol.* 99, 135–146. doi: 10.1016/j.ijfoodmicro.2004.08.011

Varin, C., Reid, N., and Firth, D. (2011). An overview of composite likelihood methods. *Stat. Sin.* 21, 5–42.

Wagner, B. D., Grunwald, G. K., Zerbe, G. O., Mikulich-Gilbertson, S. K., Robertson, C. E., Zemanick, E. T., et al. (2018). On the use of diversity measures in longitudinal sequencing studies of microbial communities. *Front. Microbiol.* 9:1037. doi: 10.3389/fmicb.2018.01037

Wang, C. L., Shi, D. J., and Gong, G. L. (2008). Microorganisms in Daqu: a starter culture of Chinese Maotai-flavor liquor. *World J. Microbiol. Biotechnol.* 24, 2183–2190. doi: 10.1007/s10295-009-0661-5

Yang, J.-G., Dou, X., Han, P.-J., Bai, F.-Y., Zhou, J., Zhang, S.-Y., et al. (2017). Microbial diversity in Daqu during production of Luzhou-flavored liquor. *J. Am. Soc. Brew. Chem.* 75, 136–144.

Zheng, X. W., Tabrizi, M. R., Nout, M. J. R., and Han, B. Z. (2011). Daqu-a traditional Chinese liquor fermentation starter. *J. Institute Brew.* 117, 82–90.

Zheng, X. W., Yan, Z., Han, B. Z., Zwietering, M. H., Samson, R. A., Boekhout, T., et al. (2012). Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiol.* 31, 293–300. doi: 10.1016/j.fm.2012.03.008

Zheng, X. W., Yan, Z., Nout, M. J. R., Boekhout, T., Han, B. Z., Zwietering, M. H., et al. (2015). Characterization of the microbial community in different types of *Daqu* samples as revealed by 16S rRNA and 26S rRNA gene clone libraries. *World J. Microbiol. Biotechnol.* 31, 199–208. doi: 10.1007/s11274-014-1776-z

Zou, W., Zhao, C., and Luo, H. (2018). Diversity and function of microbial community in Chinese strong-flavor baijiu ecosystem: a review. *Front. Microbiol.* 9:671. doi: 10.3389/fmicb.2018.00671

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.